Journal of Inorganic Biochemistry 193 (2019) 60–69

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Mir-215-5p induces autophagy by targeting PI3K and activating ROS-mediated MAPK pathways in cardiomyocytes of chicken☆

Jingzeng Cai[a], Jie Yang[a], Qi Liu[a], Yafan Gong[a], Yuan Zhang[a], Yingying Zheng[a], Dahai Yu[a], Ziwei Zhang[a,b,*]

[a] College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, PR China
[b] Key Laboratory of the Provincial Education, Department of Heilongjiang for Common Animal Disease Prevention and Treatment, PR China

ARTICLE INFO

Keywords:
Autophagy
miR-215-5p
PI3K
MAPK
Cardiomyocytes

ABSTRACT

Our previous study revealed that selenium (Se) deficiency can cause myocardial injury through triggering autophagy. MicroRNAs (miRNAs) play crucial roles in autophagic cell death. However, the relationship between miRNAs and myocardial autophagy injury caused by Se deficiency remains unclear. We selected differential microRNA-215-5p (miR-215-5p) in Se-deficient myocardial tissue using high-throughput miRNA-sequencing. To further explore the role of miR-215-5p in myocardial injury, overexpression/knockdown of miR-215-5p in primary cardiomyocyte model was established by miRNAs interference technology. In this study, we report that miR-215-5p can promote myocardial autophagy by directly binding to the 3′untranslated region (3′UTR) of phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K). Its target gene PI3K was confirmed by dual luciferase reporter assay, quantitative real-time polymerase chain reaction (qRT-PCR) and western blot in cardiomyocytes. Our results showed that overexpression of miR-215-5p could trigger myocardial autophagy through PI3K-threonine-protein kinase (AKT)-target of rapamycin (TOR) pathway. Further studies revealed that autophagic cell death was dependent on the activation of extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 kinase (p38) and generation of reactive oxygen species (ROS) in overexpression of miR-215-5p in cardiomyocytes. On the contrary, miR-215-5p inhibitor can enhance cell survival capacity against autophagy by inhibiting ROS-mitogen-activated protein kinase (MAPK) pathways and activating the PI3K/AKT/TOR pathway in cardiomyocytes. Together, our findings support that miR-215-5p may modulate cell survival programs by regulating autophagy, and miR-215-5p acts as an autophagic regulator in the regulatory feedback loop that regulates cardiomyocyte survival by modulating the PI3K/AKT/TOR pathway and ROS-dependent MAPK pathways.

1. Introduction

Selenium (Se), a trace element with antioxidant properties, plays a vital role in maintaining the normal biochemical and physiological functions of many species [1,2]. During the last decades advances in Se research and applications have proved that Se deficiency can induce a series of diseases and injuries in the heart, including myocardial injury [3], Keshan disease [4], mulberry heart of swine [5] and chronic heart failure in rats [6]. Our previous study proved that Se deficiency may cause myocardial injury and autophagy based on establishing successfully Se deficient myocardial model in chickens [7]. In the absence of Se, excessive reactive oxygen species (ROS) could cause oxidative

Abbreviations: miR-215-5p, microRNA-215-5p; PI3K, phosphatidylinositol3-kinase; 3′UTR, 3′untranslated region; AKT, threonine-protein kinase; qRT-PCR, quantitative real-time polymerase chain reaction; P-AKT, phosphorylation-threonine-protein kinase; TOR, target of rapamycin; ATG4C, autophagy related 4C; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; Se, Selenium; ZEB2, zinc finger E-box binding homeobox 2; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; JNK, c-Jun N-terminal kinase; p38, p38 kinase; AGEs, advanced glycation end products; ERK1/2, extracellular signal-regulated kinase1/2; miRNAs, MicroRNAs; RPISeq, RNA-Protein Interaction Prediction; DCFH-DA, 2, 7-dichlorofurescin diacetate; LC3–1, Light Chain 3-1; LC3–2, Light Chain 3-2; ATG5, autophagy related 5; Dynein, Dynein heavy chain 64C; P-JNK, phosphorylation-c-Jun N-terminal kinase; P-ERK, phosphorylation-extracellular signal-regulated kinase; P-P38, phosphorylation - P38; MEM, Opti-Minimum Essential Medium; HRP, horseradish peroxidase; pRL-TK, Promoter Hsv-thymidine kinase; SDS, sodium dodecyl sulfate; OD, optical density; VCD, vertical overflow drain; MDC, monodansylcadaverine; TEM, transmission electron microscopy

☆ All authors have read the manuscript and agreed to submit it in its current form for consideration for publication in the Journal.

* Corresponding author at: College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, PR China.
E-mail address: zhangziwei@neau.edu.cn (Z. Zhang).

https://doi.org/10.1016/j.jinorgbio.2019.01.010
Received 9 December 2018; Received in revised form 7 January 2019; Accepted 13 January 2019
Available online 18 January 2019
0162-0134/

damage of myocardial tissues through wrecking the biofilm system [8].

MicroRNAs (miRNAs) are ~22 nt endogenous noncoding RNAs. Their functions are to regulate the different biological reactions by targeting mRNAs of translational repression, and these functions could affect the abundance of many genes-coding protein. Numerous studies have shown that miRNAs play a crucial role in various pathological and physiological processes in the heart, such as arrhythmia [9], myocardial fibrosis of cardiovascular disease [10], cardiac hypertrophy [11], cardiac remodeling and heart failure [12]. And miRNAs are also associated with autophagy [13]. Indeed, recent studies proved that miRNAs can regulate autophagy by modulating genes related to autophagy, such as miR-376b can inhibit mammalian target of rapamycin (mTOR) to activate autophagy by targeting autophagy related 4C (ATG4C) and Beclin1 [14]. Se deficiency could cause a large number of abnormal expressions of miRNAs, but we still don't know the specific mechanism of these miRNAs in Se deficiency diseases. Previous results of transcriptional group from our laboratory indicated that expression level of miR-215-5p was rising in the heart of Se-deficient chickens with using high-throughput sequencing [3]. We predicted that phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) could be a target gene of microRNA-215-5p (miR-215-5p) using the website-based miRNA target gene prediction databases.

Great majority of heart diseases have a relationship with cardiac myocyte death, and autophagy is one of culprits for causing myocardial injury [15]. Accompanied with the increasing of autophagic lysosome, autophagy is one of the crucial cellular mechanisms for organisms that could lead to cell death [16,17]. And mTOR can regulate negatively the induction of autophagy [18], and activation of c-Jun N-terminal kinase (JNK) may trigger autophagy and aggravate myocardial injury in the heart of male Sprague–Dawley rats [19]. Additionally, increasing evidence has demonstrated that autophagy can play an important role in regulating neurodegenerative diseases, liver disease, muscle disease and cardiac disease [20]. Some researchers have reported that the PI3K/AKT/mTOR (AKT, threonine-protein kinase) pathway could be involved in processes of advanced glycation end products (AGEs) and regulate autophagy in neonate cardiomyocytes of rat [21]. Intracellular ROS is a generally small highly reactive molecule, and the accumulation of ROS may activate various defense mechanisms in intracellular processes. The high level of ROS production may cause cellular dysfunction, lipid peroxidation, mitochondria DNA damage and ultimately led to autophagic cell death by dying in the heart and skeletal muscle [22,23]. Accumulating reports revealed that autophagy was activated by ROS-dependent JNK and p38 kinase (p38) mitogen-activated protein kinase (MAPK) pathways under the influence of palmitate [24], and extracellular signal-regulated kinase1/2 (ERK1/2) of another subchannel of MAPKs also acted as an important role in autophagy of glioma cell [25].

In the present study, we aimed to elucidate whether miR-215-5p could play a regulatory role in autophagy of cardiomyocytes. Our data showed that miR-215-5p may regulate the PI3K/AKT/TOR (TOR, target of rapamycin) pathway and ROS-MAPK pathways that give rise to autophagy. We provided evidence that miR-215-5p can regulate the PI3K/AKT/TOR pathway by targeting PI3K in cardiomyocytes. Further, we observed that overexpression of miR-215-5p could induce the generation of ROS and activation of MAPK (p38, ERK1/2 and JNK) pathways to activate autophagy related genes. These results suggest that miR-215-5p is potential regulator in autophagy of cardiomyocytes. Additionally, current and future treatment strategies targeting autophagy in diseased myocardium as well as emerging miRNA-based therapies will be showed.

2. Materials and methods

2.1. RNA-Protein Interaction Prediction (RPISeq)

To investigate the connection between the miRNA of miR-215-5p and the protein of PI3K, the website (*http://pridb.gdcb.iastate.edu/RPISeq/index.html*) was used to determine the interaction probabilities generated by RPISeq, the probability score indicated that the target RNA and protein were likely to interact. Using this threshold, the accuracy of classifier was ranged from 87 to 90% in cross-validation evaluation experiments on benchmark datasets, when classifiers were tested on independent (blind) datasets of RPISeq.

2.2. Primary cardiomyocytes culture

Briefly, cardiomyocytes isolated from the cardiac apical tissues of 12-day-old chicken embryos were digested with 0.1% collagenase II (Invitrogen, Carlsbad, CA, USA) at 37 °C for 5 min, and terminated by use DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, GIBCO, Grand Island, NY, USA) with 10% fetal bovine serum. Repeat that step until the small tissue fragments were completely digested. The cell suspension was centrifuged at 900 rpm for 6 min, discarded the liquid supernatants, and resuspended the cell use DMEM/F12 [26]. All supernatants were collected with 300 meshes and 500 meshes filter, respectively. The non-adherent cells were collected by differential adhesion method (the first was 1 h, second was 1.5 h). Cardiomyocytes were inoculated into 6-well culture plates (Jet, China) at a density of 3×10^5 cells/cm^2 in 5% CO_2 at 37 °C, for 24 h. All experiments were carried out at least three times independently.

2.3. Transfection of miR-215-5p mimic and miR-215-5p inhibitor

Synthetic, chemically modified short RNA oligonucleotides were purchased from Shanghai Gene Pharma Co. Ltd. The miR-215-5p mimic and mimic negative control (mimic-NC) sequences were 5′-AUGACCU AUGAAUUGACAGAC-3′ and 5′ UUUGUACUACACAAAAGUACUG-3′, respectively. The miR-215-5p inhibitor and inhibitor negative control (inhibitor-NC) sequences were 5′-mGmUmCmUmGmUmCmAmAmUmUmCmAmUmAmGmGmUmCmAmU-3′ and 5′-mCmAmGmUmAmCmUmUmUmUmGmUmGmUmAmGmUmAmCmAmAmA-3′, respectively. An m represents – Ome (Methylation modification). Cardiomyocytes were plated in 6-well culture plates at 80% confluence and cells were transfected with the miR-215-5p (100 nM), mimic-NC (50 nM), miR-215-5p inhibitor (150 nM), or inhibitor-NC (50 nM), which was separately transfected into cardiomyocytes. Lipofectamine 2000 reagent (Invitrogen) and Opti-Minimum Essential Medium (MEM) were added into cells in control group (group C). Mir-215-5p mimic (100 nM) was transfected into cardiomyocytes with Lipofectamine 2000 reagent and Opti-MEM (group M). Mir-215-5p inhibitor (150 nM) was transfected into cardiomyocytes with Lipofectamine 2000 reagent and Opti-MEM (group I). At 24 h post-transfection, cells were harvested, and the protein and RNA levels of identified target genes were analyzed by western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR), respectively.

2.4. Sections for electron microscopy

For the cell ultrastructural examination, cardiomyocytes were collected into a tube and centrifuged at 250 ×*g* for 10 min, and then the supernatant was discarded. Cells at the bottom of the tube were mixed with 2.5% glutaraldehyde phosphate-buffered saline (v/v, pH 7.2), post fixed in 1% osmium tetroxide (v/v) and dehydrated in a graded series of ethanol concentrations, followed by embedding in Embed812 resin. Next, the ultra-thin sections were cut, mounted, washed, impregnated, post-stained and incubated with lead citrate for analysis by microscopy. The microphotographs were acquired using a transmission electron microscope (GEM-1200ES, Japan).

2.5. Preparation of the luciferase construct of PI3K and luciferase activity assay

Double stranded DNA fragments were synthesized by annealing single stranded oligonucleotides of wild PI3K and mutant PI3K in which there was 3′untranslated region (3′UTR) interacting with miR-215-5p binding site (Sangon Biotech synthesis). Those DNA fragments were inserted into the pmicroRNA-report (pMIR-REPORT, Thermo Fisher) vector to generate the pMIR-PI3K constructs (WT, wild type and MUT, mutant type). Cardiomyocytes were co-transfected with the plasmid constructs of pMIR-PI3K-WT or pMIR-PI3K-MUT with Lipofectamine 2000 (Invitrogen) according to the protocol of manufacturer. The empty of pMIR-REPORT vector was used as the negative control. The luciferase assay was carried out on the extracts from the cells at 48 h post transfection and activity was measured using the Dual-Luciferase Assay System (Promega). Promoter Hsv-thymidine kinase (pRL-TK) expressing Renilla luciferase was co-transfected as an internal control. Data were normalized using the ratio of Firefly and Renilla luciferase activities.

2.6. Detection of intracellular ROS accumulation

Cardiomyocytes were collected and the level of ROS was detected by using the ROS assay kit (Nanjing Jiancheng Bioengineering institute, China). DCFH-DA (2, 7-dichlorofurescin diacetate, 10 μM) was added into the culture medium containing cell samples and cardiomyocytes were then incubated at 37 °C for 45 min. Cells were washed with phosphate buffer saline (PBS) three times. Ultimately, collect cells for detecting the activities of ROS in excitation wavelength 500 ± 15 nm and emission wavelength 530 ± 20 nm.

2.7. RNA extraction

Total RNA was isolated from myocardial tissues using Trizol reagent according to the instructions of manufacturer (Invitrogen, Shanghai, China). The dried RNA pellets were re-suspended in 50 μL of diethyl-pyrocarbonate treated water. The concentration and purity of the total RNA were determined by spectrophotometer. cDNA was synthesized from 5 μg of the total RNA using oligo dT primers and Superscript II reverse transcriptase according to the instructions of manufacturer (Promega, Beijing, China). cDNA was diluted at a ratio of 1:5 with sterile water and stored at −80 °C.

2.8. qRT-PCR analysis for mRNA and miRNA quantification

Primer Premier 6.0 Software (PREMIER Biosoft International, USA) was used to design specific primers for PI3K, AKT, TOR, Light Chain 3-1 (LC3-1), Light Chain 3-2 (LC3-2), autophagy related 5 (ATG5), Dynein heavy chain 64C (Dynein), Beclin1, ERK1/2, p38, JNK, U6 and β-actin, and all of the genes of interest were based on known chicken sequences (Tables 1 and 2). Primer specificity was confirmed by qRT-PCR. Reverse transcription was performed using the miRcute microRNA First-Strand cDNA Synthesis Kit or miRcute miRNA cDNA Synthesis Kit (Tiangen Biotech Co. Ltd., Beijing) according to the manufacturer's instructions (Roche). The levels of miR-215-5p and mRNA were detected by qRT-PCR using the Light Cycler®480 System (Roche, Basel, Switzerland) and Fast Universal Sybr Green Master Mix (Roche, Basel, Switzerland), respectively. Either β-actin or U6 was used as an internal reference. For each PCR reaction, Dissociation Curve 1.0 Software (Applied Biosystems) was used to analyze dissociation curves in order to detect possible primer-dimers. The relative abundance of each mRNA and miRNA was calculated according to the method of Pfaffl.

Table 1
microRNA primer sequence.

	Primer sequence
gga-mir-215-5p	5′-CGATGACCTATGAATTGACAGAC-3′
U6	5′-CACGCAAATTCGTGAAGCGTTCCA-3′

Table 2
The primers used in the present study.

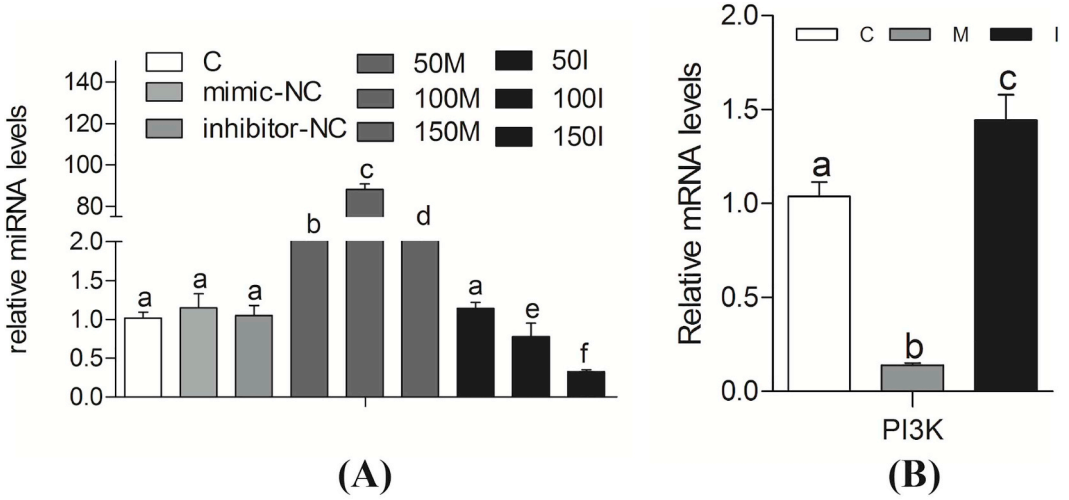
Target gene	Primer sequence (5′ - 3′)
PI3K	Forward 5′-GTCCTTGAGCCACTGATG-3′ Reverse 5′-TGTTGCCTTACGGTTGTT-3′
AKT	Forward 5′-AGGAGGAAGAGATGATGGAT-3′ Reverse 5′-GAATGGATGCCGTGAGTT-3′
TOR	Forward 5′-GGACTCTTCCCTGCTGGCTAA-3′ Reverse 5′-TACGGGTGCCCTGGTTCTG-3′
LC3-1	Forward 5′-TTACACCCATATCAGATTCTTG-3′ Reverse 5′-ATTCCAACCTGTCCCTCA-3′
LC3-2	Forward 5′-AGTGAAGTGTAGCAGGATGA-3′ Reverse 5′-AAGCCTTGTGAACGAGAT-3′
ATG5	Forward 5′-GGCACCGACCGATTTAGT-3′ Reverse 5′-GCTGATGGGTTTGCTTTT-3′
Dynein	Forward 5′-CGGCTTGACCTATGGAATCT-3′ Reverse 5′-CATCACTGCGAGGAACTGC-3′
Beclin1	Forward 5′-CGACTGGAGCAGGAAGAAG-3′ Reverse 5′-TCTGAGCATAACGCATCTGG-3′
ERK	Forward 5′-TGGTACAGGGCTCCTGAAAT-3′ Reverse 5′-GGAAAGATGGGTCTGTTGGA-3′
P38	Forward 5′-GCATCCATCTTCGTCGTCAT-3′ Reverse 5′-TCATCTACAGCAACCCAGAGG-3′
JNK	Forward 5′-CAGATAAGCAGTTAGATGAGAG-3′ Reverse 5′-GACAGATGACGACGAAGAT-3′
β-actin	Forward 5′-CCGCTCTATGAAGGCTACGC-3′ Reverse 5′-CTCTCGGCTGTGGTGGTGAA-3′

2.9. Protein extraction and western blot analysis

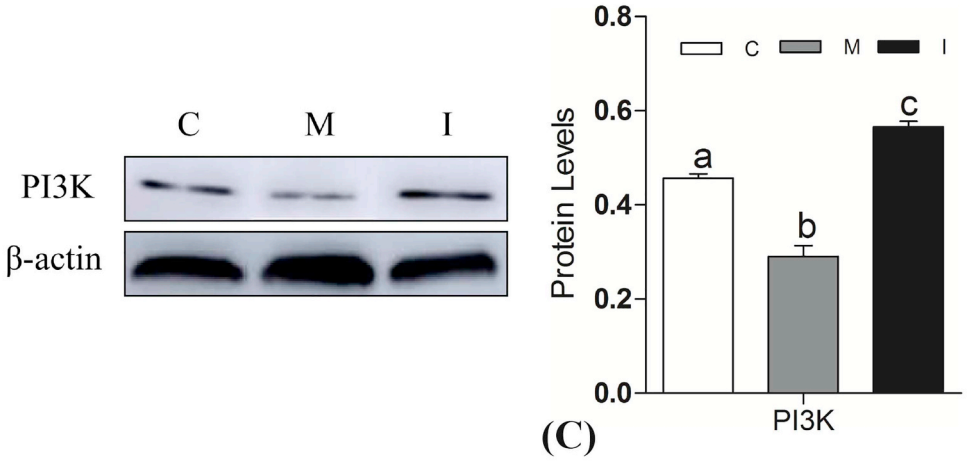
For total protein extraction, protein lysis solutions were subjected to 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions. Then the separated proteins were transferred to a nitrocellulose membrane for 1 h at 200 mA in a tank transfer apparatus containing Tris-glycine buffer and 20% methanol. Nitrocellulose membranes were blocked for 1 h with 5% bovine serum albumin at 37 °C and incubated overnight at 4 °C with diluted primary antibodies against PI3K (1:1000, Santa Cruz Biotechnology, USA), AKT (1:1000, Sigma, USA), TOR (1:500, Sigma, USA), light chain 3 (LC3, 1:500, wanleibio, china), Beclin1 (1:1000, Sigma, USA), JNK (1:2000, Sigma, USA), ERK (1:1000, Sigma, USA), p38 (1:1000, Sigma, USA), PhosphoPlus JNK (P-JNK, 1:1000, Sigma, USA), PhosphoPlus ERK (P-ERK, 1:2000, Sigma, USA) and PhosphoPlus p38 (P-p38, 1:1000, Sigma, USA) at 4 °C, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody against rabbit IgG (1:5000, Santa Cruz Biotechnology, USA). The signal was detected with X-ray films (TransGen Biotech Co, Beijing, China). The optical density (OD) of each band was determined using an Image vertical overflow drain (VCD) gel imaging system, and the relative abundance of these proteins was calculated and presented as the ratios of OD of each of these proteins to that of β-actin.

2.10. Autophagy detection

The autofluorescent agent, monodansylcadaverine (MDC) was used to analyze the formation of autophagolysosome during autophagy processes. After 24 h transfected cells were washed twice times with PBS, and 50 μM MDC was added and kept for 30 min in 5% CO_2. After 30 min, cells were washed with 3 times with PBS and the fluorescence was examined by a cell imaging station.



(A) (B)



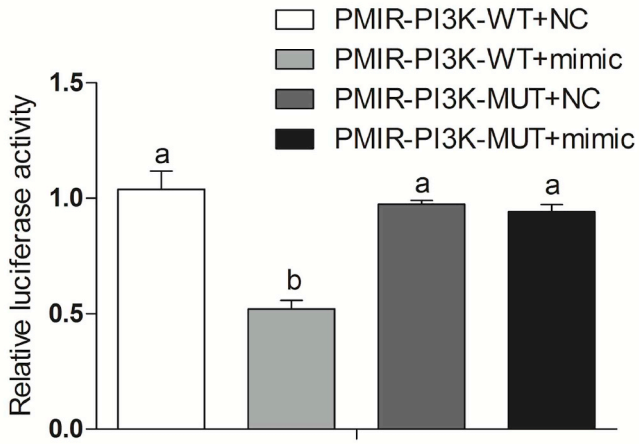
(C)

gga-mir-215-5p 5'- AUGACCUAUGAAUUGACAGAC -3'

PI3K 3'-CTTAACAGATGACAAACTGTCTC -5'

Mutation sequence 3'-CTTAACAGATGACAATATAAGTC -5'

(D)



(E)

(caption on next page)

Fig. 1. (**A**) Cardiomyocytes with transfecting different concentrations of miR-215-5p-mimic (M), miR-215-5p inhibitor (I), mimic negative control (mimic-NC) and inhibitor negative control (inhibitor-NC) for 24 h, and C indicates the control group. miR-215-5p was determined by RT-PCR. U6 was selected as the reference. Values are mean ± SEM (n = 3). (**B**) The relative mRNA levels of PI3K in groups C, M and I. β-Actin was selected as the reference. Values are mean ± SEM (n = 3). (**C**) The protein level of PI3K in groups C, M and I. (**D**) The results between PI3K and miR-215-5p in luciferase reporter gene assay. PMIR-PI3K-WT plasmids were mutated in the miRNA target sites, and designated as PMIR-PI3K-MUT. miR-215-5p-mimic inhibited PI3K-WT expression but not mutant PI3K-MUT expression. The results were calculated fromat least three independent experiments, n = 3. Bars that do not share the same letters are significantly different ($p < 0.05$) from each other. Data are expressed as mean ± SD. (**E**) The binding sequence and mutation sites of PI3K were shown.

2.11. Statistical analysis

Statistical analysis of all data was performed using GraphPad Software Prism 5 (version 5.01, GraphPad Software, Inc., La Jolla, USA). When a significant value ($P < 0.05$) was obtained by one-way analysis of variance, further analysis was done. All data showed a normal distribution and passed equal variance testing. Differences between means were assessed by Tukey's honestly significant difference test for multiple comparisons. In addition, correlation analysis was used to define the relationship between individual variations using GraphPad Software Prism 5 (version 5.01, GraphPad Software, Inc., La Jolla, USA).

3. Results

3.1. RPISeq

In performance evaluation experiments, the prediction with probabilities was 0.8 and it was considered “positive”, indicating that the corresponding miR-215-5p and PI3K was likely to interact (Fig. S1). When classifiers were tested on independent (blind) datasets of RPIs, the result of svm classifier was 97% in cross-validation evaluation experiments on benchmark datasets (Fig. S1). All predictions indicated that there was a closely connection between miR-215-5p and PI3K.

3.2. Transfection of miR-215-5p mimic and miR-215-5p inhibitor

The present results showed that we have established successfully overexpression and knockdown model of miR-215-5p in chicken cardiomyocytes (Fig. 1A). Moreover, these data indicated that the optimum concentration of miR-215-5p mimic (M) and miR-215-5p inhibitor (I) were 100 nM and 150 nM, respectively. The results showed that the level of miR-215-5p mimic (100 nM) has increased 90-fold than that of group C and it was reduced by 55% in group I (150 nM). In addition, the level of miR-215-5p in mimic negative control (mimic-NC) or inhibitor negative control (inhibitor-NC) has no significant difference, compared with that of group C.

3.3. PI3K is a direct target of miR-215-5p

These differentially expressed genes were analyzed for miR-215-5p binding sites by the TargetScan website. By this means, PI3K was selected as a novel potential target gene of miR-215-5p because the mRNA level of PI3K was downregulated 83% in group M relative to group C. In the contrary, compared with group C, the mRNA expression level of PI3K was increased 39% in group I (Fig. 1B). Concordant tendency was showed about the protein expression levels (Fig. 1C). Our lab has showed that the mRNA and protein levels of PI3K were reduced in Se-deficient myocardial tissue [7]. These results suggested that PI3K was a specific downstream target gene of miR-215-5p in myocardial autophagy induced by Se deficiency.

In addition, the luciferase reporter assay was performed to determine whether the miR-215-5p can combine with a conserved binding site in the 3′UTR of PI3K. Wild-type and mutant-type of PI3K 3′UTR sequences were constructed and inserted into PMIR vectors (Fig. 1D). As presented in Fig. 1E, the luciferase reporter represented a significant decrease in 3′UTR binding of PI3K for miR-215-5p mimic (PMIR-PI3K-WT-mimic), compared with the miR-NC (PMIR-PI3K-WT-NC). Unlike wild-type luciferase reporter, miR-215-5p mimic restrained negatively the luciferase activities driven by the binding site of PI3K mutation sequence (Fig. 1E). Therefore, PI3K was identified as a direct target gene of miR-215-5p in cardiomyocytes.

3.4. The ultrastructural observation of cardiomyocytes

Transmission electron microscopy (TEM) analysis demonstrated that there were no obviously visible ultrastructural changes in normal myocardial cells and it exhibited the normal myocardial morphology (Fig. 2A). Contrastingly, typical autophagy characteristics, such as the numerous autolysosome and autophagic vacuolization was observed in group M. Consistent with the results observed in group C, and normal myocardial morphology was showed in group I. These findings indicated that overexpression of miR-215-5p can induce autophagic myocardial death in cardiomyocytes.

3.5. MDC staining of miR-215-5p induced autophagy in cardiomyocytes

MDC is a specific marker for a detection of autophagy vacuoles formation and it appears as a dot-like structure in the green cytoplasm (Fig. 2B). In overexpression of miR-215-5p group, there was a number of a dot-like structures compared with normal cells and the level of MDC has increased 79.66% in comparison with group C (Fig. 2C). The opposite results in group I showed that autophagic vacuoles of cardiomyocytes with knockdown of miR-215-5p were decreased by 49.64% (Fig. 2C). These results indicated that the increasing miR-215-5p strongly can induce autophagy in chicken cardiomyocytes.

3.6. MiR-215-5p targeted regulation of PI3K mediated autophagy

The results showed that the effects of miR-215-5p overexpression on mRNA levels of the PI3K/AKT/TOR pathway (AKT, TOR) and the autophagy related genes (LC3-1, LC3-2, ATG5, Dynein and Beclin1) were displayed in Fig. 3A. Compared with group C, qRT-PCR results showed that the mRNA expression levels of AKT and TOR significantly decreased by 82% and 72% in group M, but the mRNA level of autophagy related genes (LC3-1, LC3-2, ATG5, Dynein and Beclin1) was increased 30%, 120%, 202%, 60% and 145%, respectively (Fig. 3A). Contrarily, the mRNA levels of AKT and TOR increased by 44% and 82% in group I and mRNA expression levels of LC3-1, LC3-2, ATG5, Dynein and Beclin1 decreased by 43%, 33%, 84%, 87% and 32%, respectively, compared with group C (Fig. 3A).

Western blot was performed to determine the protein expression of autophagic markers genes (LC3-1, LC3-2 and Beclin1) and PI3K/AKT/TOR pathway related genes (AKT, TOR) in chicken cardiomyocytes (Fig. 3B). In vitro cardiomyocytes treated with miR-215-5p mimic (group M) showed that the protein abundance of AKT, P-AKT and TOR decreased by 36%, 46% and 41%, while the protein level of Beclin1 increased by 120% and that of LC3-2/LC3-1 was increased 26%, compared to group C (Fig. 3B). Opposite results were observed in cardiomyocytes with knockdown of miR-215-5p, the protein levels of AKT, P-AKT and TOR were increased by 58%, 78% and 72%, autophagic protein Beclin1 decreased by 37% and the protein levels of LC3-2/LC3-1 decreased by 81% (Fig. 3B). Moreover, the protein level of the ratio of P-AKT and AKT was 84% in autophagic pathway in group M, and in

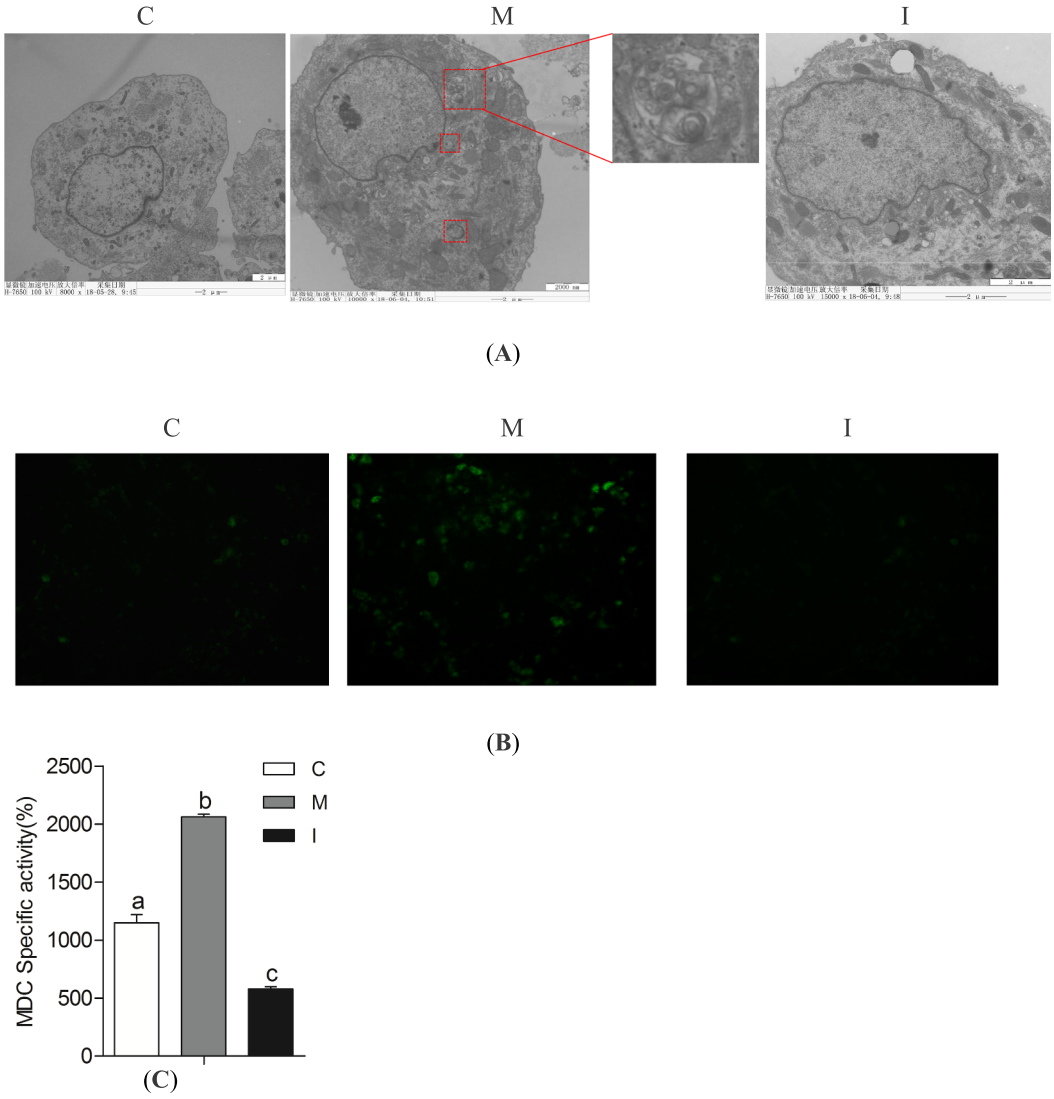


Fig. 2. (A) The ultrastructural changes of cardiomyocytes transfected with miR-215-5p mimic (M), miR-215-5p inhibitor (I). Control cardiomyocytes (C). Numerous autolysosomes (red boxes) appeared in the group treated with miR-215-5p mimic. Values are mean ± SEM (n = 3). (B) MiR-215-5p-mimic induced autophagosome formation. C indicates the control group; M indicates the miR-215-5p-mimic overexpression group. I indicates the miR-215-5p-inhibitor group. Cardiomyocytes were stained with 50μM MDC and images were taken with a cell imaging station (920). In cells treated miR-215-5p-mimic, green fluorescence particle indicates autophagosome formations. MDC fluorescence quantification data ($P < 0.05$). Values are mean ± SEM (n = 3). (C) Three wells were analysed per treatment and per time. Values are mean ± SEM (n = 3). The experiment was repeated three times. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this chapter.)

group I the ratio of that was 126% when compared to group C (Fig. 3B).

3.7. Detection of intracellular ROS accumulation

To further verify whether miR-215-5p could affect the level of ROS in cardiomyocytes, we validate the level of intracellular ROS accumulation. The production of ROS has significantly increased 153% ($P < 0.05$) in group M compared with group C, and it decreased by 32% in cardiomyocytes treated with miR-215-5p inhibitor (Fig. 4).

3.8. Mir-215-5p mediated modulation of MAPK activation

We set out to determine whether changes of MAPK signaling pathways may be associated with the mRNA (p38, ERK and JNK) and protein (p38, P-p38, ERK, P-ERK, JNK and P-JNK) levels in chicken cardiomyocytes. The results were showed in Fig. 5A, compared to group C, the mRNA expressions of p38, ERK and JNK was increased 155%, 121% and 172% respectively in group M, additionally the mRNA levels decreased by 40%, 41% and 68% in group I, respectively ($P < 0.05$).

The results showed the changes in phosphorylated protein of MAPK pathway key kinases p38, ERK and JNK in cardiomyocytes treated with miR-215-5p mimic/inhibitor ($P < 0.05$) in Fig. 5B. The protein (group M) levels of p38, P-p38, ERK, P-ERK, JNK and P-JNK increased by 23%, 61%, 41%, 61%, 25% and 35% respectively in comparison to control cells (group C), whereas the protein expression levels decreased by 25%, 42%, 24%, 40%, 41% and 40% in group I (Fig. 5B), respectively. In this study, the expression level of MAPK pathways related protein and mRNA was the same as ROS in cardiomyocytes.

4. Discussion

The most important findings of this study was that miR-215-5p can reduce the cell viability by targeting PI3K to inhibit the PI3K/AKT/TOR pathway, and it also could enhance ROS-MAPK signaling pathways to induce autophagy in cardiomyocytes.

Se is an essential micronutrient that protects the tissues and organs

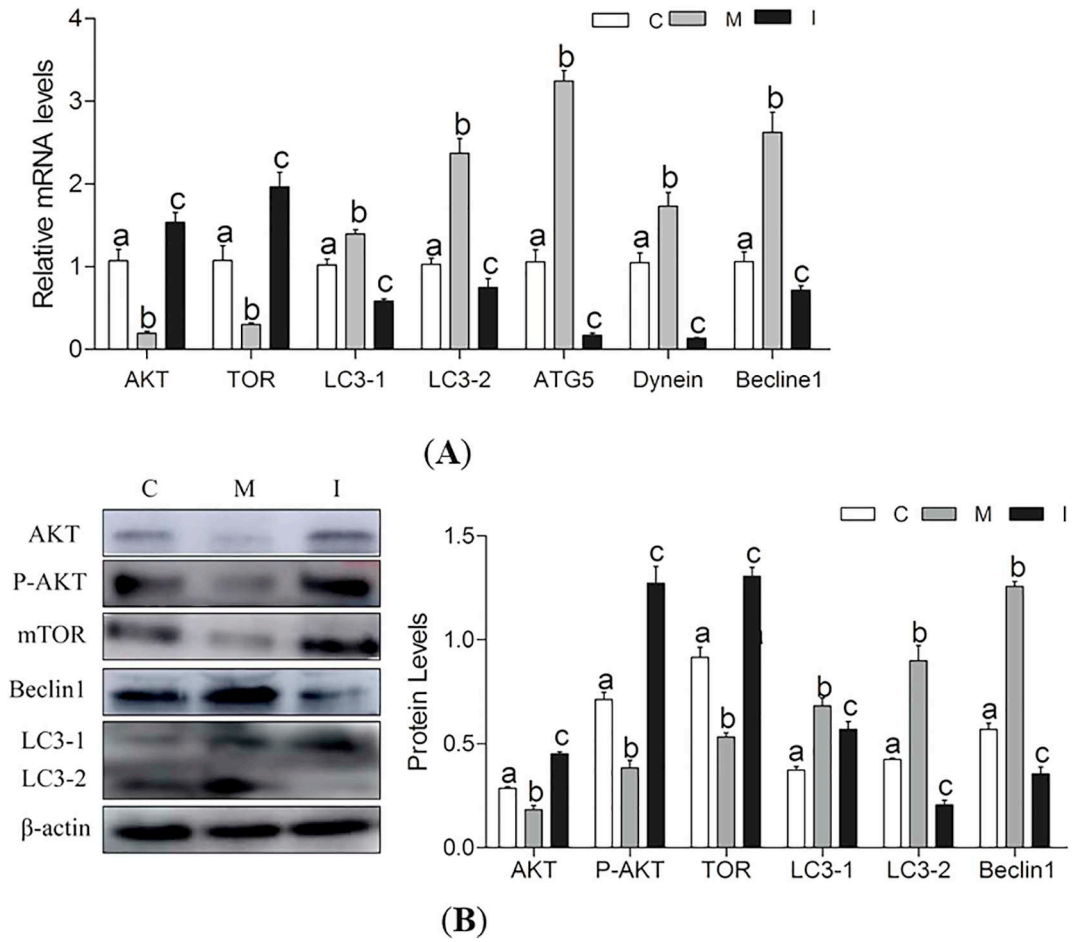


Fig. 3. (A) The autophagy-related gene mRNA levels of AKT, TOR, LC3-1, LC3-2, ATG-5, Dynein and Becline1 were determined by RT-PCR in three groups C, M and I. The results were calculated at least three times. Values are mean ± SEM (n = 3). (B) The effects of miR-215-5p-mimic and miR-215-5p-inhibitor on the expression levels of AKT, P-AKT, TOR, LC3-1, LC3-2, and Becline1 proteins in cardiomyocytes. β-Actin was selected as the reference. The data are expressed as the means ± SD (n = 3). Bars that do not share the same letters are significantly different ($P < 0.05$) from each other.

against oxidative damage. Previously, Se deficiency may cause severe oxidative stress, and excessive oxidative stress can destroy the antioxidant defense system in the body [27–29]. Myocardial injury induced by Se deficiency indicated that the importance of autophagic cell death was manifested in maintaining cardiac function. The results of miRNAomics in a Se-deficient myocardial model showed that there was a significant difference of miR-215-5p. To date, the study of miR-215 has focused primarily on cell proliferation, invasion and migration, and it can promote cell apoptosis to suppress the tumorigenicity of human non-small cell lung cancer by targeting zinc finger E-box binding homeobox 2 (ZEB2) in vitro [30,31]. We screened the Se-deficient myocardium-specific miRNA (miR-215-5p) and its target protein PI3K. And miR-215-5p has not been reported and functions remain unknown in poultry. The results showed that Se deficiency can result in the up-regulation of miR-215-5p expression level. Numerous experiments and clinical studies have confirmed that miRNAs may play a unique role during myocardial injury [32]. The disorder of miR-215-5p in myocardial tissues of chickens is caused by Se deficiency, and miR-215-5p could participate in the regulation of injury. Mir-215-5p may be involved in the regulation of ROS and the occurrence of oxidative stress, but whether or not miR-215-5p actually participates in myocardial autophagy still needs further investigation. During the up-regulation of miR-215-5p, we further examined various signaling pathways when autophagosomes formation was further increased, and identified that PI3K could be a target gene of miR-215-5p in vitro.

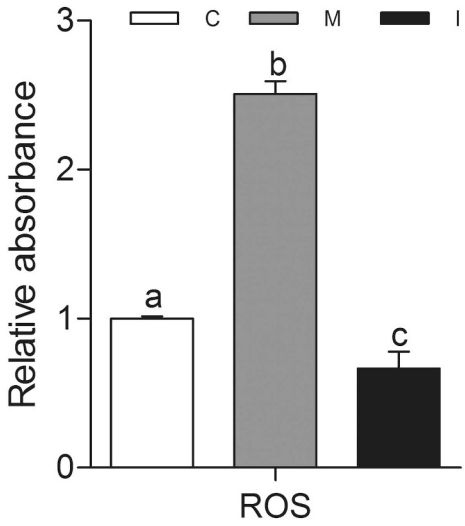


Fig. 4. The effects of groups C, M and I on the ROS levels in cardiomyocytes. The data are expressed as the means ± SD (n = 3). The results were calculated at least three times. Bars that do not share the same letters are significantly different ($p < 0.05$) from each other. The experiment was repeated three times.

Autophagy is associated with many pathophysiological processes and diseases, such as myocardial ischemia/reperfusion injury [33,34], dilated cardiomyopathy and failing myocardium [35]. However, excessive up-regulation of autophagy [36] induced by the overexpression of autophagy mark protein Beclin1 in cardiac myocytes [37,38] can

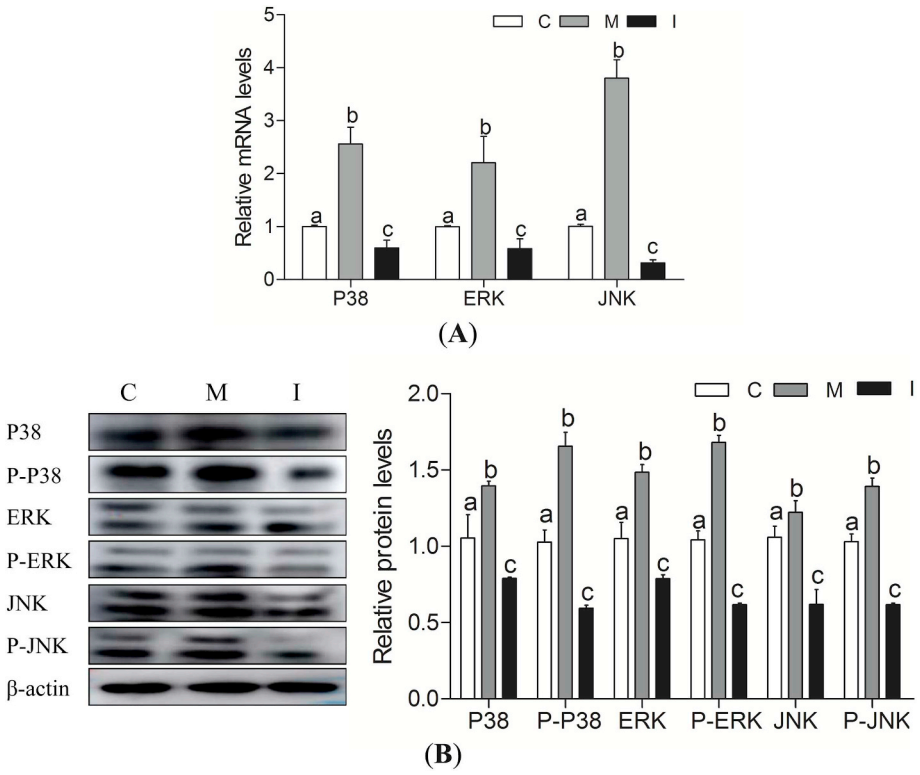


Fig. 5. (**A**) The mRNA levels of p38, P-p38, ERK, P-ERK, JNK and P-JNK were detected in groups C, M and I in vitro. β-Actin was selected as the reference. Values are mean ± SEM (n = 3). (**B**) The protein levels of p38, P-p38, ERK, P-ERK, JNK and P-JNK were detected by western blot in groups C, M and I in vitro. β-Actin was selected as the reference. The data are expressed as the means ± SD (n = 3). Bars that do not share the same letters are significantly different ($p < 0.05$) from each other. The experiment was repeated three times.

cause cell death. Previous studies have proved that histone deacetylase 6 may induce autophagic cell death through activating Beclin1 and LC3-2 [39] when moving from healthy toward autophagic area of the human myocardium. As with other tissues, a steady-state mechanism of cardiac autophagy is necessary for maintaining the size, structure and function of cardiac cells [21]. In recent years, multiple tissue injuries and diseases caused by excessive autophagy in human beings and animals have attracted more and more attention. Published data have been proved that DNA damage, nutrient starvation and growth factor deprivation could activate autophagy [40]. Many reports have shown that autophagy was accompanied by the increasing expression levels of Beclin1 and the ratio of LC3-2/LC3-1 during ischemia and reperfusion in the heart [41,42]. In this study, we found that miR-215-5p mimic can increase the number of autophagic vacuoles and impair the viability of cardiomyocytes. An increase in LC3-2/LC3-1 in group M showed that miR-215-5p mimic can activate the conversion of LC3 from LC3-1 to the autophagic membrane type LC3-2. These findings indirectly supported the results of this study that myocardial autophagy caused by Se deficiency was related with the upregulation of miR-215-5p. Interestingly, the silencing of miR-215-5p alleviated myocardial autophagy accompanied by reducing the expressions of Beclin1 and LC3-2, and ameliorated the ultra-structure of cardiomyocytes, suggesting that inhibition of miR-215-5p can promote cell survival to alleviate myocardial autophagy.

The PI3K family plays complex and extensive roles in many aspects of cell biology and metabolism [43]. The PI3K/AKT signaling pathway is an important pathway involved in the regulation of cell proliferation, differentiation in mouse myogenic C2 cells [44], cell growth, cell cycle and so on [45]. PI3K is a key signal molecule in many life activities, such as apoptosis and autophagy [46]. Du Tao et al. reported that Lipopolysaccharide can regulate macrophage autophagy through the PI3K/AKT/mTOR pathway in the macrophage cell line RAW264.7 [47]. The PI3K/AKT/TOR signaling pathway is an important pathway for regulating autophagy in the body [48]. Meanwhile, overexpression or inhibition of miR-215-5p can regulate the expression level of PI3K in the cultured primary cardiomyocytes. Further, the targeting relationship between miR-215-5p and PI3K was verified by the luciferase reporter system. At the same time, we investigated the signal transduction pathway of miR-215-5p overexpression to induce autophagy in cardiomyocytes. The decreasing of the phosphorylation of AKT and TOR showed that miR-215-5p could trigger autophagy by the PI3K/AKT/TOR signaling pathway. Although miR-215-5p could play a role in the regulation of the PI3K/AKT/TOR signaling pathway by targeting PI3K, whether it can mediate the PI3K/AKT/TOR pathway to regulate autophagy of cardiomyocytes remains unclear. Thus, autophagy-related genes were detected and the results showed that the up-regulation of expression levels of autophagy related genes of Beclin1, Atg5 and LC3-2/LC3-1 were showed in cardiomyocytes. By comparison to the autophagy modulators Beclin1, LC3-2 and Atg5, which are modulated by association with PI3K. PI3K can become activated when cells treated with miR-215-5p inhibitor. Upon activation, PI3K, AKT and TOR may induce signal transduction events that ameliorate the accumulation of autophagosomes in cardiomyocytes by decreasing expression of Atg5, Beclin1 and LC3-2. In addition, miR-215-5p inhibitor may activate the PI3K/AKT/TOR pathway to reduce myocardial autophagy and improve the cell viability by regulating its target protein, PI3K. More importantly, the modulation of this pathway could induce various functional changes in processes of myocardial injury, suggesting that miR-215-5p was able to trigger autophagy of cardiomyocytes through the PI3K/AKT mediated pathway, which may be a new miRNA molecular mechanism in the regulation of autophagy in cardiomyocytes. Last but not least, it should be pointed out that PI3K is not the only regulator of autophagy.

The downregulating of the PI3K and activation of MAPK pathways induced by Naringin may promote autophagy to inhibit advanced gastric glands (AGS) cancer cells [49]. The results suggested that acute exposure of crotonaldehyde can induce cell death mediated by autophagy through down-regulating the activity of PI3K pathway and elevating the activities of MAPK and AMP-activated protein kinase (AMPK) pathways in human bronchial epithelial cells [50]. Extensive

studies have indicated that PI3K and MAPK pathways could regulate physiological processes in various cells, thus the expression levels of genes associated with MAPK pathways were detected. MAPK pathways can regulate various physiological processes by activating phosphorylation transcription factors in cells, such as inflammation, cell growth, cell development and death [51]. Several reports have showed that the MAPK pathway was involved in the regulation of autophagy [52]. ROS has the function of an endogenous signalling molecule that may regulate multiple signalling pathways in various intracellular processes, and it is also associated with the initiation and promotion of autophagy [53]. There is growing evidence that excessive ROS can cause the damage of mitochondria and endoplasmic reticulum, even the autophagic cell death in mammalian cells or yeast [54]. Previous studies indicated that ROS was found to participate in the processes of autophagy to activate Beclin1 in rat intestinal epithelial cells [55]. In our results, direct involvement of the regulation of overproduction ROS was induced by the miR-215-5p mimic which may be involved in the occurrence of autophagy. In addition, the ROS-activated autophagy was dependent on the activation of MAPK family members (ERK1/2 and JNK) in gastric carcinoma [56]. Comprised of ERK, p38 and JNK, the MAPK pathway was the key downstream pathway associated with the generation of ROS, and MAPK pathways played a significant role in cellular survival. ROS also may activate MAPK pathways in multiple cell types, therefore we examined the possibility of whether MAPK pathways were involved in cardiomyocytes treated miR-215-5p mimic. The results of the present study revealed that the activation of autophagic proteins Beclin1 and LC3-2 with a significant phosphorylation of protein of MAPKs was confirmed by the increased autophagosomes in group M. Taking these data into consideration, we came to a conclusion that miR-215-5p mimic-induced autophagy was also dependent on the generation of ROS and activation of MAPK (p38, ERK1/2 and JNK) pathways in cardiomyocytes, evidenced by the increased expression levels of the phosphorylation of ERK1/2, p38 and JNK. An important observation in this study showed that miR-215-5p inhibitor can block autophagy by inhibiting the MAPK pathways and reduce the generation of ROS to promote cardiomyocyte survival. Thus, the results showed that the abnormal elevation of miR-215-5p could result in myocardial injury through the ROS-MAPK autophagy pathways. Indubitability, there were some other effective regulatory pathways and future researches on this topic should continue to progress. These results revealed a possible molecular mechanism for studying the cytoprotective effects of autophagy by miR-215-5p inhibitor.

5. Conclusion

In conclusion, the results showed that mir-215-5p was a new autophagy-related miRNA, and its targeting relationship with PI3K was determined in cardiomyocytes. Combined with these results, we clarified the mechanism that the up-regulation of miR-215-5p was one of the reasons of myocardial injury, which was related to regulation of the PI3K/AKT/TOR pathway and ROS-MAPK pathways to mediate autophagy. These findings shed lights on the therapeutic potential of miR-215-5p in the mechanism of myocardial autophagy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2019.01.010.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (31872531), State Key Laboratory of Animal Nutrition foundation (2004DA125184F1725), National Pig Industry Technology System Foundation (CARS-35-04), Merit-based Funding for Returned Oversea Student of Heilongjiang Province (2018QD0005) and “Academic backbone” Project of Northeast Agricultural University (17XG11).

Disclosure of potential conflicts of interest

The authors declare that there are no conflicts of interest.

References

[1] D. Behne, et al., Evidence for specific selenium target tissues and new biologically important selenoproteins, Biochim. Biophys. Acta Gen. Subj. 966 (1) (1988) 12–21.
[2] H.D. Yao, et al., Selenoprotein W serves as an antioxidant in chicken myoblasts, Biochim. Biophys. Acta Gen. Subj. 1830 (4) (2013) 3112–3120.
[3] T. Yang, et al., miR-200a-5p regulates myocardial necroptosis induced by Se deficiency via targeting RNF11, Redox Biol. 15 (2017) 159–169.
[4] C.S. Li, Selenium deficiency and endemic heart failure in China: a case study of biogeochemistry for human health, Ambio 36 (1) (2007) 90–93.
[5] H. Shen, et al., Vitamin E and selenium levels are within normal range in pigs diagnosed with mulberry heart disease and evidence for viral involvement in the syndrome is lacking, Transbound. Emerg. Dis. 58 (6) (2011) 483–491.
[6] M. De Lorgeril, P. Salen, Selenium and antioxidant defenses as major mediators in the development of chronic heart failure, Heart Fail. Rev. 11 (1) (2006) 13–17.
[7] J. Yang, et al., Interplay between autophagy and apoptosis in selenium deficient cardiomyocytes in chicken, J. Inorg. Biochem. 170 (2017) 17–25.
[8] I. Kranner, Analyses of reactive oxygen species and antioxidants in relation to Se, Palaeogeogr. Palaeoclimatol. Palaeoecol. 154 (4) (1999) 301–312.
[9] C. Ben-Zhi, et al., Role of miRNAs in cardiac arrhythmias, Chin. Bull. Life Sci. 14 (6) (2015) 31–38.
[10] H.M. Xiao, W. Yang, D.O. Cardiology, Role of miRNAs in myocardial fibrosis of cardiovascular disease, Med. Recapitulate 13 (8) (2016) 185–190.
[11] Eva van Rooij, L.B. Sutherland, Ning Liu, Andrew H. Williams, John McAnally, Robert D. Gerard, James A. Richardson, Eric N. Olson, A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure, Proc. Natl. Acad. Sci. U. S. A. 103 (48) (2006) 18255–18260.
[12] V. Divakaran, D.L. Mann, The emerging role of MicroRNAs in cardiac remodeling and heart failure, Cardiovasc. Drugs Ther. 25 (2) (2011) 171–182.
[13] Y. Kim, J. Lee, H. Ryu, Modulation of autophagy by miRNAs, BMB Rep. 48 (7) (2015) 371–372.
[14] G. Korkmaz, et al., miR-376b controls starvation and mTOR inhibition-related autophagy by targeting ATG4C and BECN1, Autophagy 8 (2) (2012) 165–176.
[15] R. Beverly, et al., Myocyte Autophagy in Heart Disease: Friend or Foe, Autophagy 3 (6) (2007) 632–634.
[16] F. Liu, et al., Cadmium disrupts autophagic flux by inhibiting cytosolic Ca^{2+}-dependent autophagosome-lysosome fusion in primary rat proximal tubular cells, Toxicology 383 (2017) 13–23.
[17] X.Y. Wang, et al., Trehalose protects against cadmium-induced cytotoxicity in primary rat proximal tubular cells via inhibiting apoptosis and restoring autophagic flux, Cell Death Dis. 8 (10) (2017) 46–47.
[18] Y. Zhu, et al., Beclin 1 cleavage by caspase-3 inactivates autophagy and promotes apoptosis, Protein Cell 1 (5) (2010) 468–477.
[19] J. Xu, et al., Mitochondrial JNK activation triggers autophagy and apoptosis and aggravates myocardial injury following ischemia/reperfusion, Biochim. Biophys. Acta 1852 (2) (2015) 262–270.
[20] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, Cell 132 (1) (2008) 27–42.
[21] X. Hou, et al., Advanced glycation endproducts trigger autophagy in cadiomyocyte via RAGE/PI3K/AKT/mTOR pathway, Cardiovasc. Diabetol. 13 (1) (2014) 78.
[22] H. Tsutsui, S. Kinugawa, S. Matsushima, Oxidative stress and heart failure, Nihon Rinsho Jpn. J. Clin. Med. 14 (2) (2011) 109.
[23] Q. Chi, et al., The effects of atmospheric hydrogen sulfide on peripheral blood lymphocytes of chickens: perspectives on inflammation, oxidative stress and energy metabolism, Environ. Res. 167 (2018) 1–6.
[24] R. Scherz-Shouval, Z. Elazar, Regulation of autophagy by ROS: physiology and pathology, Trends Biochem. Sci. 36 (1) (2011) 30–38.
[25] V. Thiyagarajan, et al., 16-Hydroxy-cleroda-3,13-dien-16,15-olide induced glioma cell autophagy via ROS generation and activation of p38 MAPK and ERK-1/2, Environ. Toxicol. Pharmacol. 45 (2016) 202–211.
[26] J. Yang, et al., Gene expression of selenoproteins can be regulated by thioredoxin (Txn) silence in chicken cardiomyocytes, J. Inorg. Biochem. 177 (2017) 118.
[27] H. Yao, et al., Selenium deficiency mainly influences the gene expressions of antioxidative selenoproteins in chicken muscles, Biol. Trace Elem. Res. 161 (3) (2014) 318.
[28] Y. Hai-Dong, et al., Gene expression of endoplasmic reticulum resident selenoproteins correlates with apoptosis in various muscles of se-deficient chicks, J. Nutr. 143 (5) (2013) 613–619.
[29] Y. Wang, et al., Alleviative effect of selenium on inflammatory damage caused by lead via inhibiting inflammatory factors and heat shock proteins in chicken testes, Environ. Sci. Pollut. Res. Int. 24 (Suppl. 3) (2017) 1–9.
[30] Y. Hou, et al., miR-215 functions as a tumor suppressor and directly targets ZEB2 in human non-small cell lung cancer, Oncol. Lett. 10 (4) (2015) 1985–1992.
[31] T. Liu, et al., MicroRNA-193b-3p regulates hepatocyte apoptosis in selenium-deficient broilers by targeting MAML1, J. Inorg. Biochem. 186 (2018) 235–245.
[32] D.M. Leistner, et al., Abstract 15280: differential release mechanisms of muscle-enriched miRNAs upon ischemic myocardial injury: insights from transcoronary gradient measurements, Circulation 21 (2012) 55–65.
[33] J. Zheng, et al., MicroRNA-30e protects the heart against ischemia and reperfusion injury through autophagy and the Notch1/Hes1/Akt signaling pathway, Int. J. Mol.

Med. 41 (6) (2018) 59–65.
[34] Y. Wang, et al., Copper (II) and/or arsenite-induced oxidative stress cascades apoptosis and autophagy in the skeletal muscles of chicken, Chemosphere (2018) 597–605.
[35] K. Xie, et al., Modulating autophagy improves cardiac function in a rat model of early-stage dilated cardiomyopathy, Cardiology 125 (1) (2013) 60–68.
[36] N. Mizushima, et al., Autophagy fights disease through cellular self-digestion, Nature 451 (7182) (2008) 1069.
[37] L. Valentim, et al., Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischaemia/reperfusion injury, J. Mol. Cell. Cardiol. 40 (6) (2006) 846–852.
[38] B.X. Chu, et al., Interplay between autophagy and apoptosis in lead(II)-induced cytotoxicity of primary rat proximal tubular cells, J. Inorg. Biochem. 182 (2018) 184.
[39] J. KH, et al., Histone deacetylase 6 functions as a tumor suppressor by activating c-Jun NH2-terminal kinase-mediated beclin 1-dependent autophagic cell death in liver cancer, Hepatology 56 (2) (2012) 644–657.
[40] J.J. Lum, R.J. Deberardinis, C.B. Thompson, Autophagy in metazoans: cell survival in the land of plenty, Nat. Rev. Mol. Cell Biol. 6 (6) (2005) 439–448.
[41] Y. Matsui, et al., Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy, Circ. Res. 100 (6) (2007) 914–922.
[42] X.B. Song, et al., Autophagy blockade and lysosomal membrane permeabilization contribute to lead-induced nephrotoxicity in primary rat proximal tubular cells, Cell Death Dis. 8 (6) (2017) 145–154.
[43] B. Vanhaesebroeck, et al., Synthesis and function of 3-phosphorylated inositol lipids, Annu. Rev. Biochem. 70 ((1):535) (2001) 95–104.
[44] D. Elia, et al., Sonic hedgehog promotes proliferation and differentiation of adult muscle cells: involvement of MAPK/ERK and PI3K/Akt pathways, Biochim. Biophys. Acta Mol. Cell Res. 1773 (9) (2007) 1438–1446.
[45] M.A. Krasilnikov, Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation, Biochemistry 65 (1) (2000) 59–67.
[46] M. Liu, et al., Celecoxib regulates apoptosis and autophagy via the PI3K/Akt signaling pathway in SGC-7901 gastric cancer cells, Int. J. Mol. Med. 33 (6) (2014) 1451–1458.
[47] D.U. Tao, et al., LPS regulates macrophage autophagy through PI3K/Akt/mTOR pathway, Chin. J. Pathophysiol. 30 (4) (2014) 675–680.
[48] X.J. Fan, et al., Salidroside induces apoptosis and autophagy in human colorectal cancer cells through inhibition of PI3K/Akt/mTOR pathway, Oncol. Rep. 36 (6) (2016) 3559.
[49] S. Raha, et al., Naringin induces autophagy-mediated growth inhibition by downregulating the PI3K/Akt/mTOR cascade via activation of MAPK pathways in AGS cancer cells, Int. J. Oncol. 47 (3) (2015) 1061–1069.
[50] L. Wang, et al., Crotonaldehyde induces autophagy-mediated cytotoxicity in human bronchial epithelial cells via PI3K, AMPK and MAPK pathways, Environ. Pollut. 228 (2017) 287.
[51] M.A. Bogoyevitch, B. Kobe, Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases, Microbiol. Mol. Biol. Rev. 70 (4) (2006) 1061–1095.
[52] S.T. Pan, et al., Plumbagin induces G2/M arrest, apoptosis, and autophagy via p38 MAPK- and PI3K/Akt/mTOR-mediated pathways in human tongue squamous cell carcinoma cells, Drug Des. Dev. Ther. 2015 (default) (2015) 1601–1626.
[53] B. D'Autréaux, M.B. Toledano, ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis, Nat. Rev. Mol. Cell Biol. 8 (10) (2007) 813–824.
[54] S. R, E. Z, ROS, mitochondria and the regulation of autophagy, Trends Cell Biol. 17 (9) (2007) 422–427.
[55] C.J. Chang, et al., Lutein induces autophagy via Beclin-1 upregulation in IEC-6 rat intestinal epithelial cells, Am. J. Chin. Med. 45 (6) (2017) 1273.
[56] K.F. Hui, P.L. Yeung, A.K.S. Chiang, Induction of MAPK- and ROS-dependent autophagy and apoptosis in gastric carcinoma by combination of romidepsin and bortezomib, Oncotarget 7 (4) (2016) 4454–4467.