NR_027324 regulates autophagy and apoptosis by combining with miR-103-3p as ceRNA to regulate ATG5 in cardiomyocytes during hypoglycemic and hypoxic injury

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Research

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Abstract

Background: Myocardial infarction (MI) refers to a fatal disease, and the border zone (BZ) of myocardial infarction is of high importance to the prognosis of myocardial infarction patients. Autophagy and apoptosis can significantly impact cardiovascular diseases. In the previous studies conducted by the authors, plenty of differential expressions of long non-coding RNA (lncRNA) were reported in the border zone of myocardial infarction. As revealed from the results of bioinformatics analysis, LncRNA may take up a vital part in the pathological process of cardiovascular disease by regulating apoptosis and autophagy. This study aimed to firstly conduct bioinformatics analyses to predict that NR_027324, as a competitive endogenous RNA (ceRNA), binds to miR-103-3p and subsequently regulates the expression of downstream target gene ATG5, to secondly verify the mentioned regulatory relationship by molecular biological experiments, and thirdly to investigate that the above pathways transmit apoptosis and autophagy signals in cardiomyocytes during hypoglycemic and anoxic injuries.

Methods: The binding of NR_027324 to miR-103-3p was predicted by the bioinformatics analysis, and then the expression of downstream ATG5 was regulated. By the dual luciferase assay, the binding of NR_027324 to miR-103-3p was confirmed. H9c2 cells underwent the culture under low glucose and hypoxia to simulate cardiomyocytes under ischemia. NR_027324 siRNA and overexpression plasmid vector and miR-103-3p (mimics and inhibitors) were adopted to transfect cells to examine its function in cardiomyocytes. By the reverse transcription-quantitative polymerase chain reaction (RT-PCR), the expression levels of NR_027324, miR-103a-3p and ATG5 were assessed. Cell viability was measured by the MTT assay, and cell injury was analyzed by the lactate dehydrogenase assay (LDH). The western blotting assay was performed to detect the expressions of Bcl2, Bax, Atg5, cleaved-caspase3 and cleaved-caspase9. Moreover, the immunofluorescence assay was used to detect the expression of LC3-I/II as a marker of autophagy.

Results: In hypoglycemic and anoxic H9C2 cells, the expressions of NR_027324 and ATG5 were up-regulated, while miR-103-3p showed a down-regulated expression. By the dual luciferase assay, the binding of NR_027324 to miR-103-3p was confirmed. The overexpressed NR_027324 could enhance the reduced cell viability of hypoglycemic and anoxic H9C2 cells and reduce cell damage. Interfering with the expression of NR_027324 could further reduce the viability of hypoglycemic anoxic H9C2 cells, increase injury, inhibit protective autophagy and accelerate apoptosis of hypoglycemic anoxic H9C2 cells. The mentioned pathophysiological processes were achieved by NR_027324 binding to miR-103-3p and then regulating the expression of target gene ATG5.

Conclusion: In hypoglycemic and anoxic H9C2 cells, the expressions of NR_027324 and ATG5 are up-regulated, while the expression of miR-103-3p decreases. NR_027324 acts as ceRNA combined with miR-103-3p to regulate ATG5 and control autophagy and apoptosis in cardiomyocytes during hypoglycemic and anoxic injuries.
Myocardial infarction refers to a fatal disease. The existing treatment of myocardial infarction is primarily to save the dying cardiomyocytes in the border zone of myocardial infarction[1]. In the process of pathophysiological variations in the border zone of myocardial infarction, autophagy and apoptosis of cardiomyocytes are critical to disease progression and prognosis. The autophagy and apoptosis of cardiomyocytes show a close relationship to the regulation of gene expression[2]. Long non-coding RNA (lncRNA) belongs to a type of non-coding RNA exhibiting a transcription length > 200 nt without coding protein[3]. It significantly impacts the pathophysiological process of myocardial infarction[4–8]. Their regulatory functions are primarily determined by various epigenetic regulatory mechanisms (e.g., transcriptional regulation, post-transcriptional gene regulation, competing endogenous RNAs (CeRNA), protein post-translational gene transcription regulation, as well as nuclear compartmentalization)[9]. According to the research findings previously achieved by the authors [10], lncRNA has considerable differential expressions in the border zone of myocardial infarction in rats. Besides, by the bioinformatics analysis, lncRNA was found to play a potential role in various pathological mechanisms. To simulate myocardial ischemia in the border zone of myocardial infarction, H9C2 cells were administrated with hypoglycemia and hypoxia. Among the differentially expressed lncRNAs screened by lncRNA microarray, NR_027324 with a significant differential expression and an up-regulated expression in the border zone of rat myocardial infarction was taken as the research object. The aim was to explore the mechanism by which NR_027324 regulates the expression of miR-103-3p as CeRNA and then regulates the expression of downstream target ATG5 to control cardiomyocyte autophagy and apoptosis.

Materials And Methods

2.1 Cell culture and treatment

The H9C2 and 293T cells used in this study were provided by Wan Lei Biotechnology Co., Ltd. (Shenyang, China). In the control group, high glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM; Gibco; ThermoFisher Scientific, Inc., Waltham, MA, USA) was supplemented with 10% fetal bovine serum (FBS; EverGreen, Zhejiang Tianhang Biological Technology Co., Ltd. China) at 37°C and 5% CO2. To simulate ischemia and hypoxia, the cells underwent the culture in hypoglycemia (1.0g/L) DMEM (Gibco; ThermoFisher Scientific, Inc. USA), and then they were placed in a hypoxic incubation chamber with 1% O2, 94%N2 and 5% CO2 for 6 h.

2.2 Transfection

H9C2 cells were plated in 6-well plates (1x105 per well) and subsequently incubated at 37 ºC for 24 h under 5% CO2. By complying with the manufacturer's protocol, the cells underwent the transient transfection with a final 20 nM dose of overexpression plasmid of LncRNA-NR_027324 (Lncm), empty plasmid/overexpression plasmid control (EPC), LncRNA small interfering RNA (si-Lnc), LncRNA small interfering RNA control (si-Lnc-NC), miR-103-3p mimics (miRm) or miR-103-3p mimics control (miR-NC) with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After the cells were incubated for 24 h, the different cell groups were subsequently analyzed.
2.3 Dual luciferase reporter assay.

293T cells were plated in 12-well plates (2.5×10^5 per well) at 37 °C for 24 h with 5% CO2. NR_027324 was ligated into pmirGLO dual luciferase reporter vector (wt); the mutated NR_027324 was classified as the control(mut). Subsequently, miR-103-3p-mimic, miR-103-3p-mimic-NC and the mentioned recombinant plasmids were co-transfected into 293T cells with Lipofectamine2000 (Invitrogen; Thermo Fisher Scientific, Inc. USA). After the cells were incubated for 48 h, the luciferase activity was measured with the dual luciferase reporter gene assay kit (cat. No. KGAF040, Dual Luciferase Reporter Gene Assay Kit, KeyGEN BioTEH, China) following the manufacturer's protocol. Such measurement was performed three times for each per sample.

2.4 Reverse transcriptional quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted with TRIpure reagent (Cat.No.RP1001;BioTeke Corporation, Beijing, China). The concentration and purity of RNA were measured with Nanodrop2000 system (ThermoFisher Scientific, Inc.). In accordance with the manufacturer's protocol, complementary DNAs (cDNAs) were synthesized with Super M-MLV reverse transcriptase (Cat.No.PR6502; BioTeke Corporation, Beijing, China). Complying with the manufacturer's protocol, SYBRGREEN mastermix (Cat.No.SY1020; Solarbio technology co.LTD, Beijing, China) was adopted to amplify the cDNA samples. Besides, the fluorescence quantitative analysis was conducted with the ExicyclerTM96 fluorescence quantitative instrument produced by BIONEER company in Korea. In brief, the PCR reaction conditions of mRNA and LncRNA included 94 °C for 5 min, followed by 40 cycles of 94 °C for 10 sec and 60 °C for 20 sec. The PCR reaction conditions of miRNA included 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 sec and 60 °C for 15 sec. Moreover, ddH_2O was used as a non-template control for each plate. 5S was used to normalize the expression levels of miR-103-3p. β-actin was employed for normalizing the expression levels of NR_027324 and Atg5. The relative expression was quantified by 2^-△△CT method, which was repeated three times. The median of the three results was obtained to calculate the relative expression level. The sequence list of PCR reaction primers is presented in Table 1.

2.5 Western blot analysis

The total protein was lysed with a whole protein extraction kit (Cat.No.WLA019; wanleibio Co., Ltd., Shenyang, China). The BCA protein concentration determination kit (Cat.No. WLA004; wanleibio Co., Ltd.) was employed to measure the concentration and purity of the protein. 40 μg of protein samples per lane were separated on SDS-PAGE gels (10%) and subsequently transferred to polyvinylidene difluoride membranes (EMDMillipore, Bedford, MA, USA). At ambient temperature, the membrane was blocked in 5% skim milk for 2 h; subsequently, it underwent the incubation with the primary antibodies (Atg5, Cat.No. WL02411, 1:500; Bax, Cat.No. WL01637, 1:400; Bcl-2, Cat.No. WL01556,1:1000;cleavedcaspase-3, Cat.No. WL02117,1:500;cleavedcaspase-9, Cat.No. WL03421,1:500;βactin, Cat.No. WL01845,1:1000; Wanleibio Co., Ltd., Shenyang, China) overnight at 4°C. Next, the membrane was washed 3 times with TBST and incubated with horseradish peroxidase-labeled secondary antibody (Cat.No. WLA023, 1:5,000; wanleibio
Co., Ltd., Shenyang, China) at 37°C for 45 min; afterwards, it was further washed 6 times with TBST.

Besides, the ECL chemiluminescence kit (Cat.No. WLA003; wanleibio Co., Ltd., Shenyang, China) was employed to detect the blot, and the gel image processing system (Gel-Pro-Analyzer software) was adopted to analyze the optical density of the target band value.

2.6 Immunofluorescence

1-2 drops of cell suspension were dropped on the glass slides that were washed in advance, sterilized by pure alcohol and sterilized under high pressures, and the cell adhesion was observed the next day. After the cells were adhered to the wall and exhibited the required density, they were washed with 1x phosphate buffer (PBS) for 5min x 3 times and then fixed in 4% paraformaldehyde for 15 min. Next, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. The samples were blocked with goat serum at ambient temperature for 15 min and subsequently incubated with primary antibody (LC3-I/II) overnight at 4 °C. On the following day, the samples were incubated with secondary antibodies for 1 h at ambient temperature in the dark. Lastly, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich; Merck KGaA) at ambient temperature for 5 min. After each step, the slides were washed with PBS for 5min x 3 times. The anti-fluorescence quenching agent was dropped on the glass slide, and the slide upside down on the glass slide dripped with anti-fluorescence quenching agent was blocked. Olympus BX53 fluorescence microscope (Olympus corporation, Tokyo, Japan) was used to capture images.

2.7 Lactate dehydrogenase (LDH) assay

H9C2 cells were cultured with 10% fetal calf serum and were inoculated with 24-well plates (1x10^5 per well). After the cells were transfected, the LDH leakage assay was performed to determine cell injury with the LDH cytotoxicity assay kit (Cat.No. WLA072; wanleibio Co.,Ltd., Shenyang, China) by complying with the manufacturer's protocol. The absorbance was measured at 490nm enzyme-linked immunosorbent assay (ELX-800, BIOTEK, USA), which was repeated three times in each group.

2.8 MTT assay

The H9C2 cells were plated in 96-well plates (5×10^3 per well). In each group, 5 repeats were set, and a blank control was set with the culture medium only. After the cells were adhered to the wall, the cells received the transfection and culture at 37 °C for 24 h. The cells were administrated with hypoglycemia and hypoxia according to different groups. After reaching the time point, the culture medium was removed; 20 μl of MTT reagent was added to each well and then incubated at 37 °C and 5%CO2 for 4 h; the supernatant was rigorously discarded; afterwards, 150 μl dimethyl sulfoxide (DMSO) was added and maintained in the dark for 10 min. The absorbance of each well was measured with a microplate reader at 570nm, and the average value of 5 wells was determined.

2.9 Statistical analysis
Data were analyzed with SPSS version 16.0 (IBM corp., Armonk., NY, USA). All data are expressed as the mean ± standard deviation. Significant differences were identified by Student's t-test or one-way analysis of variance followed by Tukey's test. P<0.05 was considered to exhibit a statistically significant difference.

Results

Variations of NR_027324, miR-103-3p and ATG5 in hypoglycemic and hypoxic H9C2 cells

To simulate the state of ischemia and hypoxia in the border zone of myocardial infarction, H9C2 cells were cultured under high glucose (4.5g/L) and normoxic conditions for 24 h; then, they were cultured in low glucose (1g/L glucose) DMEM and in an hypoxic incubator at 37°C, 1% O₂, 94% N₂, and 5% CO₂ for 6 h. High glucose (4.5g/L) and normoxic conditions were classified as controls. As revealed from the results, the cell viability was reduced, and LDH increased in hypoglycemia and hypoxia group. The expression of NR_027324 was significantly up-regulated, complying with the results of microarray. The expression of miR-103-3p decreased, while the expression of ATG5 was up-regulated, complying with the results of Zhang et al[11]. Fig. 1.

NR_027324 binds to miR-103-3p and regulates the expression of miR-103-3p

As indicated from the dual luciferase reporter gene analysis, pmirGLO-NR_027324wt 3’UTR+miR-103-3p-mimic exhibited a significantly lower luciferase activity than pmirGLO-NR_027324wt 3’UTR+miR-103-3p-NC, indicating that the binding of pmirGLO-NR_027324 3’UTR plasmid to miR-103-3p can inhibit dual luciferase activity. In the experimental group with site-directed mutation of pmirGLO-NR_027324mut3’UTR plasmid + miR-103-3p-mimic, no significant difference was identified in luciferase activity between pmirGLO-NR_027324mut 3’UTR+miR-103-3p-NC group and pmirGLONR_027324wt 3’UTR+miR-103-3p-NC group. As revealed from the results, the pmirGLO-NR_027324mut 3’UTR plasmid could not bind to miR-103-3p after site-directed mutation, i.e., the binding sites of NR_027324 3’UTR and miR-103-3p have been fully mutated, which cannot affect the variation of dual luciferase activity. As verified by subsequent experiments, the expression of NR_027324 was respectively up-regulated and down-regulated by plasmid transfection technology; however, it was found that miR-103-3p varied in an inconsistent manner. Fig. 2.

Effect of overexpression of NR_027324 on viability of hypoglycemic and hypoxic H9C2 cells

Fig. 3 shows that H9C2 cells were transfected with NR_027324 overexpression plasmid for 24 h and then cultured in hypoglycemia and hypoxia for 6 h. Compared with the control group, the expression of NR_027324 in the hypoglycemia and hypoxia group was up-regulated; compared with the hypoglycemia and hypoxia + transfection empty plasmid group, the NR_027324 in the hypoglycemia and hypoxia + NR_027324 overexpression group was further up-regulated, while the expression of miR-103-3p decreased. Compared with the control group, the relative viability of cells in the hypoglycemia and hypoxia group was reduced significantly. Compared with the hypoglycemia and hypoxia + transfection empty plasmid group, the cell viability in the hypoglycemia and hypoxia + NR_027324 overexpression
group increased compared with the control group, while the LDH exhibited the corresponding variations. (P < 0.001)

**Effect of interfering with NR_027324 expression on the viability of hypoglycemic and hypoxic H9C2 cells**

As suggested in the mentioned experiment, compared with the control group, the viability of H9C2 cells was reduced, and LDH increased after cultured for 6 h in hypoglycemia and hypoxia group. 24 h after transfection of si-NR_027324, the cells were cultured for 6 h in hypoglycemia and hypoxia group. Compared with the hypoglycemia and hypoxia + si-NR_027324-NC group, the expression of NR_027324 was down-regulated, while that of miR-103-3p increased and that of ATG5 decreased in the hypoglycemia and hypoxia + si-NR_027324 group. After interfering with the expression of NR_027324, the relative viability of (MTT) was further reduced, and LDH showed a further increase in the hypoglycemia and hypoxia group. (P < 0.001) Fig. 4.

**Interference with NR_027324 expression regulates autophagy and apoptosis in hypoglycemic and hypoxic H9C2 cells**

As presented in Fig. 5, compared with the control group, Bax, Cleaved-caspase3, Cleaved-caspase9 (i.e., apoptosis-promoted proteins) increased in hypoglycemia and hypoxia group, while the expression of apoptosis inhibitor protein Bcl2 was down-regulated, suggesting that the apoptosis was activated. After hypoglycemia and hypoxia, compared with si-NR_027324-NC, the expression of ATG5 was down-regulated after interfering with the expression of NR_027324, while those of Bax, Cleaved-caspase3 and Cleaved-caspase9 showed further up-regulations (P < 0.001), and that of Bcl2 further decreased (P < 0.001). It was therefore suggested that interfering with the expression of NR_027324 can down-regulate the expression of Atg5, inhibit cell protective autophagy and promote apoptosis. No significant change was identified in the mentioned apoptosis-promoted genes between Gdh group and si-Lnc-NC+Gdh group (P > 0.05). As revealed from the results of LC3-I/II immunofluorescence, compared with the control group, LC3-I/II protein in the hypoglycemia and hypoxia group (Gdh group) exhibited an increased expression, while in the Gdh+ si-NR_027324 group, the expression of LC3-I/II was down-regulated. (Fig. 6)

**NR_027324 regulates ATG5 expression by binding mir-103-3p as CeRNA**

As shown in Fig. 7, compared with the control group, the expression of miR-103-3p decreased, and that of ATG5 was up-regulated after transfection with overexpression of NR_027324 (P < 0.001). After NR_027324 showed an up-regulated expression, compared with miR-103-3p-mimic-NC, transfection with miR-103-3p-mimic led to the increased expression of miR-103-3p (P < 0.001), while the expression level of ATG5 decreased (P < 0.001). No significant variation was identified in the above expression between Con group and Epc group, and between Lncm group and Lncm+miR-NC group. P>0.05

**Discussion**
In the previous research of the authors [10], the lncRNA expression profile was analyzed in the border zone of myocardial infarction. It was reported that lncRNAs were differentially expressed in the border zone of myocardial infarction. Unlike the results of other existing studies on myocardial infarction area[5, 7], the results achieved by the authors revealed that the total number of differentially expressed lncRNAs in the border zone was more than that of the myocardial infarction area, demonstrating more occurrences of active and more complex biological processes. For instance, LncRNA: AY212271, showing a co-expression relationship to Alox5ap, might be involved in various inflammatory reactions[12–16].

EF424788 and MRAK088538 were co-expressed with Itgb2, and Itgb2 was reported to be a risk factor for myocardial infarction and atherosclerotic thrombotic cerebral infarction[17–19]. Moreover, Itgb2 was involved in the reduction of the risks of myocardial infarction by statins[20]. BC166504 was co-expressed with 4 mRNAs to participate in myocardial infarction, which included B4galt1, Eln, Il1b and Nfkbiz. B4galt1 and Il1b significantly impacted inflammation[21]. Eln (elastin), expressed as tropoelastin in smooth muscle, was associated with the remodeling of the extracellular matrix (ECM) of the vessel wall, which is considered a vital step in atherosclerosis and likely to predict potential cardiovascular events[22]. Nfkbiz refers to a nuclear inhibitor of NF-κB protein(IκB), which may reduce the susceptibility of myocardial infarction by reducing NFκB, i.e., a critical factor of potentially activated inflammation[23].

Both GO analysis and Pathway analysis suggested that LncRNA is involved in the apoptosis signaling pathway.

LncRNA could significantly regulate the expression of miRNAs by acting as competing endogenous RNAs, and then it could regulate the expression of target genes at the level of post-transcriptional translation[5, 24, 25]. MiRNAs refers to a type of small RNA that has been newly discovered in recent years, and some miRNAs are involved in the pathophysiological process of cardiovascular disease[26–28]; MiRNAs may also be a novel target for treatment. MiRNAs exhibits the function of regulating gene expression at the post transcriptional translation level. Mature miRNAs are not fully complementary to the target mRNA, thereby inhibiting the expression at the protein translation level, which may affect the stability of mRNA and cause the degradation of target mRNA. Existing studies reported that various miRNAs regulate apoptosis in cardiomyocytes. For instance, miR-25 prevented oxidative damage of cardiomyocytes by inhibiting mitochondrial apoptosis[29]. MiR-21 was significantly up-regulated during heart failure and inhibited cardiomyocyte apoptosis by suppressing PDCD4[30, 31]. MiR-1 could promote cardiomyocyte apoptosis after myocardial infarction, while miR-133 could select caspase-9 as the target gene to inhibit cardiomyocyte apoptosis after myocardial infarction [32]. MiRNA acts as the target of intervention therapy, and there may be too many pathways regulated by the identical miRNA simultaneously, causing too much influence. However, the conservatism of LncRNA is poor, and the impact is significantly smaller. Thus, as a target of gene therapy, LncRNA may be more suitable.

Apoptosis and autophagy participate in the overall pathophysiology in the border zone of myocardial infarction. Abnormal expression of many apoptosis-related genes could promote or inhibit cardiomyocyte apoptosis and autophagy (e.g., ATG5, Bax, Bcl-2, Caspase-3 and Caspase-9) [33].

As indicated from the reannotation analysis, NR_027324 was a segment of H9 gene, and mir-103-3p was evaluated as its binding target. MiR-103-3p could down regulate the protein expression of Beclin1, ATG5,
and inhibit autophagy[11]. The authors inferred that NR_027324, a section of the H9 gene, may target miR-103-3p via CeRNA binding, adjust its expression, and subsequently regulate the downstream target gene ATG5 of miR-103-3p. Based on the dual luciferase reporter system, the inference of targeted binding of NR_027324 to miR-103-3p was directly confirmed. Through the overexpression of NR_027324 and by interfering with the expression of NR_027324 in hypoglycemic and hypoxic H9C2 cells, it was verified that NR_027324 could regulate the viability, apoptosis and autophagy.

Existing studies suggested that after the primary rat cardiomyocytes was administrated with OGD (oxygen-glucose deprivation) for 4 h, the cell survival rate decreased, LDH release was improved, and autophagy was activated[34]. The same method was adopted in this study to simulate ischemia and hypoxia in the border zone of myocardial infarction in rats, and the role of NR_027324 in the zone was studied. According to some existing studies, autophagy of H9C2 cells was activated at 4 h and inhibited from 8 h to 12 h after being administrated with OGD. Administration of rapamycin, an autophagy activator, could inhibit the activation of NFκB [35]. Some studies reported that NR_027324 can protect the hypoxic injury of cardiomyocytes[36, 37]. Through the results of this study, it was inferred that NR_027324 promotes autophagy. Autophagy increases after the overexpression and decreases after interfering with the expression. After the intervention of hypoxia and hypoglycemia for 6 h, the expression of Atg5 was up-regulated, and the stress level of autophagy rose, which might be the self-protective mechanism of cardiomyocytes. After the overexpression of NR_027324, autophagy was further activated, which could protect the cells. The cell survival rate increased, and the release of LDH decreased. After transfection with interfering NR_027324, the expression of Atg5 was down-regulated, protective autophagy was inhibited, apoptosis was promoted, and the expressions of Bax and cleaved-caspase3 were up-regulated; thus, the cell survival rate decreased, and the release of LDH increased.

In brief, this study reported that NR_027324 binds to miR-1033p by acting as CeRNA, and then regulates the downstream target gene ATG5 to control cardiomyocyte apoptosis and autophagy in rats H9C2 cells with hypoglycemia and hypoxia. In hypoglycemic and hypoxic rat cardiomyocytes, LncRNA is likely to act as a possible therapeutic target to promote cardiomyocyte autophagy, reduce cardiomyocyte apoptosis and save ischemic and hypoxic cardiomyocyte. It cannot be ignored that with the time evolution of myocardial infarction, autophagy and apoptosis also have dynamic changes, and the role of LncRNA may also show dynamic variations, which should be confirmed by further research. The microarray analysis suggested that more LncRNAs may play a part by regulating inflammatory response, abnormal contraction and reducing scarring and other different pathways. More research and evidence are required to delve into the functions of other LncRNAs.

**Conclusions**

In hypoglycemic and anoxic H9C2 cells, the expressions of NR_027324 and ATG5 are up-regulated, while the expression of miR-103-3p decreases. NR_027324 acts as ceRNA combined with miR-103-3p to regulate ATG5 and control autophagy and apoptosis in cardiomyocytes during hypoglycemic and anoxic injuries.
### Abbreviations

| LncRNAs          | long non-coding RNAs                                      |
|------------------|-----------------------------------------------------------|
| miRNAs           | microRNAs                                                 |
| BZ               | border zone                                               |
| MI               | myocardial infarction                                     |
| CeRNA            | competitive endogenous RNA                               |
| RT-PCR           | reverse transcription-quantitative polymerase chain reaction |
| LDH              | lactate dehydrogenase assay                              |
| Lncm             | overexpression plasmid of LncRNA-NR_027324                |
| EPC              | empty plasmid/overexpression plasmid control              |
| si-Lnc           | LncRNA small interfering RNA                              |
| si-Lnc-NC        | LncRNA small interfering RNA control                      |
| miRm             | miR-103-3p mimics                                         |
| miR-NC           | miR-103-3p mimics control                                 |
| OGD              | oxygen-glucose deprivation                               |
| ECM              | extracellular matrix                                      |
| Eln              | elastin                                                   |
| Nfkbiz           | nuclear factor-kappa B inhibitor zeta                     |
| IκB              | nuclear inhibitor of NF-κB                                |

### Declarations

**Ethics approval and consent to participate:** This investigation was performed according to the protocols approved by the Medical Research and New Technology Ethical Committee of the Shengjing Hospital of China Medical University (approval no. 2015PS295K).

**Consent for publication:** Written informed consent for publication was obtained from all participants.

**Availability of data and material:** We declare that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

**Competing interests:** Not applicable
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Author contribution statement: Qingkun Meng and Zhijun Sun designed experiments; Qingkun Meng, Hao Tan and Hui Gu carried out experiments; Qingkun Meng and Hao Tan analyzed experimental results. Jiaying Luo and Jingjing Wang took the bioinformatics analysis. Qingkun Meng, Chuanhe Wang and Su Han wrote the manuscript.

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References

1. Feiring AJ, Johnson MR, Kioschos JM, Kirchner PT, Marcus ML, White CW: The importance of the determination of the myocardial area at risk in the evaluation of the outcome of acute myocardial infarction in patients. Circulation 1987, 75:980-987.

2. Dabek J, Owczarek A, Gasior Z, Ulczok R, Skowerski M, Kułach A, Mazurek U, Bochenek A: Oligonucleotide microarray analysis of genes regulating apoptosis in chronically ischemic and postinfarction myocardium. Biochem Genet 2008, 46:241-247.

3. Batista PJ, Chang HY: Long noncoding RNAs: cellular address codes in development and disease. Cell 2013, 152:1298-1307.

4. Zangrando J, Zhang L, Vausort M, Maskali F, Marie PY, Wagner DR, Devaux Y: Identification of candidate long non-coding RNAs in response to myocardial infarction. BMC Genomics 2014, 15:460.

5. Ounzain S, Micheletti R, Beckmann T, Schroen B, Alexanian M, Pezzuto I, Crippa S, Nemir M, Sarre A, Johnson R, et al: Genome-wide profiling of the cardiac transcriptome after myocardial infarction identifies novel heart-specific long non-coding RNAs. Eur Heart J 2015, 36:353-368a.

6. Ishii N, Ozaki K, Sato H, Mizuno H, Susumu S, Takahashi A, Miyamoto Y, Ikekawa S, Kamatani N, Hori M, et al: Identification of a novel non-coding RNA, MIAT, that confers risk of myocardial infarction. J Hum Genet 2006, 51:1087-1099.

7. Liu Y, Li G, Lu H, Li W, Li X, Liu H, Li T, Yu B: Expression profiling and ontology analysis of long noncoding RNAs in post-ischemic heart and their implied roles in ischemia/reperfusion injury. Gene 2014, 543:15-21.

8. Wang P, Fu H, Cui J, Chen X: Differential IncRNA-mRNA co-expression network analysis revealing the potential regulatory roles of IncRNAs in myocardial infarction. Mol Med Rep 2016, 13:1195-1203.

9. Rinn JL, Chang HY: Genome regulation by long noncoding RNAs. Annu Rev Biochem 2012, 81:145-166.

10. Meng Q, Sun Z, Gu H, Luo J, Wang J, Wang C, Han S: Expression profiles of long noncoding RNAs and messenger RNAs in the border zone of myocardial infarction in rats. Cell Mol Biol Lett 2019, 24:63.

11. Zhang C, Zhang C, Wang H, Qi Y, Kan Y, Ge Z: Effects of miR-103a-3p on the autophagy and apoptosis of cardiomyocytes by regulating Atg5. Int J Mol Med 2019, 43:1951-1960.
12. Helgadottir A, Manolescu A, Thorleifsson G, Gretarsdottir S, Jonsdottir H, Thorsteinsdottir U, Samani NJ, Gudmundsson G, Grant SF, Thorgerisson G, et al: The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. Nat Genet 2004, 36:233-239.

13. Kajimoto K, Shioji K, Ishida C, Iwanaga Y, Kokubo Y, Tomoike H, Miyazaki S, Nonogi H, Goto Y, lwai N: Validation of the association between the gene encoding 5-lipoxygenase-activating protein and myocardial infarction in a Japanese population. Circ J 2005, 69:1029-1034.

14. Linsel-Nitschke P, Götz A, Medack A, König IR, Bruse P, Lieb W, Mayer B, Stark K, Hengstenberg C, Fischer M, et al: Genetic variation in the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) is associated with myocardial infarction in the German population. Clin Sci (Lond) 2008, 115:309-315.

15. Zhang SY, Xu ML, Zhang CE, Qu ZY, Zhang BB, Zheng ZY, Zhang LM: Association of ALOX5AP gene single nucleotide polymorphisms and cerebral infarction in the Han population of northern China. BMC Med Genet 2012, 13:61.

16. Zintzaras E, Rodopoulou P, Sakellaridis N: Variants of the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene and risk of stroke: a HuGE gene-disease association review and meta-analysis. Am J Epidemiol 2009, 169:523-532.

17. May AE, Schmidt R, Kanse SM, Chavakis T, Stephens RW, Schöming A, Preissner KT, Neumann FJ: Urokinase receptor surface expression regulates monocyte adhesion in acute myocardial infarction. Blood 2002, 100:3611-3617.

18. Lehmkühl H, Horn C, von der Driesch P, Kämmerer U, Müller T, von der Emde J, Olbrich HG, Kunkel B, Bachmann K: Analysis of adhesion molecules in myocardial biopsies of cardiac allografts and coronary artery disease with CABG. J Cardiovasc Surg (Torino) 1996, 37:65-70.

19. Yamaguchi S, Yamada Y, Metoki N, Yoshida H, Satoh K, Ichihara S, Kato K, Kameyama T, Yokoi K, Matsuo H, et al: Genetic risk for atherothrombotic cerebral infarction in individuals stratified by sex or conventional risk factors for atherosclerosis. Int J Mol Med 2006, 18:871-883.

20. Peters BJ, Rodin AS, Klungel OH, Stricker BH, de Boer A, Maitland-van der Zee AH: Variants of ADAMTS1 modify the effectiveness of statins in reducing the risk of myocardial infarction. Pharmacogenet Genomics 2010, 20:766-774.

21. Qian J, Cheng C, Liu H, Chen J, Yan M, Niu S, Qin J, Sun L, Liu L, Gu J, Shen A: Expression of beta-1,4-galactosyltransferase-I in rat during inflammation. Inflammation 2007, 30:59-68.

22. Kong CH, Lin XY, Woo CC, Wong HC, Lee CN, Richards AM, Sorokin VA: Characteristics of aortic wall extracellular matrix in patients with acute myocardial infarction: tissue microarray detection of collagen I, collagen III and elastin levels. Interact Cardiovasc Thorac Surg 2013, 16:11-15.

23. Boccardi V, Rizzo MR, Marfella R, Papa M, Esposito A, Portoghese M, Paolissio G, Barbieri M: -94 ins/del ATTG NFKB1 gene variant is associated with lower susceptibility to myocardial infarction. Nutr Metab Cardiovasc Dis 2011, 21:679-684.

24. Kung JT, Colognori D, Lee JT: Long noncoding RNAs: past, present, and future. Genetics 2013, 193:651-669.
25. Schonrock N, Harvey RP, Mattick JS: Long noncoding RNAs in cardiac development and pathophysiology. *Circ Res* 2012, 111:1349-1362.

26. van Rooij E: Introduction to the series on microRNAs in the cardiovascular system. *Circ Res* 2012, 110:481-482.

27. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Dovendans PA, Mummery CL, Borlak J, Haverich A, et al: MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* 2007, 116:258-267.

28. Bostjancic E, Zidar N, Glavac D: MicroRNA microarray expression profiling in human myocardial infarction. *Dis Markers* 2009, 27:255-268.

29. Pan L, Huang BJ, Ma XE, Wang SY, Feng J, Lv F, Liu Y, Liu Y, Li CM, Liang DD, et al: MiR-25 protects cardiomyocytes against oxidative damage by targeting the mitochondrial calcium uniporter. *Int J Mol Sci* 2015, 16:5420-5433.

30. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, et al: MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature* 2008, 456:980-984.

31. Cheng Y, Liu X, Zhang S, Lin Y, Yang J, Zhang C: MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4. *J Mol Cell Cardiol* 2009, 47:5-14.

32. Katz MG, Fargnoli AS, Williams RD, Kendle AP, Steuerwald NM, Bridges CR: MiRNAs as potential molecular targets in heart failure. *Future Cardiol* 2014, 10:789-800.

33. Levine TB, Levine AB, Bolenbaugh J, Green PR: Reversal of heart failure remodeling: is it maintained? *Clin Cardiol* 2003, 26:419-423.

34. Miao H, Qiu F, Huang B, Liu X, Zhang H, Liu Z, Yuan Y, Zhao Q, Zhang H, Dong H, Zhang Z: PKCα replaces AMPK to regulate mitophagy: Another PEDF role on ischaemic cardioprotection. *Journal of Cellular and Molecular Medicine* 2018, 22:5732-5742.

35. Wu X, He L, Chen F, He X, Cai Y, Zhang G, Yi Q, He M, Luo J: Impaired autophagy contributes to adverse cardiac remodeling in acute myocardial infarction. *PLoS One* 2014, 9:e112891.

36. Yuan L, Yu L, Zhang J, Zhou Z, Li C, Zhou B, Hu X, Xu G, Tang Y: Long noncoding RNA H19 protects H9c2 cells against hypoxia-induced injury by activating the PI3K/AKT and ERK/p38 pathways. *Mol Med Rep* 2020, 21:1709-1716.

37. Gong LC, Xu HM, Guo GL, Zhang T, Shi JW, Chang C: Long Non-Coding RNA H19 Protects H9c2 Cells against Hypoxia-Induced Injury by Targeting MicroRNA-139. *Cell Physiol Biochem* 2017, 44:857-869.

**Tables**

Table 1, sequence of primers for PCR
| Target gene       | Sequence                                      |
|------------------|----------------------------------------------|
| 5S               | Forward 5'-GATCTCGGAAGCTAAGCAGG-3'            |
|                  | Reverse 5'-TGGTGCAGGGTCCGAGGTAT-3'           |
| β-actin          | Forward 5'-GGAGATTACTGCCCTGGCTCCTAGC-3'      |
|                  | Reverse 5'-GGCCGGACTCATCGTACTCCTGCTT-3'      |
| rno-LncRNA-NR_027324 | Forward 5'-GCACTGTATGCCCTAACC-3'            |
|                  | Reverse 5'-ACCCACCTCCCTCCCTA-3'             |
| rno-miR-103-3p   | Forward 5'-GCAGCATTGTACAGGGCT-3'             |
|                  | Reverse 5'-GCAGGGTCGGAGGTATT-3'             |
| ATG5             | Forward 5'-CCGTGCAAGGATGCAGTTGA-3'           |
|                  | Reverse 5'-TGAGTTTCCGGTTGATGGTC-3'           |

Figures
Figure 1

Expression of NR_027324, miR-103-3p, ATG5, cell viability and LDH in hypoglycemic and anoxic H9C2 cells. A, The relative expression of NR_027324 was analyzed by RT-PCR. B, The relative expression of miR-103-3p was studied by RT-PCR. C, The relative expression of ATG5 was analyzed by RT-PCR. D, The cell viability was detected by MTT assay (%). E, LDH (U/L) was detected to analyze the cell damage. Con. Normal control group, hyperglycemia and normoxia; Gdh. Low glucose and hypoxia group. ATG5, autophagy-related 5. LDH, lactate dehydrogenase. n=3, *P<0.05, **P<0.01, ***P<0.001.
Figure 2

Luciferase reporter assays were used to evaluate the interaction between NR_027324 and miR-103-3p. A, Quantitative analysis results: pmiR-GLO-NR_027324wt, cells co-transfected with dual luciferase reporter wild type vector and miR-103-3p mimic or miR-103-3p mimic-NC; pmiR-GLO-NR_027324mut, cells co-transfected with dual luciferase reporter target mutant vector and miR-103-3p mimic or miR-103-3p mimic-NC. B, The relative expression levels of NR_027324 were determined to determine the transfection efficiency of overexpressed plasmid. C, The relative expression levels of miR-103-3p after transfection of overexpressed plasmid. D, The relative expression levels of NR_027324 was obtained to calculate the transfection efficiency of si-NR_027324. E, The relative expression of miR-103-3p after transfection of siNR_027324. F, The binding site of NR_027324wt to miR-103-3p and the mutation site of NR_027324mut binding site. Con, normal control group; Epc, empty plasmid group; Lncm, LncRNA overexpression plasmid group; si-Lnc-NC, LncRNA interference control group; si-Lnc, LncRNA interference group. (n=3, *P<0.05, **P<0.01, ***P<0.001)
Figure 3

Overexpression of NR_027324 in hypoglycemic and anoxic H9C2 cells affected the expression of miR-103-3p and ATG5, cell viability and LDH. A, The transfection efficiency of LncRNA-NR_027324 overexpression plasmid was analyzed by the reverse transcription-quantitative polymerase chain reaction analysis. B, The relative expression of miR-103-3p was analyzed by the reverse transcription-quantitative polymerase chain reaction analysis. C, MTT assays for the detection of cell viability. D, Cell injury was determined by the leakage of LDH(U/L). Con, normal control group, high glucose normoxic group; Gdh, hypoglycemic hypoxia group; Epc, empty plasmid group/LncRNA overexpression control group; Lncm,LncRNA overexpression group. (n=5, *P<0.05; **P<0.01; ***P<0.001)
Figure 4

Interfering with the expression of NR_027324 in hypoglycemic and anoxic H9C2 cells affects the expression of miR-103-3p and ATG5, variations in cell viability and LDH. A, The transfection efficiency of si-LncRNA was analyzed by the reverse transcription-quantitative polymerase chain reaction analysis. B, The relative expression of miR-103-3p was analyzed by the reverse transcription-quantitative polymerase chain reaction analysis. C, The relative expression of ATG5 was analyzed by the reverse transcription-quantitative polymerase chain reaction analysis. D, MTT assays to measure cell viability. E, Cell injury was determined via the leakage of LDH(U/L). Con, normal control group; high glucose normoxic group; Gdh, hypoglycemic hypoxia group; si-Lnc-NC, LncRNA interference control group; si-Lnc, LncRNA interference group. (n=5, *P<0.05; **P<0.01; ***P<0.001)
Figure 5

Expression of apoptosis-related proteins and autophagy-related proteins in H9C2 cells under hypoglycemia and hypoxia, and transfected with si-NR_027324 or si-NR_027324-control. A-E, Quantitative evaluation of protein expression levels of ATG5, Bax, Bcl2, Cleaved-caspase3 and Cleaved-caspase9 proteins was quantitatively studied. F, Protein expression levels of ATG5, Bax, Bcl2, Cleaved-caspase3 and Cleaved-caspase9 were determined by the western blotting assay. Con, normal control group, high glucose normoxic group; Gdh, hypoglycemic hypoxia group; si-LncNC, LncRNA interference control group; si-Lnc, LncRNA interference group. n=3, *P<0.05, **P<0.01, ***P<0.001
Induction of autophagy was associated with the expression of LncRNA-NR_027324. The immunofluorescence of LC3-I/II was detected in four groups of H9C2 cells. Con, normal control group, high glucose normoxic group; Gdh, hypoglycemic hypoxia group, si-Lnc-NC+Gdh, transfected with siLncRNA control in H9C2 cells under hypoglycemia and hypoxia; si-Lnc+Gdh, transfected with si-LncRNA in H9C2 cells under hypoglycemia and hypoxia. Scale bar: 50μm.
Figure 7

After transfection with NR_027324 overexpression plasmid, the expression of miR-103-3p decreased, and that of ATG5 was up-regulated. After transfection with miR-103-3p-mimics, the expression of ATG5 was down-regulated. A, The relative expression of NR_027324 was analyzed by RT-PCR; B, the relative expression of miR-103-3p was analyzed by RT-PCR; C, The relative expression of mRNA of ATG5 was analyzed by RT-PCR; Con, normal control group; Epc, empty plasmid group/LncRNA overexpression control group; Lncm, LncRNA overexpression group; miR-NC, miRNA-mimics control group; miRm, miRNA-mimics group. n=3, *P<0.05, **P<0.01, ***P<0.001, Con vs. Lncm; #P<0.05, ##P<0.01, ###P<0.001, Lncm+miR-NC vs. Lncm+miRm.