Inhibition of lncRNA NEAT1 protects endothelial cells against hypoxia/reoxygenation-induced NLRP3 inflammasome activation by targeting the miR-204/BRCC3 axis

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Received February 26, 2020; Accepted August 27, 2020

DOI: 10.3892/mmr.2021.12548

Abstract. Cardiovascular ischemia/reperfusion (I/R) injury is primarily caused by oxygen recovery after prolonged hypoxia. Previous studies found that the long non coding RNA (lncRNA) nuclear enriched abundant transcript 1 (NEAT1) was involved in cardiovascular pathology, and that NOD-like receptor protein 3 (NLRP3) inflammasome activation-dependent pyroptosis played a key role in cardiovascular I/R injury. The present study aimed to explore the molecular mechanism of I/R pathogenesis in order to provide novel insights for potential future therapies. Cell viability and lactate dehydrogenase enzyme activity assays were used to detect cell injury after human umbilical vein endothelial cells (HUVECs) were subjected to hypoxia/reoxygenation (H/R). The expression of the NEAT1/microRNA (miR)-204/BRCA1/BRCA2-containing complex subunit 3 (BRCC3) axis was examined by reverse transcription-quantitative PCR, and the associations among genes were confirmed by luciferase reporter assays. Western blotting and ELISA were used to measure the level of NLRP3 inflammasome activation-dependent pyroptosis. The results demonstrated that NEAT1, BRCC3 expression and NLRP3 inflammasome activation-dependent pyroptosis were significantly increased in H/R-injured HUVECs, whereas silencing BRCC3 or NEAT1 attenuated H/R-induced injury and pyroptosis. NEAT1 positively regulated BRCC3 expression via competitively binding with miR-204. Moreover, NEAT1 overexpression counteracted miR-204 mimic-induced injury, BRCC3 expression and NLRP3 inflammasome activation-dependent pyroptosis. Taken together, these findings demonstrated that inhibition of lncRNA NEAT1 protects HUVECs against H/R-induced NLRP3 inflammasome activation by targeting the miR-204/BRCC3 axis.

Introduction

Emerging evidence suggests that cardiovascular disease is the leading cause of death worldwide (1). Myocardial infarction, the most fatal type of cardiovascular disease, has attracted widespread attention because of its increasing incidence, complications, poor outcomes and limited treatment. A complex issue is that clinical treatment for myocardial infarction to restore cardiovascular perfusion can cause secondary damage. There is a large body of work examining various regulators in ischemia/reperfusion (I/R), however, the molecular mechanisms underlying I/R injury remain unclear. Therefore, there is a clear need to study the molecular etiology of I/R injury in order to develop more effective treatment strategies and improve prognosis.

Pyroptosis, also known as cellular inflammatory necrosis, is a form of programmed cell death in which cells swell until the plasma membrane ruptures, causing the release of cellular contents to activate a strong inflammatory response (2). Pyroptosis is an important natural immune response in the body and plays a significant role in combating infection (3). It was previously demonstrated that NOD-like receptor protein 3 (NLRP3) inflammasome activation-dependent pyroptosis was involved in cardiovascular I/R injury (3) and that the BRCA1/BRCA2-containing complex subunit 3 (BRCC3), a deubiquitinating enzyme, is a critical regulator of NLRP3 activity (4). This indicated that the regulation of BRCC3 expression, and its direct interaction with NLRP3, could be a potential strategy for the treatment of cardiovascular I/R injury.

Long non-coding RNAs (lncRNAs) are non-coding RNAs that are >200 nucleotides in length (5). Studies have reported that lncRNAs play an important role in numerous biological processes, such as dose compensation effect, epigenetic regulation, cell cycle regulation and cell differentiation regulation, and have become an important area of interest in genetics (6). It was found that the lncRNA nuclear enriched abundant
transcript 1 (NEAT1) aggravated cardiovascular I/R injury (7), but the potential regulatory mechanisms have not yet been elucidated. At present, no reports have examined NEAT1 regulated NLRP3 inflammasome activation-dependent pyroptosis in cardiovascular I/R injury.

MicroRNAs (miRNA/miRs) are a class of endogenous regulatory non-coding RNAs found in eukaryotes that are 20-25 nucleotides in length (8). Emerging evidence has suggested a functional role of lncRNAs by acting as competing endogenous RNAs (ceRNAs) that regulate specific RNA transcripts through competing for shared miRNAs (9). To the best of our knowledge, no previous studies have explored miR-204 and NLRP3 inflammasome activation-dependent pyroptosis in cardiovascular I/R injury.

In the present study, the regulatory effect of NEAT1 and related miR-204/BRCC3 axis in NLRP3 inflammasome activation-dependent pyroptosis was investigated in human umbilical vein endothelial cells (HUVECs) exposed to hypoxia/reoxygenation (H/R).

Materials and methods

Cell culture. HUVECs (cat. no. aBFN6021424) were obtained from Shanghai Institute of Biochemistry and Cell Biology and cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C, 5% CO₂.

Cell transfection. Short hairpin RNAs (shRNAs) against NEAT1 (NEAT1-shRNA, 5'-CCGTGTGGTGTTGTGTTGGA ATCTGTG-3')/BRCC3 (BRCC3-shRNA, 5'-GUACUGGGUU UGUUACAGAUU-3') and corresponding negative control (con-shRNA: NEAT1-con-shRNA, 5'-CCGTGTGGTGTTGTGTTGGA ATCTGTG-3'; BRCC3-con-shRNA, 5'-UCAUCUGCCG UCAGCAGUTT-3'), pcDNA-NEAT1 and corresponding negative control (pcDNA-con), miR-204 mimics (5'-TTCCCTT TGTCATCTCATGCT-3') and corresponding negative control (miR-con, 5'-CGATCGCATCAGCATCGATTGC-3'), were constructed by Shanghai GenePharma Co., Ltd. The constructs (50 nM) were transfected into 1x10⁶ HUVECs using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Transfection efficiency was observed, and cells were used for subsequent experiments 48 h after transfection.

Establishment of H/R cell model. A cellular H/R model was constructed based on the methods of Deng et al (10). Briefly, medium containing 4.5 g/l glucose and 10% FBS was replaced with a sugar-free, serum-free medium before exposure to hypoxic conditions. HUVECs were cultured for 12 h at 1% oxygen, 5% CO₂ and 94% nitrogen to induce hypoxia. Following this, cells were exposed to reoxygenation conditions for 4 h, at 37°C and 5% CO₂, in medium containing 4.5 g/l sugar and 10% FBS.

Cell Counting Kit (CCK)-8 cell viability assay. CCK-8 was used according to the manufacturer's protocol. Transfected or untransfected HUVECs were seeded in a 96-well plate at a density of 1x10⁵ cells/100 µl. After undergoing conventional H/R operations, 10 µl detection reagent (Dojindo Molecular Technologies, Inc.) was added to HUVECs for 2-4 h at 37°C. The OD value at a wavelength of 450 nm was measured with a microplate reader.

Lactate dehydrogenase (LDH) assay. Transfected or untransfected HUVECs were seeded in a 6-well plate at a density of 1x10⁶ cells/2 ml. After undergoing conventional H/R operations, LDH activity was determined using commercial kits (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

 Luciferase reporter assay. Wild-type (WT) or mutant (MUT) NEAT1 and BRCC3 were inserted into a pGL3 promoter vector (ShanghaiGenePharmaCo.,Ltd.), HUVECs were transfected with pGL3-NEAT1/BRCC3-WT or pGL3-NEAT1/BRCC3-MUT and miR-mimics or mimics control (Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. The luciferase activity was evaluated using a Luciferase Reporter Assay System (Shanghai GenePharma Co., Ltd.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from 1x10⁶ HUVECs using TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The first strand cDNA was synthesized from 1 µg total RNA using a Prime Script RT kit and gDNA Eraser (Takara Bio, Inc.). The relative mRNA levels were analyzed using the 2−ΔΔCq method (11) and normalized to the internal reference genes U6 and GAPDH (12).

Western blot analysis. Total protein was extracted from 1x10⁵ HUVECs using RIPA lysis buffer (BestBio). The proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% gel, and subsequently transferred to PVDF membranes. The membranes were incubated with 5% fat-free milk at room temperature for 1 h. Anti-BRCC3 antibodies (cat. no. aPA5-20426, 1:1,000, Invitrogen; Thermo Fisher Scientific, Inc.), anti-NLRP3 antibodies (cat. no. aPA5-20426, 1:1,000, Invitrogen; Thermo Fisher Scientific, Inc.), anti-cleaved gaddermnin D (GSDMD) antibodies (cat. no. ab215203, 1:1,000, Abcam) and anti-β actin antibodies (cat. no. ab8226, 1:1,000, Abcam) were used as the primary antibodies at 4°C for 12 h. The secondary antibodies (cat. nos. 31430 and G-21234, 1:10,000, Invitrogen; Thermo Fisher Scientific, Inc.) was added for 2 h at room temperature. Protein bands were detected using Pierce ECL Western blot analysis substrate (Thermo Fisher Scientific, Inc.).

Statistical analyses. Data are presented as the mean ± SD. All statistical analyses were conducted using SPSS 19.0 software (IBM Corp.). The normal distribution and homogeneity of variance of data were tested first. Differences between two groups were compared by an unpaired Student's t-test, while multiple groups were compared by one-way analysis of variance (ANOVA) when data were normally distributed and had homogeneity of variance. Bonferroni correction was used to assess multiple comparisons following one-way ANOVA. The rank-sum test was used to test the data that was not normally distributed or lacked homogeneity of variance.
P<0.05 was considered to indicate a statistically significant difference.

Results

H/R evokes damage and NLRP3 inflammasome activation-dependent pyroptosis in HUVECs. The HUVECs were subjected to an in vitro model of hypoxia for 12 h and reoxygenation for 4 h. As a result, H/R significantly decreased cell viability (Fig. 1A) and increased LDH activity (Fig. 1B), protein expression of BRCC3, NLRP3, ASC, cleaved CASP1 and cleaved GSDMD (Fig. 1F), mRNA expression of interleukin (IL)-1β and IL-18 (Fig. 1G and H) and secreted levels of IL-1β and IL-18 (Fig. 1I and J). Meanwhile, the expression of NEAT1 (Fig. 1C) and BRCC3 (Fig. 1E) were significantly increased, whereas miR-204 expression was significantly decreased (Fig. 1D) in the H/R group as measured by RT-qPCR.

Knockdown of BRCC3 represses NLRP3 inflammasome activation-dependent pyroptosis and cell damage in HUVECs. To investigate the expression of BRCC3 and the interaction between NLRP3 and BRCC3 in H/R-induced pyroptosis and cell damage, BRCC3 expression was depleted by the transfection of BRCC3-shRNA into cells prior to induction of hypoxia (Fig. 2A). As a result, BRCC3-shRNA treatment significantly reversed the H/R-induced reduction in cell viability (Fig. 2B) and H/R-induced increase in LDH activity (Fig. 2C). Protein expression of BRCC3, NLRP3, ASC, cleaved CASP1 and cleaved GSDMD (Fig. 2D) were all reduced following transfection with BRCC3-shRNA but not in con-shRNA. Similarly, mRNA expression and secreted protein levels of IL-1β and IL-18 (Fig. 2E-H) were reduced by depletion of BRCC3 prior to H/R.

Knockdown of NEAT1 reverses H/R-induced cell damage and NLRP3 inflammasome activation-dependent pyroptosis. HUVECs were transfected with either NEAT1-shRNA or shRNA-con before the induction of hypoxia to observe the function of NEAT1 (Fig. 3A) in H/R-induced cell damage and NLRP3 inflammasome activation-dependent pyroptosis.
As shown in Fig. 3, NEAT1-shRNA significantly reversed the H/R-induced reduction in cell viability (Fig. 3B) and H/R-induced increase in LDH activity (Fig. 3C). Additionally, NEAT1-shRNA transfection reduced the H/R-induced increase in protein expression of BRCC3, NLRP3, ASC, cleaved CASP1 and cleaved GSDMD (Fig. 3G). Similar to BRCC3 depletion, mRNA expression and secreted protein levels of IL-1β and IL-18 (Fig. 3H-K) were significantly decreased by depletion of NEAT1 before H/R. Of note, the expression of miR-204 was significantly increased (Fig. 3E), whereas the expression of BRCC3 was significantly reduced (Fig. 3F), following NEAT1-shRNA transfection.
Figure 3. Knockdown of NEAT1 reverses H/R-induced cell damage by reducing NLRP3 inflammasome activation-dependent pyroptosis. (A) The expression of NEAT1 mRNA was examined by RT-qPCR. Detection of (B) cell viability and (C) LDH activity in human umbilical vein endothelial cells following H/R. RT-qPCR analysis of (D) NEAT1, (E) miR-204 and (F) BRCC3 expression. (G) The expression levels of BRCC3, NLRP3, ASC, cleaved CASP1 and cleaved GSDMD protein were examined by western blotting. The expression levels of (H) IL-1β and (I) IL-18 mRNA were examined by RT-qPCR. The levels of secreted (J) IL-1β and (K) IL-18 were evaluated by ELISA. Data are shown as the mean ± SD from five different experiments. **P<0.01. H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; IL, interleukin; NLRP3, NOD-like receptor protein 3; ASC, apoptosis speck-like protein; CASP1, caspase-1; GSDMD, gasdermin D; NEAT1, nuclear enriched abundant transcript 1; BRCC3, BRCA1/BRCA2-containing complex subunit 3; miR, micro RNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; CCK-8, Cell Counting Kit-8; shRNA, short hairpin RNA.
NEAT1 functions as a sponge of miR-204. Prior research has indicated that lncRNAs act as miRNA sponges in multiple diseases including I/R injury (7). In the present study it was found that miR-204 was a target of NEAT1 (Fig. 4A). To further explore the association between NEAT1 and miR-204, luciferase reporter assays were performed (Fig. 4B-C). This demonstrated that NEAT1-WT, but not NEAT1-MUT, could be specifically reduced by miR-204 mimics (Fig. 4D). In addition, miR-204 expression was significantly decreased by transient transfection of pcDNA-NEAT1, but not pcDNA-con (Fig. 4E).

NEAT1 aggravates H/R-induced cell damage and NLRP3 inflammasome activation-dependent pyroptosis and can be rescued by miR-204 overexpression. To further confirm whether NEAT1 regulates H/R-induced injury by sponging miR-204, pcDNA-NEAT1 or pcDNA-con was co-transfected into HUVECs with miR-204 mimics. It was found that miR-204 mimics significantly increased cell viability (Fig. 5A) and decreased LDH activity (Fig. 5B) following H/R compared with the control group. The relative mRNA levels of NEAT1, and BRCC3 were reduced by overexpression of miR-204, while the relative mRNA levels of miR-204 were increased (Fig. 5C-E). Protein expression of NLRP3, ASC, cleaved CASP1 and cleaved GSDMD (Fig. 5F) were all reduced by overexpression of miR-204. Both mRNA expression and secreted protein levels of IL-1β and IL-18 (Fig. 5G-J) were significantly reduced in cells transfected with miR-204 mimic. However, overexpression of NEAT1 by transient transfection of pcDNA-NEAT1 reversed the aforementioned changes induced by miR-204 expression, further demonstrating the role of NEAT1 as a miR-204 sponge.

NEAT1 serves as a ceRNA for miR-204 to target BRCC3. To ensure whether NEAT1 functioned as a ceRNA for miR-204 to target BRCC3, luciferase reporter systems driven by
Figure 5. NEAT1 aggravates H/R-induced cell damage and NLRP3 inflammasome activation-dependent pyroptosis and is rescued by miR-204 overexpression. HUVECs were co-transfected with miR-204 or miR-con and pcDNA-NEAT1 or pcDNA-con before H/R. Detection of (A) cell viability and (B) LDH activity in HUVECs following H/R. RT-qPCR analysis of (C) NEAT1, (D) miR-204 and (E) BRCC3 expression. (F) The expression levels of BRCC3, NLRP3, ASC, cleaved CASP1 and cleaved GSDMD protein were examined by western blotting. The expression levels of (G) IL-1β and (H) IL-18 mRNA were examined by RT-qPCR. The levels of secreted (I) IL-1β and (J) IL-18 were evaluated by ELISA. Data are shown as the mean ± SD from five different experiments. *P<0.01, H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; IL, interleukin; NLRP3, NOD-like receptor protein 3; ASC, apoptosis speck-like protein; CASP1, caspase-1; GSDMD, gasdermin D; NEAT1, nuclear enriched abundant transcript 1; BRCC3, BRCA1/BRCA2-containing complex subunit 3; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; HUVEC, human umbilical vein endothelial cell; CCK-8, Cell Counting Kit-8.
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BRCC3-WT and BRCC3-MUT were constructed to verify predicted miR-204 binding sites (Fig. 6A). It was found that miR-204 mimics significantly decreased the luciferase activity of BRCC3-WT but not BRCC3-MUT in HUVECs. This effect was reversed by co-transfection of pcDNA-NEAT1 and miR-204 mimics, but not co-transfection of pcDNA-con and miR-204 mimics (Fig. 6B and C). In addition, the expression of BRCC3 was also confirmed this tendency (Fig. 6D).

Discussion

Emerging evidence has suggested that lncRNAs can function as ceRNAs that regulate specific RNA transcripts by competing for shared miRNAs. In the present study, it was found that NEAT1 and BRCC3 expression was significantly increased in H/R-induced injury, whereas miR-204 expression was decreased. Furthermore, it was demonstrated that NEAT1 positively regulated BRCC3 expression and NLRP3 inflammasome activation-dependent pyroptosis by competitively binding to miR-204. To the best of our knowledge, the present study was the first to demonstrate that NEAT1 and miR-204 regulated NLRP3 inflammasome activation-dependent pyroptosis in H/R injury, and provides a potential mechanism and novel therapeutic targets for cardiovascular I/R damage.

Recent studies have revealed that lncRNAs acts as a ceRNA for miRNA to target mRNA to regulate cardiovascular disease (12). Several lncRNAs, including H19 (13), metastasis-associated lung adenocarcinoma transcript 1 (14) and taurine-upregulated gene 1 (15) have been confirmed to regulate cardiovascular I/R injury through the ceRNA network. Moreover, previous studies partially support the results of the present study. It was previously revealed that NEAT1 was overexpressed in myocardial I/R injury cells compared with normal myocardial cells, and downregulation

Figure 6. NEAT1 serves as a ceRNA for miR-204 to target BRCC3. (A) The binding sites between BRCC3 and miR-204 and the luciferase reporter constructs containing WT-BRCC3 or MUT-BRCC3. (B-D) The luciferase reporter vectors (WT-BRCC3 and MUT-BRCC3) were co-transfected with miR-204 mimics, pcDNA-NEAT1 and matched controls into human umbilical vein endothelial cells, and luciferase activity was detected by a luciferase reporter assay. Data are shown as the mean ± SD from five different experiments. **P<0.01. ceRNA, competing endogenous RNA; NEAT1, nuclear enriched abundant transcript 1; BRCC3, BRCA1/BRCA2-containing complex subunit 3; miR, microRNA; WT, wild-type; MUT, mutant.
of NEAT1 enhanced cell proliferation while inhibiting cell apoptosis via targeting miR-193a in I/R injury H9C2 cells (16). Luo et al (9) revealed that NEAT1 contributed to I/R injury via the modulation of miR-495-3p and MAPK6 in H9C2 cells. In addition, it was previously reported that NEAT1 contributed to myocardial I/R injury by activating the MAPK pathway (7). All of these studies confirmed that NEAT1 may deteriorate myocardium in I/R damage; however, the role of NEAT1 in endothelial I/R injury and cardiovascular pyroptosis has not been reported at present. In the present study, it was reported that the regulation and potential mechanism of NEAT1 in cardiovascular I/R injury was via the modulation of NLRP3 inflammasome activation-dependent pyroptosis, and could provide a theoretical basis for the clinical treatment of I/R injury.

miRNA links upstream lncRNA with downstream mRNA with a shared competitive binding site for lncRNA and mRNA. It was found that upregulation of miR-204 alleviated ventricular remodeling and improved cardiac function in mice following myocardial I/R injury (12). Furthermore, the function of miR-204 to protect against I/R injury has also been confirmed to be applicable to the kidney (17) and diabetic myocardial I/R (18). However, there is currently no research demonstrating a relationship between NEAT1 and miR-204, and no studies have reported that miR-204 provides a shared competitive binding site for NEAT1 and mRNA. Therefore, the present study was the first to show that NEAT1 served as a ceRNA for miR-204 to target BRCC3. In addition, it was reported that miR-204 played a key role in endothelial cell I/R injury and pyroptosis, and elucidated its ability to regulate BRCC3 expression and activity.

Conversely, other studies have demonstrated contrary evidence to those presented in the present study. Yan et al (19) found that NEAT1 functioned as a miRNA sponge to inhibit cardiomyocyte apoptosis and may be a novel therapeutic target for cardiomyocyte apoptosis associated heart diseases. These findings are consistent with other studies reporting that NEAT1 knockdown improves LPS-induced myocardial injury in mice by inhibiting the toll-like receptor 2/NF-kB signaling pathway (20). It was previously reported that inhibition of miR-204 exerts spinal cord protection against I/R injury, possibly via promotion of autophagy and anti-apoptotic effects (21). The reason for these differences is likely to be due to differences in experimental models and differences in the ceRNA network studied. This also suggests that NEAT1 is likely to play multiple roles in cardiovascular I/R injury and can act as a double-edged sword.

However, it should be noted that there are some limitations to the research in the present study. Although the data in the present study validated the key role and novel mechanism of the NEAT1/miR-204/BRCC3 axis in the regulation of NLRP3 inflammasome activation-dependent-pyroptosis in vitro, the absence of in vivo animal experiments or human tissue samples reduces the clinical application potential of the conclusions. In addition, some other important forms of regulatory cell death, including ferroptosis, apoptosis, autophagy and necroptosis, have also been indicated to serve an important role in myocardial I/R injury (22-26). However, the present study did not focus on the effects of NEAT1/miR-204/BRCC3 signals on these forms of regulatory cell death.

The present study focused on NLRP3 inflammasome activation-dependent pyroptosis in cardiovascular I/R injury and demonstrated that NEAT1 positively regulated BRCC3 expression and NLRP3 inflammasome activation-dependent pyroptosis by competitively binding with miR-204. Taken together, these findings demonstrated that inhibition of lncRNA NEAT1 may protect HUVECs against H/R-induced NLRP3 inflammasome activation by targeting the miR-204/BRCC3 axis.

Acknowledgements
Not applicable.

Funding
This work was supported by grants from the Social Welfare Project in Bao'an District, Shenzhen, China (grant no. 2020JD360 to TY; grant no. 2021JD070 to SL).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
TY and XY conceived and designed the project. TY performed the experiments with the help of YS, SL and JG. YS analyzed the data. TY and XY conceived and designed the project. TY performed the experiments with the help of YS, SL and JG. YS analyzed the data. TY wrote the manuscript. TY and XT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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