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Different roles of a novel shrimp microRNA in white spot syndrome virus (WSSV) and *Vibrio alginolyticus* infection

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ABSTRACT

In this study, *Marsupeneaus japonicus* microRNA-S5 (miR-S5) was found to be up-regulated 24 h post white spot syndrome virus (WSSV) or *V. alginolyticus* infection. The loss of function using an *anti*-microRNA oligonucleotide (AMO-miR-S5) showed that expression levels of multiple innate immune-related genes were affected. The expression of p53 and tumor necrosis factor-α (TNF-α) were significantly down-regulated, expression of myosin was significantly up-regulated. The miR-S5 knockdown delayed WSSV-induced death for 48 h, but the final mortality was not affected, while *V. alginolyticus*-induced mortality was increased by 30%. The effect of miR-S5 knockdown on phagocytosis and apoptosis rates showed that miR-S5 knock down significantly decreased phagocytosis rate of WSSV from 27.8% to 7.0%, and phagocytosis rate of *V. alginolyticus* from 27.2% to 21.4%, separately. WSSV-induced apoptosis decreased from 60.83% to 51.25%, but no effect on *V. alginolyticus*-induced apoptosis (43.72%–45.04%). We concluded that miR-S5 could be used by WSSV via regulating hemocyte phagocytosis and apoptosis processes, but helps to defend against bacterial infection by regulating the proPO system, superoxide dismutase activity and phagocytosis.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that generally function by base pairing with sequence-specific messenger RNAs to regulate their expression, consequently influencing various biological processes (Bartel, 2004). Since their first identification in *Caenorhabditis elegans*, numerous miRNAs have been identified in multiple organisms, including mammals, plants, insects and viruses by computational or experimental methods (Taganov et al., 2007). Mammals have been reported to use cellular miRNAs to combat viral infections through the interferon system (Pedersen et al., 2007). Thirty-five miRNAs were first identified in the shrimp *Marsupenaeus japonicus* (Ruan et al., 2011), some signature miRNAs have been shown to be involved in the innate immune system of invertebrates (e.g. phagocytosis, apoptosis, and phenol oxidase (PO) (Yang et al., 2012)). Sixty-three miRNAs were identified in *M. japonicus* following white spot syndrome virus (WSSV) challenge (Huang et al., 2012), and shrimp miR-7 was found to inhibit WSSV replication by targeting the viral early gene (Huang and Zhang, 2012). We also recently identified 55 miRNAs from *M. japonicus* that either were up-regulated or down-regulated following *Vibrio alginolyticus* infection (Zhu et al., 2015).

In our previous study, the expression level of miR-S5 in *M. japonicus* was affected following WSSV or *V. alginolyticus* infection (Wang and Zhu, 2017). In this study, the role of miR-S5 in the shrimp innate immune system was further explored.

2. Materials and methods

2.1. Materials preparation

Healthy shrimps were purchased from local seafood market in Hangzhou, Zhejiang, China. Three to four shrimps were randomly selected for virus detection, then the shrimps were maintained under experimental conditions for 1 week. WSSV (GenBank accession no. AF332093) was purified and applied to virus challenge experiments as described previously (Wang and Zhu, 2016). *V. alginolyticus* (ATCC17749) was cultured and applied to bacteria challenge experiments as described previously (Wang et al., 2017). Healthy shrimps were divided into five groups: three groups were treated with PBS, WSSV or *V. alginolyticus*, respectively, while the other two groups were treated with a mixture of AMO-miR-S5 and

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pathogens (WSSV + AMO-miR-S5 or *V. alginolyticus* + AMO-miR-S5, respectively). For apoptosis analysis, an AMO-miR-S5 only group was included. At different time post pathogen treatments, shrimp hemolymph and/or gills were collected for subsequent experiments.

2.2. Sequencing of small RNAs

Total RNA was extracted from hemocytes of infected or uninfected shrimps at 24 and 48 h post-infection using miRNA isolation kit (Ambion, CA, USA) in accordance with the manufacturer's protocol. The following steps were performed as described previously (Huang et al., 2012).

2.3. In vitro loss-of-function of miRNA

The miR-S5 expression was knocked down by injection of the *anti*-microRNA-S5 oligonucleotide (AMO-miR-S5) at 0.15 mM. The specificity of AMO-miR-S5 was confirmed by scrambling the sequence (swapping T and C; AMO-miR-S5-scramble). AMO-miR-S5-scramble (0.15 mM) or high-saline PBS were used as controls, respectively. To avoid degradation of AMO-miR-S5 and AMO-miR-S5-scramble, the respective oligonucleotides were injected sequentially into the same shrimp twice within a 24 h interval. Hemocytes were collected from at least three random shrimp individuals (as technical repeat) 24 h post the last injection, mixed, and subjected to further assays. All the assays described above were repeated biologically three times.

2.4. qPCR of mRNA and miRNA

The qPCR of mRNA was performed as described before (Wang and Zhu, 2016). The method of miRNAs qPCR was designed based on stem-loop real-time PCR technology and was performed as described before (Wang et al., 2017; Hellens, 2011). Primer sequences were listed in Table 1.

2.5. Shrimp mortality

Shrimps (>20 individuals) were injected with AMO-miR-S5 in the lateral area of the fourth abdominal segment, followed 12 h later by 100 μL of WSSV (10^5 copies/mL)/*V. alginolyticus* (10^6/mL) containing AMO-miR-S5, using a syringe with a 29-gauge needle. WSSV/*V. alginolyticus* groups received injections of 100 μL WSSV (10^5 copies/mL)/*V. alginolyticus* (10^6/mL) in PBS. Shrimps of control (>20 individuals) were injected with PBS or WSSV only. Shrimp mortality was monitored every 12 h after the last injection. Mortality experiment was biologically repeated for three times; an average mortality was represented as result.

2.6. Detection of WSSV copies

Shrimp gills were taken at 0 h, 12 h, 24 h, 48 h, 72 h, 96 h post treatment for whole-genome extraction using DNA extraction Kit (Tiangen, China). The extraction process of gDNA was performed according to manual. WSSV copies were detected by TaqMan vp28 probe in Two Color Real-Time PCR Detection System (Bio-Rad, CA, USA) as described before (Wang and Zhu, 2016). Standard curve was made based on previous experiment (Gong et al., 2015).

2.7. The immune parameters analysis

Immune parameters including total hemocyte count (THC), PO activity, and SOD activity were determined. At 24 h, 48 h post treatment, shrimp hemolymph from at least three shrimps of each group was collected for immune parameter analysis, each experiment was biologically repeated for three times. Shrimp anticoagulant (450 mM NaCl, 10 mM EDTA-Na_2, 10 mM HEPES, pH 7.3, 850 mOsm/kg) was prepared according to previous study (Wang et al., 2017). Approximately 1.0–1.5 mL shrimp hemolymph was collected using an anticoagulant soaked syringe, together with equal volume of anticoagulant. The mixture was kept on ice. Add 50 μL 4% paraformaldehyde in 150 μL mixture to immobilize the hemocyte for THC as described before (Wang et al., 2017). Based on the calculate method of the hemocytometer plate, the THC of each group represented the total hemocyte count in 1 mL hemolymph. 500–1000 μL hemolymph was centrifuged at 300 g for 10 min to separate the hemocyte cells and the serum for PO activity analysis. Equal volumes of serum (50 μL) of each group were incubated with 50 μL of L-DOPA for 30 min at 40 °C. The incubated serums were applied to measure the PO activity using a BioRad spectrophotometer (Bio-Rad, CA, USA) at a wavelength of 490 nm. 100 μL hemolymph was fully mixed with 0.5 mL phosphate buffer (PB, 50 mM, pH7.8), the homogenate was applied to SOD activity detection. SOD extraction method and the improved Nitro blue tetrazolium (NBT) detection method were described in our early research (Zhu et al., 2015; Campa-Córdova et al., 2002).

2.8. Phagocytosis detection by flow cytometry

Healthy shrimps were randomly separated into two groups, each group contained at least three shrimps as technical repeat. One group was treated with high saline PBS as control, the other group was treated with AMO-miR-S5 as knock down treatment group. 24 h post injection, hemolymph of PBS group and AMO-miR-S5 group were draw separately for phagocytosis experiment as described before (Zhu et al., 2015).

2.9. Apoptosis analysis with Annexin V by flow cytometry

Apoptosis assay of shrimps with/without miR-S5 knockdown was conducted with Annexin V (Invitrogen, CA, USA) accord to the manufacturer's protocol. Briefly, shrimp hemolymph was draw with equal volume anticoagulant, centrifuged at 300 g × 5 min to collect hemocytes, and washed once in pre-cold sterilized high-salt PBS. Gently suspend hemocytes in 100 μL 1 × Annexin-Binding buffer containing 5 μL of Alexa Flour 488 Annexin V and 1 μg/mL of PI (propidium iodide). A non-stained empty control, a PI-only negative control, and an FITC-only positive control were prepared to help setting up thresholds of PI and FITC fluorescence channel. After incubation at room temperature for 15 min or at 4 °C for 30 min, add 400 μL Annexin-binding buffer to end reaction. Centrifuge again to wash away residual dye and suspend hemocytes with 500 μL Annexin-Binding buffer. Filter cell fragments using throwaway filter (provided by flow cytometry detection platform). The fluorescence emission of the stained samples was examined by flow cytometry at the wavelengths of 530 nm and 575 nm.

2.10. Statistical analysis

Means and standard deviations of the results of three biologically repeated assays were analyzed by one-way analysis of variance. Differences between treatments at different times were analyzed using two-way ANOVA multiple comparisons.

Table 1
Primer names, sequences, and purpose of all primers and probes used in this research.

Primer Name	Primer Sequence (5′ to 3′)	Used for
miR-S5 RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTCACTGGATACGACGGCTGGG	miRNA reverse transcription primer
U6 RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTCACTGGATACGACCTCACTT	
miR-S5 real time F	CGCCGTTATTGCACTTTC	miR-S5 Stem-Loop real time Q-PCR primer
miRNA Universal-R	TGCAGGGTCCGAGGTCACTG	
U6 real time F	TTCACGAATTTGCGTGTCAT	Stem-Loop real time PCR internal control primer
U6 real time R	CGCTTCGGCAGCACATATAC	
AMO-miR-S5	CTGGGGAAAGTGCAAT	miRNA knock down
AMO-miR-S5 scrambled	TCGGGGAAAGCGTAAC	
GAPDH-F	GGTGCCGAGTACATCGTTGAGTC	Real-time PCR primers of innate immunity related pathway genes
GAPDH-R	GGCAGTTGGTAGTGCAAGAGGC	
hemocyanin-F	AACCCTGAACAAAGAGTTGCCTAT	
hemocyanin-R	AACGGACGGTAAGTTGATGATGT	
IMD-F	ATTCATCCGTCTACCTCCCTACA	
IMD-R	GAGCTGAGTCTGTCTTAATGTTATCC	
myosin -F	GCCCAGGTCAAGAAGGACAAGGA	
myosin -R	AAGACGCTCACCAAGGGACAGGA	
*p*53-F	TTCCTGCCTGGCTGACTCTA	
*p*53-R	CACCCAATCTTCCAACATCACAT	
MAPK-F	CGCATCACTGTTGAGGAGG	
MAPK-R	GCAGGTCATCAAGTTCCATCT	
*pro*PO-F	TTCTACCGCTGGCATAAGTTTGT	
*pro*PO-R	TATCTGCCTCGTCGTTCCTCAC	
Rab7-F	TCATTAGGTGTTGCATTTTATCGC	
Rab7-R	AGGCTTGAATTAGGAACTCGTC	
Rho-F	GTGATGGTGCCTGTGGTAAA	
Rho-R	GCCTCAATCTGTCATAGTCCTC	
QM-F	CGTCACAAGGAGCAGGTTATT	
QM-R	GGGACCATGTTCAGGGAGA	
L-type lectin-F	ATGTTATGCCATCTGCCTCGTATTT	
L-type lectin-R	CTTTCGCTGCTGCTCTTTCTGTT	
STAT-F	TGGCAGGATGGATAGAAGACAAG	
STAT-R	TGAATAAGCTGGGATACGAGGGA	
Toll-like receptor-F	CCACCTAAAGTCATCATCGCCAGTA	
Toll-like receptor-R	TCTTCATTCACCACAGCCCACAA	
TNF-alpha-F	ACAGACGGTCCAGAGTCCCAAAG	
TNF-alpha-R	GCGACGAAGTGAGCCACAGTAA	

3. Results

3.1. Identification of miRNA response to WSSV and V. alginolyticus infection in shrimps

We previously found that the expression levels of some miRNAs were significantly up-regulated or down-regulated 24 h after WSSV and/or *V. alginolyticus* infection. Microarray analysis showed that miR-S5 expression was significantly up-regulated at 24 h post-WSSV and -*V. alginolyticus* infection, but not at 48 h post-infection. Quantitative stem-loop real-time polymerase chain reaction (qPCR) was performed to confirm miR-S5 expression. The miR-S5 expression was significantly up-regulated compared with the phosphate-buffered saline (PBS) control at 24 h post-challenge, but its expression was down-regulated at 48 h post-infection.

3.2. miR-S5 knockdown using anti-miRNA oligonucleotide

MiR-S5 expression was knocked down using an *anti*-miRNA oligonucleotide (AMO) to explore its function in the shrimp innate immune system. The sequence of AMO-miR-S5 is shown in Table 1 and Fig. 2A. The T and C in the AMO-miR-S5 sequence were swapped to generate a scrambled control to verify the specificity. High-saline PBS was used as a control. MiR-S5 expression was significantly inhibited at 24 h post-injection, compared with the scrambled control or PBS control, and was strongly inhibited from 24 to 48 h (Fig. 2B and C). Expression levels of several genes involved in multiple innate immune pathways (Fig. 2D) was affected. Myosin was significantly up-regulated by AMO-miR-S5

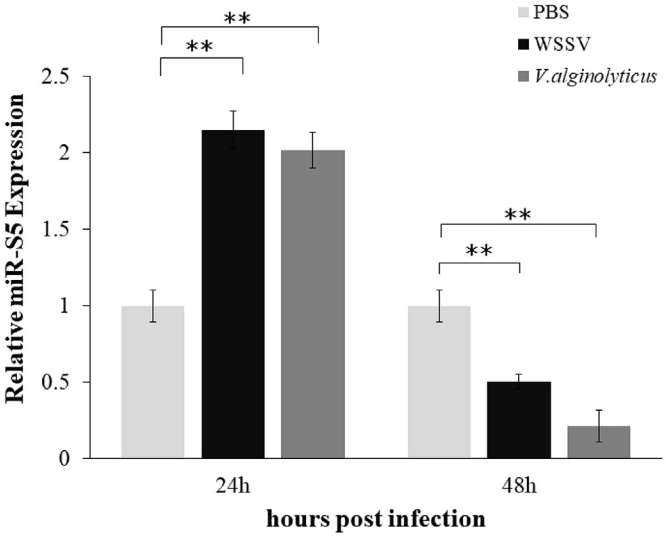

Fig. 1. miR-S5 response to WSSV or *V. alginolyticus* infection. Total RNA was extracted from hemocytes of uninfected or infected shrimps. Quantitative stem-loop real-time PCR was performed to reveal the post-infection expression of miR-S5. MiR-S5 expression was normalized to U6 expression level, miR-S5 expression of PBS was normalized as one. Data are shown as mean ± standard deviation of three separate individuals. Asterisks indicate significant difference ($P < 0.01$) between two samples.

($P < 0.01$), while the p53 and tumor necrosis factor (TNF)-α genes were significantly ($P < 0.01$) down-regulated after miR-S5 inhibition. Other genes with acknowledged immunity function showed

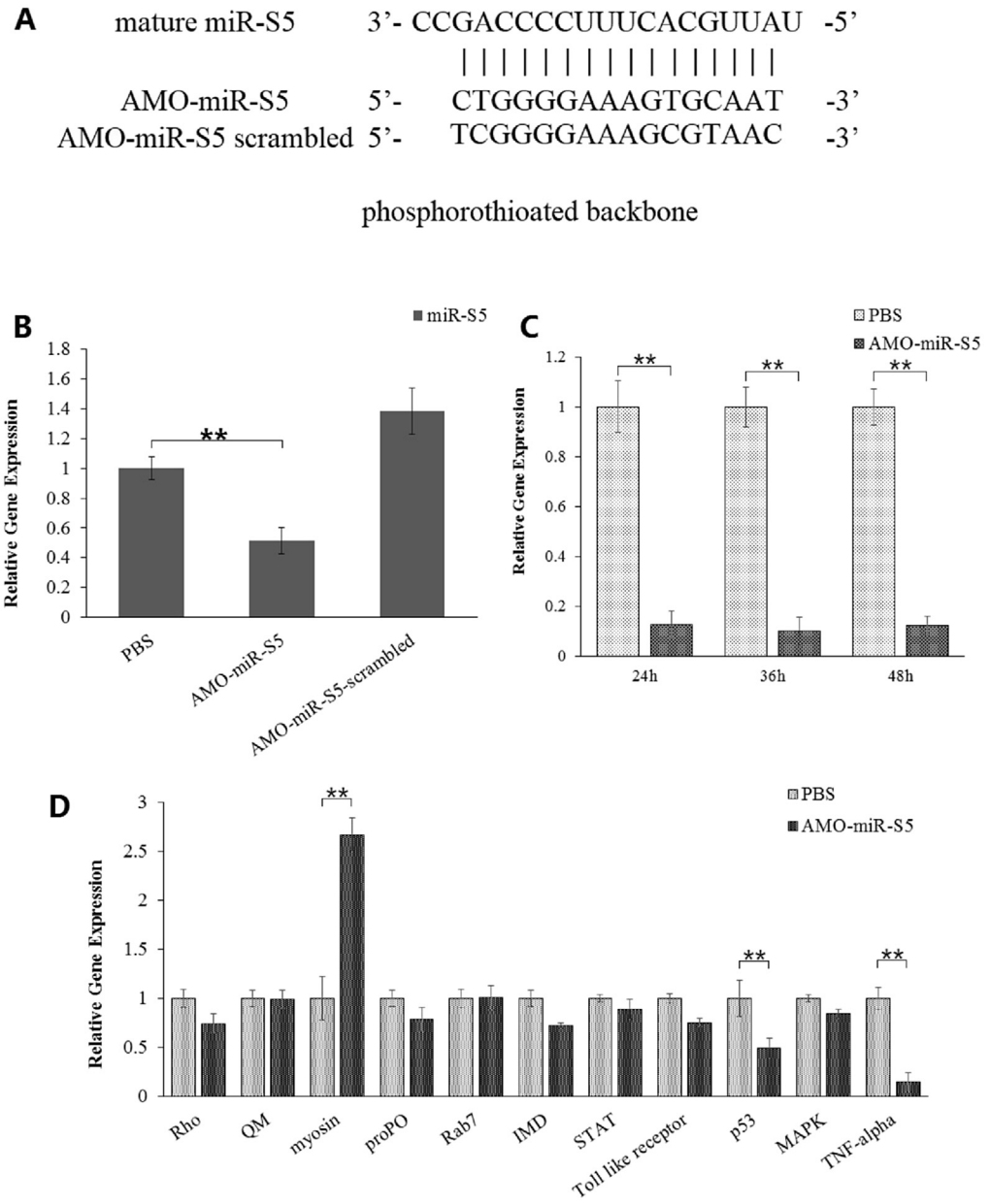

Fig. 2. Loss function of shrimp miR-S5. AMO-miR-S5 was used to inhibit shrimp miR-S5 expression, a scrambled primer, AMO-miR-S5 -scrambled was used to verify the specificity of AMO-miR-S5. (A) Sequences of AMO-miR-S5, scrambled control and mature miR-S5. (B) To confirm the knock down effect of AMO-miR-S5 primer, shrimps were treated with high saline PBS, AMO-miR-S5, or AMO-miR-S5 scrambled, separately. 24 h post treatment, hemocyte RNA was extracted from each treatment and was analyzed by quantitative Stem-Loop real-time PCR to detect miR-S5 expression. MiR-S5 expression level was normalized to U6 expression level, and miR-S5 expression of PBS treatment was used as index 1. (C) Prolonged miR-S5 knockdown by AMO-miR-S5 was detected at 24, 36 and 48 h. (D) Expression levels of eleven immune genes (Rho, QM, proPO, Rab7, IMD, STAT, Toll-like receptor, p53, MAPK, myosin and TNF-α) in shrimp hemocytes were detected by SYBR Green qPCR. Expression levels of mRNA in qPCR were normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA levels. Data are shown as mean ± standard deviation of three separate individuals. Asterisks indicate significant difference ($P < 0.01$) between two samples.

no expression change.

3.3. Effects of miR-S5 knockdown on WSSV or V. alginolyticus infection

WSSV infection affected miR-S5 expression immediately. Shrimps started to show symptoms of WSSV infection at 12 h, and mortality increased rapidly from 20% to 100%. Shrimps treated with WSSV + AMO-miR-S5, in which miR-S5 expression was inhibited, did not develop symptoms of WSSV infection nor did the mortality count increased until 60 h post-injection. Both treatments reached 50% mortality at 96 h, and the mortality of the WSSV + AMO-miR-S5 group was about 10% lower when the mortality of WSSV-alone group reached 100%. Shrimps injected with *V. alginolyticus* alone started to die at 60 h, and the mortality increased above 50% at 108 h. In contrast, shrimps started to die much earlier at 24 h, and the mortality increased above 50% at 96 h in shrimps treated with *V. alginolyticus* + AMO-miR-S5.

These results demonstrated that AMO-miR-S5 could delay shrimp death caused by WSSV, reduce the final mortality (Fig. 3A). Inhibition of miR-S5 by AMO-miR-S5 could also significantly ($P < 0.01$) increase shrimp mortality caused by *V. alginolyticus*

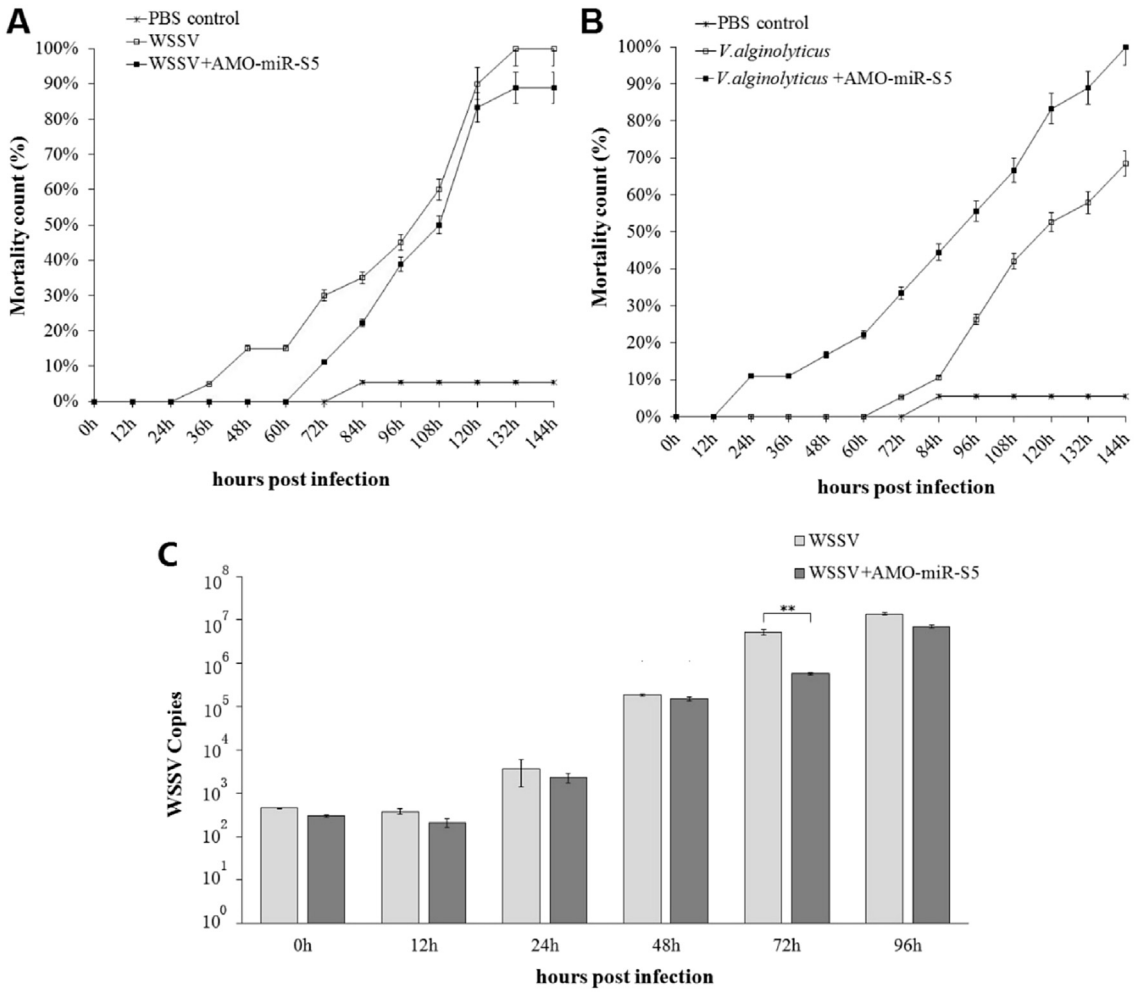

Fig. 3. Effects of miR-S5 knock down on WSSV or *V. alginolyticus* infection. (A) Effects of WSSV challenge and miR-S5 inhibition on shrimp mortality. (B) Effects of *V. alginolyticus* challenge and miR-S5 inhibition on shrimp mortality. Healthy shrimps treated with high-saline PBS were used as controls. Each group contained at least 20 shrimps to ensure adequate confidence levels, and treatments were repeated biologically three times to avoid any influences of weather, individual body condition, and injection-operation error. (C) WSSV copies detected by TaqMan VP28 probe using SYBR Green q PCR at different times post-infection.

compared with *V. alginolyticus* challenge alone (Fig. 3B). These data suggest that miR-S5 may contribute to the anti-*Vibrio* immune response and affect the mortality of *V. alginolyticus*-infected shrimps, whilst playing a different role in response to WSSV infection. We confirmed the role of miR-S5 in WSSV infection by measuring the number of WSSV vp28 copies after WSSV injection, and showed that the number of WSSV copies in the WSSV + AMO-miR-S5 group was lower than in the WSSV-alone group (Fig. 3C).

3.4. Influence of miR-S5 knockdown on shrimp immune parameters

The total hemocyte count (THC) in the WSSV-alone group was lower than that in the PBS group at 24 h and 48 h, while the THC in the WSSV + AMO-miR-S5 group was slightly higher than in the WSSV group at 24 h, but lower than the WSSV group at 48 h (Fig. 4A). The THC in the *Vibrio*-alone group was lower than in the PBS group at 24 h and 48 h, while the THC in the *V. alginolyticus* + AMO-miR-S5 group was slightly lower than in the *Vibrio*-alone group (Fig. 4B). These results indicated that the THC decreased in shrimps 24 h after WSSV infection, but inhibition of miR-S5 expression also inhibited the THC decrease caused by WSSV. However, miR-S5 inhibition could not save the virus-induced hemocyte loss at 48 h. Meanwhile, *Vibrio* infection also led to a decrease in THC, but inhibition of miR-S5 expression resulted in a further decrease in THC, indicating that loss of miR-S5 could exacerbate *Vibrio* infection.

PO activity in shrimp hemolymph was significantly increased from 0.2U to 0.7U at 24 h following WSSV infection compared with the PBS control group, and was significantly decreased to 0.3U in the WSSV + AMO-miR-S5 group. At 48 h, PO activity in WSSV group increased from 0.3U to 0.8U, after WSSV + AMO-miR-S5 treatment PO activity dropped to 0.4U (Fig. 4C). In bacteria treatment groups, PO activity increased from 0.2U to 0.9U at 24 h following *Vibrio* infection, in the *V. alginolyticus* + AMO-miR-S5 group, PO activity continue to increase from 0.9U to 1.6U. A similar result was observed at 48 h, the PO activity increased from 0.3U to 0.6U, after *V. alginolyticus* + AMO-miR-S5 treatment, PO activity increased to 1.2U, significantly higher than other two treatment groups (Fig. 4D).

Relative superoxide dismutase (SOD) activity was calculated according to a previous study (Zhu et al., 2015), with SOD activity in the PBS group taken as the reference level with a value of 1U. Relative SOD activity following WSSV infection increased significantly to 1.45U at 24 h, then dropped slightly to 1.34U at 48 h. Relative SOD activities in the WSSV + AMO-miR-S5 group were 1.35U at 24 h and 1.31U at 48 h (Fig. 4E). Relative SOD activities increased to 1.26U and 1.48U at 24 h and 48 h, respectively, after

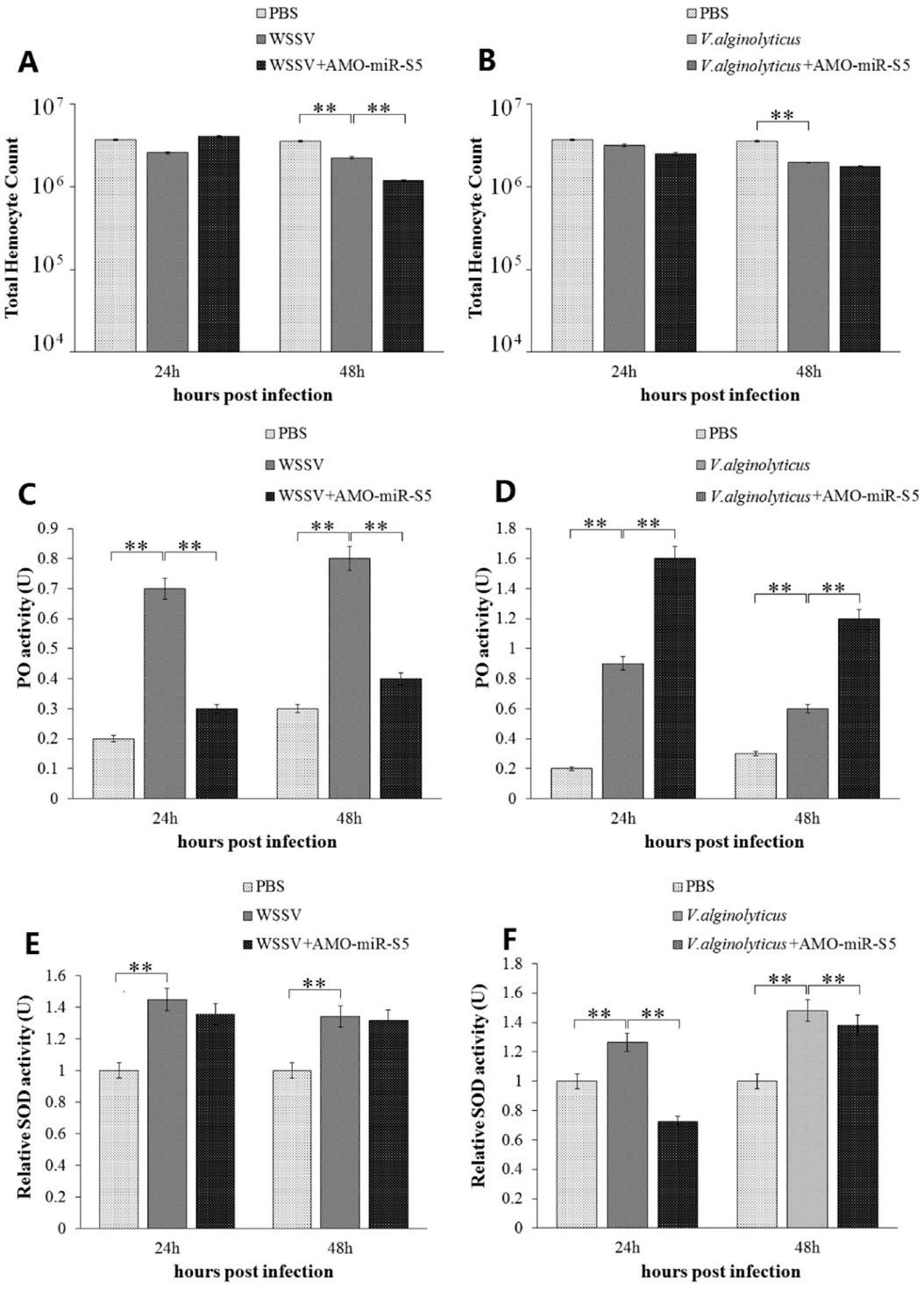

Fig. 4. Effects of miR-S5 knock down on shrimp immune parameters. Shrimps were separated into five groups: two groups were treated with the respective pathogens (WSSV or *V. alginolyticus*) alone and two groups were treated with the respective pathogens plus AMO-miR-S5. Shrimps in the other group were treated with high-saline PBS as a control. Samples were taken at 24 h or 48 h post-injection. (A) THC after WSSV or WSSV + AMO-miR-S5 treatment; (B) THC after *V. alginolyticus* or *V. alginolyticus* + AMO-miR-S5 treatment; (C) hemolymph PO activity after WSSV or WSSV + AMO-miR-S5 treatment; (D) hemolymph PO activity after *V. alginolyticus* or *V. alginolyticus* + AMO-miR-S5 treatment. Each unit of PO activity represents the oxidative activity in 50 μL hemolymph per min. (E) Relative SOD activity after WSSV or AMO-miR-S5+WSSV treatment; (F) relative SOD activity after *V. alginolyticus* or AMO-miR-S5+*V. alginolyticus* treatment. SOD activity in the PBS control was taken as 1, and each column represents the relative exvalue compared to PBS. All treatments at each time point included at least three shrimp individuals, and all experiments were repeated biologically three times. Each column represents the mean value of triplicate assays.

V. alginolyticus infection, while the equivalent activities in the *V. alginolyticus* + AMO-miR-S5 group were significantly lower at 24 h (0.72U), and slightly lower at 48 h (1.38U) (Fig. 4F). Inhibition of miR-S5 expression thus slightly reduced the high SOD activity caused by WSSV at 24 h and 48 h, but the difference was not significant ($P > 0.05$). In contrast, *V. alginolyticus* infection also significantly enhanced SOD activity in shrimp hemolymph at 24 h and 48 h, but inhibition of miR-S5 expression significantly ($P > 0.05$) reduced the high SOD activity at 24 h and 48 h.

3.5. Influence of miR-S5 knockdown on hemocyte phagocytosis

The percentage of fluorescein isothiocyanate (FITC)-positive hemocytes in the WSSV group was significantly down-regulated from 27.8% to 7.0% after miR-S5 inhibition (Fig. 5). A similar result was seen in the *Vibrio* group, in which the percentage of FITC-positive hemocytes was down-regulated from 27.2% to 21.4% (Fig. 5). The decrease in FITC-positive cells represents a decrease in hemocyte phagocytosis, as indicated by flow cytometry, which showed that inhibition of miR-S5 was associated with inhibition of phagocytosis. These results demonstrate that miR-S5 was involved in the phagocytosis of shrimp hemocytes, and its effect on phagocytosis was greater following WSSV compared with *V. alginolyticus* infection.

3.6. Influence of miR-S5 knockdown on hemocyte apoptosis

No significant difference was observed in the rates of apoptosis between the PBS and AMO-miR-S5 control groups. The apoptosis rate in the WSSV-treated group was 60.83%, decreased to about 10% following inhibition of miR-S5. In contrast, the rates of apoptosis in the *V. alginolyticus*- and *V. alginolyticus* + AMO-miR-S5-treated groups showed no significant difference (Fig. 6). These results indicated that miR-S5 was involved in virus-induced apoptosis, but apoptosis induced by bacteria was unaffected by miR-S5 inhibition. The down-regulation of virus-induced apoptosis suggested that miR-S5 could positively regulate virus-induced apoptosis.

4. Discussion

We previously identified a group of miRNAs that was up-regulated and/or down-regulated following pathogen challenge (Zhu et al., 2015). Some miRNAs are highly conserved among invertebrates and vertebrates, while others are more specific, including some only discovered in shrimps. The miR-S5 is a shrimp-specific miRNA that is affected by pathogen challenge. In this study, the role of miR-S5 in the shrimp innate immune system was investigated. Microarray experiments revealed that miR-S5 was up-regulated at 24 h and 48 h after *V. alginolyticus* infection (data not showed), stem-loop real-time qPCR confirmed that miR-S5 was up-regulated at 24 h post-WSSV or -*V. alginolyticus* infection, but down-regulated at 48 h (Fig. 1). The expression changes in response to pathogen challenge indicated a role for miR-S5 in anti-pathogen processes in shrimps. MiR-S5 expression was knocked down using an *anti*-miRNA oligonucleotide (Fig. 2A). Stem loop real-time PCR (Fig. 2B and C) confirmed that miR-S5 expression in shrimp

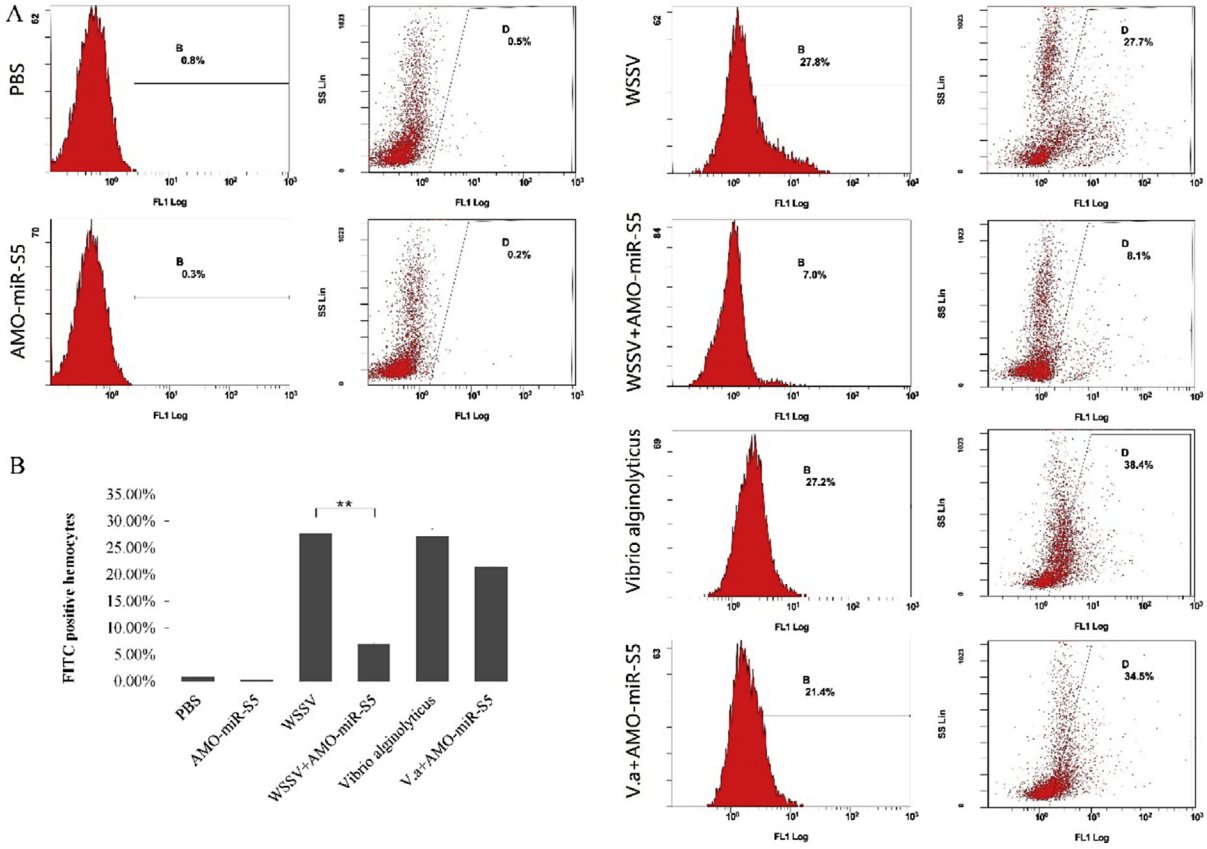

Fig. 5. Effects of miR-S5 knock down on hemocyte phagocytosis. Hemocyte phagocytosis was detected in hemolymph from healthy shrimps treated with high-saline PBS or AMO-miR-S5. FITC-labeled WSSV virions and *V. alginolyticus* were added to healthy or miR-S5-inhibited hemocytes. (A) Fluorescence peak area represents the fluorescence intensity in hemocytes, indicating the percentage of pathogen-engulfed hemocytes. The scatter plot represents the granularity of hemocytes, with an increment in cell granularity suggesting the ability of a single cell to engulf the pathogen. (B)The influence of AMO-miR-S5 was illustrated by transforming the data into a bar chart. All treatments included at least three shrimp individuals, and all experiments were repeated biologically three times. Each column represents the mean value of triplicate assays.

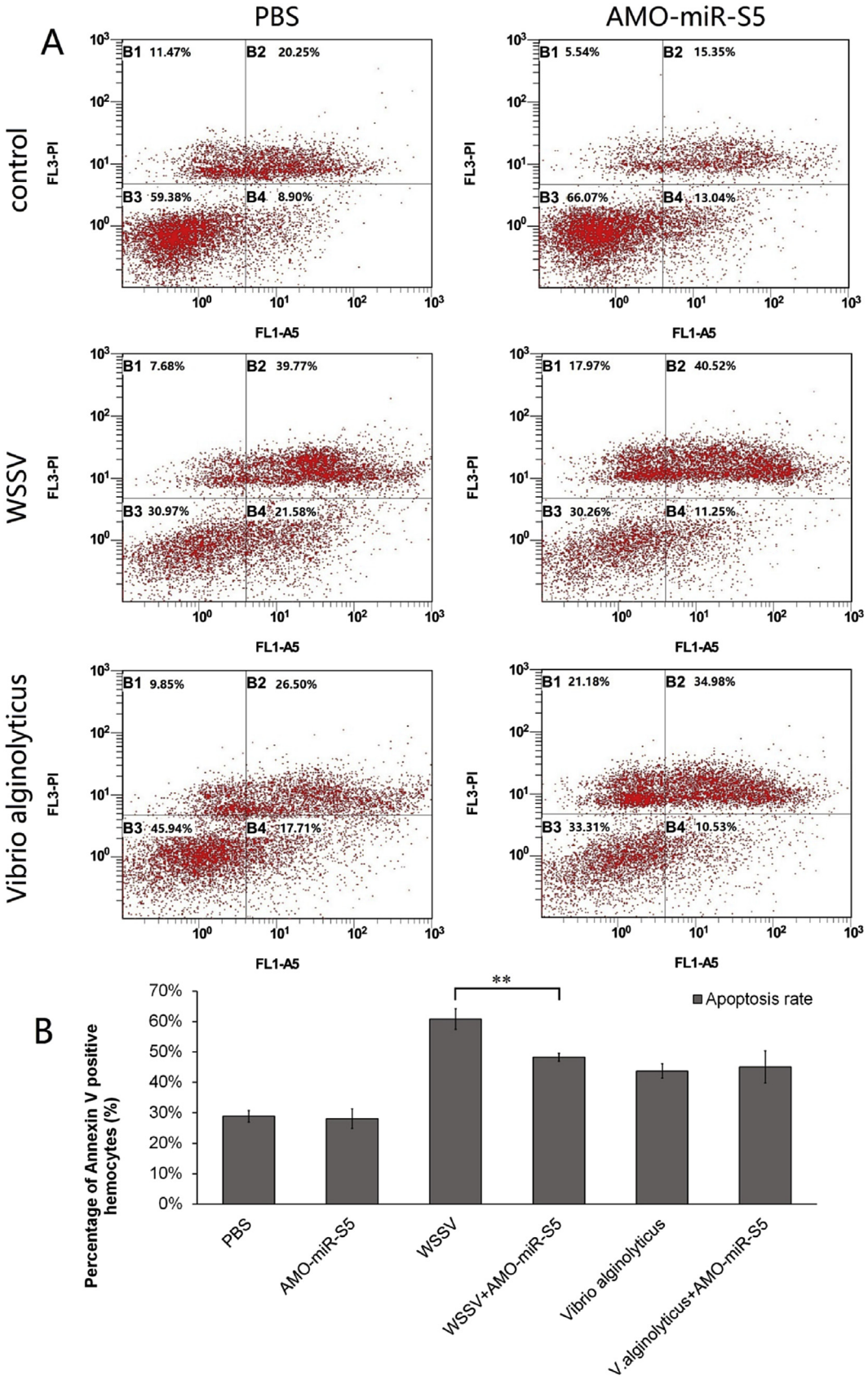

Fig. 6. Effects of miR-S5 knock down on hemocyte apoptosis. Shrimps were separated into six groups: two groups were treated with the respective pathogens (WSSV or *V. alginolyticus*) alone, two groups were treated with the respective pathogens + AMO-miR-S5 (WSSV + AMO-miR-S5 or *V. alginolyticus* + AMO-miR-S5), and the other two groups were treated with high-saline PBS or AMO-miR-S5 as controls. Samples were taken at 24 h post-injection. (A) scatter chart by flow cytometry, threshold of FL3-PI was set based on PI-only negative control, threshold of FL1-A5 (FL1-Annexin V) was set based on FITC-only positive control. Non-stained empty control helped to adjust the thresholds. B1 area represents false positives caused by cell damage, B2 represents late-stage apoptotic hemocytes, B3 represents negative (normal) hemocytes, and B4 represents late-stage apoptosis. (B) The percentage of annexin V-positive hemocytes represents the apoptosis rate in each group. All treatments included at least three shrimp individuals, and all experiments were repeated biologically three times. Each column represents the mean value of triplicate assays.

hemolymph was significantly inhibited by this procedure from 24 h to 48 h, healthy or miR-S5-inhibited shrimps were then subjected to further experiments.

Inhibition of miR-S5 expression resulted in significant down-regulation of p53, and TNF-α and up-regulation of myosin, Rho, proPO, IMD, Toll like receptor, MAPK, QM and Rab7 expression levels remained unaffected (Fig. 2D). Myosins comprise a superfamily of ATP-dependent motor proteins. Myosin molecules release phosphate following ATP hydrolysis to form a power stroke during muscle contraction. Myosin could bind to actin in the immune system (Stendahl et al., 1980) to form a myosin–actin binding complex, which is believed to be responsible for cytoskeleton formation and cell movement, and to participate in phagocytosis and apoptosis. Shrimp myosin may thus respond to viral infection and regulate hemocyte phagocytosis (Huang et al., 2015). The p53 is a known tumor suppressor with crucial roles in multicellular organisms, can help to prevent cancer via several mechanisms, including DNA damage recognition, DNA repair, and (in the event of failed DNA repair) by initiating programed cell death, i.e. apoptosis (Roos and Kaina, 2013). TNF-α is a cell signaling protein produced chiefly by activated macrophages, the primary function of which is to induce inflammation, apoptotic cell death, cachexia, and inflammation, and to inhibit tumorigenesis and viral replication and respond to sepsis via interleukin-1 and interleukin-6-producing cells. Changes in the expression levels of the above three genes indicated that miR-S5 expression is likely to be associated with cellular immunity include phagocytosis and/or apoptosis in shrimp innate immunity, and may be involved in antiviral processes. Other genes with acknowledged immunity functions like MAPK, IMD, proPO showed no expression change, indicating the humoral immunity pathways like IMD pathway, proPO pathway were not affected by miR-S5 regulation.

The results of pathogen-challenge experiments revealed that inhibition of miR-S5 reduced shrimp mortality induced by WSSV (Fig. 3A), but promoted mortality induced by *V. alginolyticus* (Fig. 3B), indicating that miR-S5 may have a negative role in antiviral process and a positive role in anti-bacterial process. Decrease of WSSV copies at 72 h after miR-S5 inhibition seconded this conclusion (Fig. 3C). In immune parameters analysis, WSSV and *V. alginolyticus* both induced high PO activity, after miR-S5 inhibition, PO activity of WSSV infection decreased, while PO activity of *V. alginolyticus* increased (Fig. 4C and D). PO was the final enzyme in proPO pathway (Chiu et al., 2007), responsible for eliminating pathogens, high PO activity represented an adverse host condition. PO activity reduction after miR-S5 inhibition indicated miR-S5 was beneficial to virus, meanwhile the PO activity increase after miR-S5 inhibition indicated miR-S5 was beneficial to host. WSSV and *V. alginolyticus* infection both induced high SOD activities, after miR-S5 inhibition, SOD activity of WSSV infection showed no change, while SOD activity of *V. alginolyticus* significantly reduced (Fig. 4E and F). SOD was responsible for eliminating reactive oxygen species (ROS) produced in inflammation process to prevent ROS induced cell damage (Simon et al., 2000). The reduction of SOD activity after miR-S5 inhibition suggesting miR-S5 could help the defense system of host.

Both WSSV and *V. alginolyticus* induced phagocytosis (Fig. 5), after miR-S5 inhibition, phagocytosis of WSSV decreased, no significant change in phagocytosis of *V. alginolyticus*. Same result was obtained in apoptosis experiments (Fig. 6), meanwhile, without pathogen treatment, miR-S5 showed no influence on hemocyte apoptosis. These results indicated that miR-S5 could promote phagocytosis and apoptosis under WSSV infection situation, under healthy or *V. alginolyticus* infection situation, phagocytosis and apoptosis were not affected by miR-S5 expression. Virus genome has a minimal structure with maximal function. Viruses can produce proteins and noncoding RNAs in their hosts, thus infiltrating host processes to ensure their own proliferation. miRNAs are crucial regulators of host defenses, and it has been suggested that, as taking advantage of host proteins, viruses may also exploit host immune pathways to promote their own proliferation by destroying, boosting, or hijacking host miRNAs (Guo and Steitz, 2014). Mahajan et al. (2009) showed that viruses, especially rapidly evolving viruses, could utilize host miRNAs for immune evasion and persistent infection. The current results provide further evidence to support this theory. WSSV has been proved to possess this ability to use host phagocytosis and apoptosis to escape host recognition system. The different consequences of miR-S5 knock down in viral/bacterial infection indicated that miR-S5 could enhance host defense system like PO activity and SOD activity, play a positive role in innate immune system, but it could be used by virus to promote apoptosis and/or phagocytosis through regulating *myosin*, *p53*, *TNF-α* to avoid innate immune system and contribute to shrimp death. In a previous research on shrimp miroRNA-100 (Wang et al., 2017), we explored the role of miR-100 in shrimp innate immune system. Shrimp miR-100 could positively regulate myosin expression, inhibit hemocyte apoptosis and phagocytosis. MiR-100 has been proven to induce cell apoptosis in several types of tumor cells, but in shrimps, miR-100 functions as an apoptosis inhibitor. In this study, the shrimp specific microRNA, miR-S5, was found to negatively regulate myosin expression, functions as a promotor in apoptosis progress. The different effect of these two microRNAs in shrimp suggesting that there might be a specific regulation method of shrimp microRNAs which could be same, similar, or converse compared with mammalian microRNAs to complete the regulatory work.

In conclusion, we revealed a role for the shrimp-specific miRNA, miR-S5, in the innate immune system. This host miRNA could regulate the host's defense against bacterial infection, but could also be used by viruses to hijack the host's defense mechanisms to enhance virus replication.

Conflict of interest

The authors have no patents, products in development, or marketed products to declare. This does not alter our adherence to gene policies on sharing data and materials.

Author contribution statement

Professor Fei Zhu and postgraduate Zhi Wang designed the experiments, postgraduates of our lab including Zhi Wang, Baozhen Sun, Xiongchao Ma and Ziyan Wang carried out the experiments; Zhi Wang and Fei Zhu analyzed the experimental results; Zhi Wang and Fei Zhu wrote the manuscript.

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