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Research Article

miR-9a Induces Apoptosis in Shrimp Primary Cells Through Directly Targeting Host Hsc70 Gene

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Abstract

Previous studies indicate shrimp Heat shock protein Hsc70 play key roles in White spot syndrome virus infection, but its regulatory mechanism remains unclear. In the present study, we identified Hsc70 was a direct target gene of miR-9a in shrimp, overexpression of miR-9a in shrimp primary cells was sufficient to inhibit the mRNA and protein expression of Hsc70 and miR-9a could directly target the coding region of Hsc70, further investigation indicated that the apoptosis- inducing effect of miR-9a in shrimp primary cells was mediated by reducing the production of Hsc70. These findings revealed the possible regulation mechanism of Hsc70 in shrimp and provided novel evidences that miRNAs mayalso target CDS ofmRNAsto play their post-transcriptional regulatory effects.

Keywords: miR-9a; Shrimp; Apoptosis; Hsc70

Introduction

White Spot Disease (WSD), which is caused by a double-stranded DNA virus White spot syndrome virus (WSSV) [1], has become the major threat to global crustacean aquaculture industries [2]. To date nearly 100 potential host species for WSD have been identified, including shrimp, crabs, lobsters, prawns, crayfishes, and copepods [3]. However, despite growing understanding of the underlying molecular biology, cost-effective vaccinations or treatments for the disease remain elusive [4].

Molecular chaperones from the family of 70kDa heat shock proteins (Hsp70s) are conserved among many kinds of organisms. The constitutively expressed members of this family, heat shock cognate proteins (Hsc70s), have been shown to be involved in protein folding in the cytoplasm, protein import into the endoplasmic reticulum, mitochondria and chloroplasts, and trafficking of receptors and coated vesicles [5-7]. In the case of animal viruses, interactions with Hsc70 appear to be involved in cell entry, virion assembly and disassembly, cell transformation, and DNA replication [8,9]. Our previous studies showed shrimp Heat shock protein Hsc70 play key roles in WSSV infection, immune evasion, host cell apoptosis and virus package [10]. However, its regulatory mechanism remains unclear.

Growing evidence has indicated that miRNAs play important roles in gene regulation. MiRNAs are endogenous, non-coding RNA molecules of approximately 19-25 nucleotides in length. They functioned as gene regulator by blocking of mRNA translation or causing mRNA degradation. Depending on their target genes, miRNAs have been shown to be involved in many cellular processes, including virus infection [11]. In this study, we identified Hsc70 was a direct target gene of miR-9a in shrimp, while upregulation of shrimp miR-9a in shrimp primary cells induced apoptosis through repressing Hsc70 expression.

Materials and Methods

Primary shrimp cell culture

Lymphoid organs were obtained from fresh adult P. monodon (100g) and then washed. The lymphoid organs were minced into fragments as small as possible, which were the filtered and transferred to the wells of a 24-well plate, and 1ml of the culture medium containing 2 9L-15 (Invitrogen) supplemented with 15% FCS, 1% glucose, 5g/l NaCl, 100IU/ml penicillin, 100 μg/ml streptomycin, and 5μg/ml gentamicin was added to each well. The plate was then sealed and incubated at 28°C until 70–80% confluent monolayers were formed. These cells were then ready for use.

Quantitative real-time PCR of miRNA

Total RNA was extracted by mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, USA) following the protocol of manufactures. Less than 200μg total RNA was used for cDNA synthesis by TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). A SYBR Green qRT-PCR assay was carried out in an ABI Vix7 Real-Time PCR Detection System, the data were calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method, and U6 was used as an internal control.

The silencing or overexpression of miR-9a

The oligonucleotides miR-9a (miR-9a-mimic) (5'-TCTTTGGTGATCTAGCTG-3'), anti-miR-9a (AMO-miR-9a) (5'-CAGCTAGATCACCAAAGA-3') and the scrambled sequences miR-9a-mimic-scrambled (5'-TGTATCGAGTCGTTCGTT-3'), AMO-miR-9a-scrambled (5'-AACGAACGACTCGATACA-3') were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China).

To determine the dosage of oligonucleotides, 10pM, 50pM, 100pM, 200pM, 500pM or 1000pM of miR-9a-mimic was transfected into cells. Then the miR-9a expression in cells was detected with quantitative real-time PCR. It was indicated that miR-9a-mimic at 100pM or more could efficiently overexpress miR-9a in cells, so

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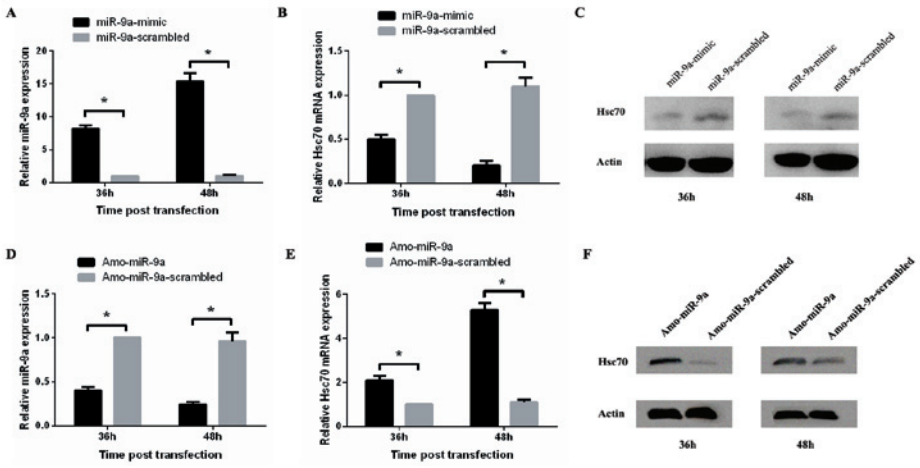

Figure 1: Expression of Hsc70 was regulated by miR-9a. A) miR-9a expression was up-regulated by miR-9a-mimic, then B) mRNA and C) protein expression levels of Hsc70 were detected by qPCR or Western blot respectively; D) miR-9a expression was silenced by Amo-miR-9a, then E) mRNA and F) protein expression levels of Hsc70 were detected by qPCR or Western blot respectively.

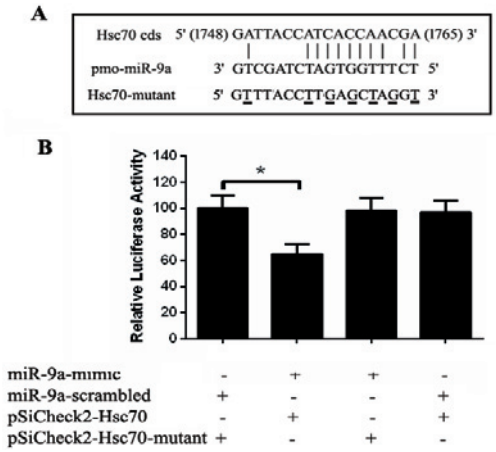

Figure 2: Interaction between miR-9a and Hsc70. A) The predicted sequence of Hsc70 coding targeted by miR-9a and the corresponding mutant sequence; B) Luciferase reporter assay showed the direct interaction between miR-9a and Hsc70 in HEK293 cells.

concentration of 100pM was used in further study.

Immunoblot analysis

Cells were prepared by washing with PBS. Protein extraction and immunoblot analysis was performed as previously described [10]. Primary antibodies included Hsc70 (1:500 dilutions; StressGen Biotechnologies) and GAPDH (1:1000 dilution; Cell Signaling Technology).

Luciferase reporter assay

A 30 bp DNA sequence containing the predicted miR-9a-Hsc70 binding site and the corresponding mutant constructs were synthetized and cloned into psiCHECK-2 Luciferase vector. The constructs were verified by sequencing. For luciferase reporter assays, HEK293T cells were seeded in 24-well plates and transiently co-transfected with appropriate reporter plasmid and miRNA. After 48h, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) in a multi-function micro-plate reader (Tristar2 LB942, Berthold, Germany). Firefly-luciferase was used for normalization.

Apoptosis analysis with Annexin V

Shrimp primary cells were collected and washed with PBS and then gently suspended in 1 × Annexin-Bindingbuffer. Then, Apoptosis assay was conducted with Annexin V (Invitrogen, USA) accord to the manufacturer's protocol and detected by flow cytometry as previously described [12].

Statistical analysis

All statistical analyses were carried out using SPSS 18.0 statistical software. Continuous data were compared using Student's 2-tailed t test. Data are represented as mean ± SEM. In all cases, $P < 0.05$ was considered statistically significant.

Results

miR-9a regulated Hsc70 expression in shrimp primary cells

To predict the potential miRNAs targeting Hsc70, three algorithms, including Target Scan (http://www.targetscan.org), miRanda (http://www.microrna.org/), and Pictar (http://pictar.mdc-berlin.de/) were conducted, *Penaeus monodon* Hsc70 mRNA (GenBank: AF474375.1) sequence was used in the analysis. The data predicted by the three algorithms were overlapped and calculated, and the prediction results indicated that the shrimp miR-9a could target Hsc70 CDS region.

Then the effects of ectopically expressed miR-9a on the mRNA and protein levels of Hsc70 were evaluated by qPCR and Western blot analysis, the results showed that overexpression of miR-9a in shrimp primary cells was sufficient to inhibit the mRNA and protein expression of Hsc70 (Figure 1A-C), while inhibition of miR-9a expression in shrimp primary cells by Amo-miR-9a would remarkably increase the endogenous expression of Hsc70 (Figure 1D-F).

miR-9a could directly target the codingregion of Hsc70

To validate the interaction between miR-9a and Hsc70, the

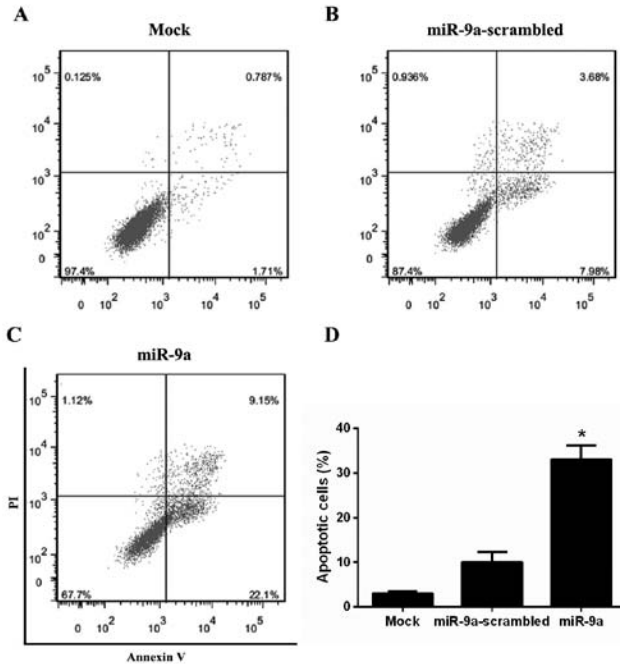

Figure 3: Effects of miR-9a overexpression on apoptosis in shrimp primary cells. Cells were treated with A) Mock, B) miR-9a-mimic-scrambled and C) miR-9a-mimic respectively and then stained with Annexin V and PI and detected by flow cytometry. D) The percentages of Annexin V-positive cells of three independent experiments.

luciferase reporter analysis was conducted in HEK293T cells. A DNA sequence containing the predicted miR-9a-Hsc70 binding site were inserted into psiCHECK-2 Luciferase vector, and the corresponding mutant constructs was used as negative control (Figure 2A). The results showed that co-transfection of miR-9a-mimic and psiCHECK2-Hsc70 caused significant decrease of luciferase activity compared with control groups (Figure 2B), which confirmed the direct interaction between miR-9a and Hsc70.

miR-9a upregulation induced host cell apoptosis

Apoptosis assay of shrimp primary cell with/without miR-9a overexpression was performed and detected by flow cytometry. Our results indicated that the apoptosis of shrimp primary cells increased substantially after the treatment with miR-9a-mimic, both the early (Annexin V^+PI^-) and late apoptosis (Annexin V^+PI^+) percentage significantly increased (Figure 3C and D), indicating that the miR-9a along can induce apoptosis.

miR-9a induced apoptosis through Hsc70

We then assessed if targeting Hsc70 wouldphenocopy miR-9a induced cell apoptosis. Firstly, by using siRNAs designed for Hsc70 [10], we demonstrated that down-regulation of Hsc70 could efficiently inducing cell apoptosisin shrimp primary cells (Figure 4A), which correlated with the effect of overexpressed miR-9a. Subsequently, the Annexin V apoptosis assaywas repeated with Amo-miR-9a and si-Hsc70 co-transfected. As shown in Figure 4B, silencing of Hsc70 induced cell apoptosis was largely eliminated upon the down-regulation of miR-9a. All these data presented here strongly suggest that the apoptosis- inducing effect of miR-9a in shrimp primary cells

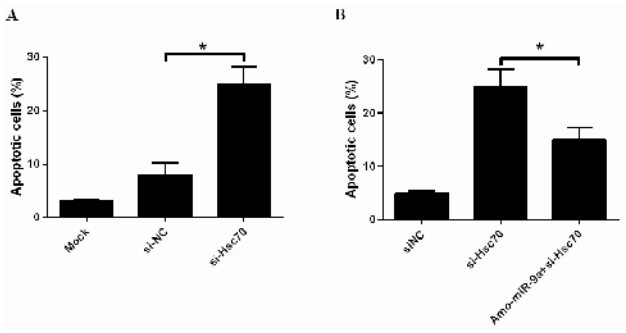

Figure 4: MiR-9a induced apoptosis through Hsc70. A) Down-regulation of Hsc70by siRNA could efficiently inducing cell apoptosis in shrimp primary cells. B) Silencing of Hsc70 induced cell apoptosis was largely eliminated upon the down-regulation of miR-9a.

was mediated by reducing the production of Hsc70 asits predominant target.

Discussion

Growing evidences indicate the important role of miRNAs as post-transcriptional regulators of gene expression. Initially miRNAs were characterized to regulate gene expression by targeting the miRNA binding sites located in the 3' untranslated regions (UTR) to degrade or repress the translation of mRNAs. However, recent increasing evidence suggests that miRNAs can also bind in the coding region (CDS) of mRNA [13]. For example, miR-15/107 group of miRNAs post-transcriptional regulate of BRCA1 through its coding sequence [14]. MicroRNA-9 induces defective trafficking of Nav1.1 and Nav1.2 by targeting Navβ2 protein coding region in rat with chronic brain hypo perfusion [15]. Cgi-miR-92d indirectly regulates TNF expression by targeting CDS region of lipopolysaccharide-induced TNF-a factor 3 (CgLITAF3) inoyster *Crassostreagigas* [16]. MiR-932 targets the coding region fits hostgene, Drosophilaneuroligin2 [17]. MiRNAs targeting CDS have a smaller impact on mRNA stability compared with miRNAs targeting 3' UTRs [13], but individual miRNAs differ in their preference for targeting the CDS or the 3' UTR of mRNAs and CDS-targeting may speed up silencing of stable mRNAs [18].

MiR-9a belongs to the conserved miR-9 family, it plays important role in embryo development [19]. Our previous study demonstrated that shrimp miR-9a was significantly upregulated in response to the WSSV infection, and it could promote the antiviral apoptosis of shrimp to against the virus infection, but the underlying mechanism remains unclear. In the present study, we identified overexpression of miR-9a in shrimp primary cells was sufficient to inhibit the mRNA and protein expression of Hsc70 and miR-9a could directly target the coding region of Hsc70, further investigation indicated that the apoptosis- inducing effect of miR-9a in shrimp primary cells was mediatedby reducing the production of Hsc70. These findings revealed the possible regulation mechanism of Hsc70 in shrimp and provided novel evidences that miRNAs may target also CDS to play their post-transcriptional regulatory effects.

Acknowledgement

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