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MicroRNA-182-3p negatively regulates cytokines expression by targeting TLR5M in orange-spotted grouper, *Epinephelus coioides*

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ABSTRACT

Toll-like receptors (TLRs) as essential pattern recognition receptors in innate immunity, can recognize pathogens and trigger immune response to eliminate invading pathogens. MicroRNAs regulates multiple biological processes by suppressing mRNA translation or resulting in mRNA degradation. MiR-182 has previously been implicated in DNA repair, disease and cancer aspects. The potential role of miR-182-3p in TLR signaling pathway against pathogens is unclear. In this study, we found that the expression of miR-182-3p was up-regulated after *Vibrio parahaemolyticus* flagellin stimulation in grouper spleen (GS) cells, and negatively correlated with the expression of orange-spotted grouper (*Epinephelus coioides*) TLR5M (*Ec*TLR5M). Then we found that miR-182-3p could directly target *Ec*TLR5M by using bioinformatic analysis and dual-luciferase reporter assay. Dual-luciferase reporter assay also showed that miR-182-3p down-regulated the wild-type *Ec*TLR5M 3′UTR in luciferase activity rather than the mutant group in HEK 293T cells. We further verified the effect of miR-182-3p on the activation of Nuclear factor-κB (NF-κB) signaling pathway, and found that miR-182-3p inhibitors significantly augmented flagellin-induced NF-κB phosphorylation. Additionally, we also demonstrated that the increased expression of miR-182-3p significantly suppressed the flagellin-induced *Ec*TLR5M, pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) mRNA expression. And the endogenous miR-182-3p knockdown experiments reversely verified the regulatory effect of miR-182-3p. These results suggested that miR-182-3p post-transcriptionally controls *Ec*TLR5M expression and thereby suppresses the expression of pro-inflammatory cytokines. This study is the first to demonstrate that miR-182-3p suppresses pro-inflammatory cytokines expression by regulating the TLR signaling pathway.

1. Introduction

The innate immune system is the first to recognize and resist pathogens invasions, using pattern recognition receptors (PRRs) to recognize danger signals and thereby induce innate immune responses against pathogens [1,2]. For fish as lower vertebrates, innate immune system plays an important role in the host response to external stimuli and pathogen invasion [3]. Toll-like receptor (TLR), as an important PRR, can specifically recognize pathogen-related molecular patterns (e.g., LPS, flagellin, DNA, etc.), and induce the secretion of pro-inflammatory cytokines or interferon (IFN) through the transmission of intracellular signaling pathways [4–6]. However, excessive or uncontrolled activation of TLR pathways are associated with inappropriate inflammation [7], autoimmune diseases and even tissue damage, so living organisms have evolved a sophisticated regulatory network to monitor the activation process of TLR signaling pathways at multiple levels, among which microRNAs (miRNAs) are important negative regulatory molecules [8–10].

MicroRNAs are a class of non-coding single-stranded small RNA with a length of about 23 nucleotides, binding to the 3′ untranslated

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region (3′ UTR) of their specific target mRNAs, leading to instability of mRNAs and suppresses of translation, resulting in significant reduction in protein levels [11]. This complex and sophisticated regulatory network can not only regulate the expression of multiple genes through one miRNA, but also precisely regulates the expression of a certain gene through the combination of several miRNAs [12]. Due to the ubiquity and diversity of miRNAs, it is possible that miRNAs have a wide variety of biological functions [13]. Relevant studies have shown that miRNAs are involved in the physiological process of resisting bacterial and viral infection, and generally have a protective effect on the host [14–16]. In mammals, previous studies have shown that miRNAs regulate TLR-signaling pathways by targeting different genes, including TLRs, signaling proteins, regulatory molecules, transcription factors, cytokines, etc. [14]. In teleost fish, the roles of miRNAs in immune responses have been studied in several species, including commercially important species such as miiuy croaker (*Miichthys miuuy*), grass carp (*Ctenopharyngodon idella*), orange-spotted grouper (*Epinephelus coioides*), focusing on the differential expression of miRNAs between the control and challenge groups [15,16]. However, the complex regulation of miRNAs and TLR signaling pathways needs to be further studied.

Orange-spotted grouper is one of the important mariculture fish in tropical and subtropical regions, which is often suffered from pathogens invasion, such as vibrio and streptococcus [17–19]. *Vibrio parahaemolyticus* is one of the important pathogenic bacteria of vibriosis, which is widely distributed and lives in sea water, fish, shrimp and shellfish. *V. parahaemolyticus* infection may cause gastrointestinal infection, tissue damage and decreased immunity in the host [20,21]. In mammals, miR-182 has previously been implicated in DNA repair, disease and cancer aspects. The potential role of miR-182-3p in regulating TLR signaling pathway against pathogens is unclear. In this study, we found that stimulation with *V. parahaemolyticus* flagellin significantly increased miR-182-3p expression in grouper spleen (GS) cells. So, we explored the potential immune regulation mechanism of miR-182-3p in orange-spotted grouper stimulated by *V. parahaemolyticus* flagellin, proving that miR-182-3p negatively regulates flagellin-induced cytokines expression by targeting *Ec*TLR5M. Our study is the first to prove that miR-182-3p is involved in the regulation of TLR signaling pathway by targeting TLR5M, which will enrich the sophisticated regulation networks for the immune responses against pathogens in fish.

2. Materials and methods

2.1. Cell culture

Grouper spleen (GS) cells were a gift from Prof. Qiwei Qin, South China Agricultural of University, and cultured in L15 medium (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Hyclone, USA) at 27 °C without CO_2.

HEK 293T cells were purchased from Cell Bank, Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM medium (Hyclone, USA) containing 10% FBS (Hyclone, USA) at 37 °C with 5% CO_2.

2.2. MiRNA transfection

MiR-182-3p mimics, miR-182-3p specific inhibitors, negative control of scrambled miRNA (NC) and negative control of miRNA inhibitors (NC inhibitors) were purchased from GenePharma (Shanghai, China). To overexpress or knockdown of miR-182-3p, GS cells were transfected with miR-182-3p mimics or miR-182-3p specific inhibitors using Lipofectamine™ 3000 (Invitrogen, USA). The sequences of mimics and inhibitors were all listed in Table 1.

2.3. Real-time qPCR

Using miRNA isolation kit (OMEGA, China) to extract RNA (more than 200 nt) and small RNA (less than 200 nt), reverse transcription of RNAs and miRNAs were performed by using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Japan) and Mir-X™ miRNA First-Strand Synthesis kit (Takara, Japan), respectively. Then, we utilized LightCycler 480 SYBR Green I Master kit and LightCycler 480 Detection System (Roche, Switzerland) to conduct RT-qPCR analysis. These assays were amplified in triplicate wells, EF-1α and U6 were detected as internal control. The Mir-X™ miRNA First-Strand Synthesis kit contains mRQ 3′primer, U6 forward primer and reverse primer for RT-qPCR.

For RT-qPCR analysis cytokines expression, extraction of total RNA and synthesis of cDNA were using SuperPrep Cell Lysis & RT Kit for qPCR Reagents (Toyobo, Japan), and performed on the LightCycler 480 Detection System using LightCycler 480 SYBR Green I Master kit (Roche, Switzerland). These assays were amplified in triplicate wells. The data were calculated using the $2^{-\Delta\Delta Ct}$ method, and EF-1α was detected as internal control. Primer sequences used for RT-qPCR were listed in Table 2.

2.4. MiR-182-3p target prediction analysis

According to the grouper ESTs sequencing database in our laboratory, we obtained the 3′UTR region sequence of *Ec*TLR5M through RACE experiment. To determine the regulatory relationship between miR-182-3p and *Ec*TLR5M, Targetscan (http://www.targetscan.org/) and MiRanda (http://www.microrna.org/) programs were used to predict the putative miR-182-3p binding sites from 3′UTR of *Ec*TLR5M.

2.5. Luciferase activity assay

Wild-type 3′UTR of *Ec*TLR5M (*Ec*TLR5M-3′UTR-WT) that contained the predicted target binding site of miR-182-3p was amplified with *Ec*TLR5M-3′UTR-WT-*Xho*I-F primer and *Ec*TLR5M-3′UTR-WT-*Xho*I-R primer. Then, the *Ec*TLR5M 3′UTR fragment was cloned into the psiCHECK-2 luciferase vector (Promega, USA) with the *Xho*I restriction enzyme sites. We constructed a *Ec*TLR5M mutant 3′UTR (*Ec*TLR5M-3′UTR-MUT) recombinant plasmid with missense mutation of miR-182-3p target site by using Mut Express II Fast Mutagenesis Kit (Vazyme, China). The *Ec*TLR5M 3′UTR mutant fragment was amplified with *Ec*TLR5M-3′UTR-MUT-F primer and *Ec*TLR5M-3′UTR-MUT-R primer. Recombinant plasmids were verified by DNA sequencing (Sangon Biothch, China). Sequences of primer used for recombinant plasmids construction were listed in Table 3.

To verify whether miR-182-3p could affect *Ec*TLR5M gene expression through binding to the predicted target site, HEK 293T cells were seeded into 48-well plates and cultured overnight, then co-transfected with either 1.2 μL miR-182-3p mimics or NC (20 nM) along with 400 ng psiCHECK-2-*Ec*TLR5M-3′UTR-WT or psiCHECK-2-*Ec*TLR5M-3′UTR-MUT plasmids by using Lipofectamine™ 3000 reagent (Invitrogen, USA), respectively. After transfection with 24 h, cells were collected and lysed to detect the luciferase activity by dual-luciferase reporter assay system (Promega, USA). These assay groups were performed in quadruplicate and each experiment was repeated three time.

2.6. Western blot

GS cells were seeded into 12-well plates and cultured overnight, then transfected with either NC, miR-182-3p mimics, or miR-182-3p inhibitors by using Lipofectamine™ 3000 reagent (Invitrogen, USA). After transfection with 24 h, cells were stimulated with *V. parahaemolyticus* flagellin for 60 min, and then lysed with RIPA lysis buffer (Beyotime Ins. Bio., China) containing 1% protease inhibitor cocktail (Sigma, USA) and 1% phosphatase inhibitor cocktail (Sigma, USA) to collect total intracellular protein. *V. parahaemolyticus* flagellin was extracted and purified directly from bacteria that was isolated from diseased orange-spotted grouper, refer to our previous article for detailed

Table 1
Sequences of miR-182-3p mimics and inhibitors.

Named	Sense (5′–3′)	Antisense (5′–3′)
NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
miR-182-3p mimics	UGGUUCUAGACUUGCCAACUA	GUUGGCAAGUCUAGAACCAUU
NC inhibitors	CAGUACUUUUGUGUAGUACAA	
miR-182-3p inhibitors	UAGUUGGCAAGUCUAGAACCA	

Table 2
Sequences of primers used for RT-qPCR.

Primer	Accession No.	Sequence (5′–3′)
miR-182-3p-RT-F		TGGTTCTAGACTTGCCAACTA
*Ec*TLR5M-RT-F	KM282522.1	TAGCCACTCCAGACCCAAG
*Ec*TLR5M-RT-R		GAGACGGGCGTAAACAATC
IL-6-RT-F	AFE62919.1	CAATCCCAGCACCTTCCAC
IL-6-RT-R		CCTGACAGCCAGACTTCCTCT
TNF-α-RT-F	ACM46004.1	CCTGGTGATGTGGAGATG
TNF-α-RT-R		GTCCGACTTGATTAGTGCTT
EF-1α-RT-F	AEG78376.1	GGTCGTCACCTTCGCTCCAT
EF-1α-RT-R		TCCCTTGGGTGGGTCATTCT

extraction and identification procedures [22]. Total proteins were resolved by 12% SDS-PAGE gels following standard procedures. Anti-total and anti-phosphorylated NF-κB-p65 rabbit antibodies were purchased from Santa cruz Biotechnology (Canada) (1:1000, Catalogue No. sc-372) and Cell Signaling Technology (USA) (1:1000, Catalogue No. 3033), respectively. Anti-β-actin mouse antibody, Goat anti-mouse IgG and Goat anti-rabbit IgG second antibodies were obtained from Proteintech (USA). Immunoreactive bands were visualized by Alliance MINI HD9 system (UVITEC, USA). β-actin was used as loading control.

2.7. Statistical analysis

Results are reported as means values ± SEM. All data were analyzed by GraphPad Prism 5.0 software, carried out by two-way ANOVA analysis of variance.

2.8. Key resources table

Resources	Source	Identifier
Antibodies		
anti-phosphorylated NF-?B-p65 rabbit	Santa cruz Biotechnology	sc-372
Anti-β-actin mouse	Proteintech	
anti-total NF-?B rabbit antibodies	Cell Signaling Technology	3033
Goat anti-mouse IgG	Proteintech	
goat anti-rabbit IgG	Proteintech	
Antibodies		
anti-phosphorylated NF-?B-p65 rabbit	Santa cruz Biotechnology	sc-372
Anti-β-actin mouse	Proteintech	
anti-total NF-?B rabbit antibodies	Cell Signaling Technology	3033
Goat anti-mouse IgG	Proteintech	
goat anti-rabbit IgG	Proteintech	
CellLine		
HEK 293T	Cell Bank, Chinese Academy of Sciences	

3. Results

3.1. V. parahaemolyticus flagellin increased expression of mir-182-3p and EcTLR5M in GS cells

To explore whether *V. parahaemolyticus* flagellin affected the expression of endogenous miR-182-3p, we detected the relative expression patterns of miR-182-3p by RT-qPCR in 1 μg/mL *V. parahaemolyticus* flagellin-stimulated GS cells at multiple time points. As shown in Fig. 1A, miR-182-3p expression significantly down-regulated at 3h and 6h, compared with control group. After stimulation for 12h and 24h, the expression of miR-182-3p was significantly up-regulated, revealing that miR-182-3p was involved in *V. parahaemolyticus* flagellin-induced immune response in GS cells. Meanwhile, the expression of *Ec*TLR5M was peaked at 6h, and then the expression was significantly down-regulated. In general, miRNA expression trends to be opposite to that of the target gene. These results proposed that miR-182-3p might regulate *Ec*TLR5M expression after flagellin stimulation in orange-spotted grouper.

3.2. MiR-182-3p suppressed V. parahaemolyticus flagellin-induced EcTLR5M expression

To verify the potential regulatory role of miR-182-3p on *Ec*TLR5M expression, we transfected miR-182-3p mimics and miR-182-3p inhibitors into GS cells and detected the relative expression of *Ec*TLR5M after 1 μg/mL *V. parahaemolyticus* flagellin stimulation. As shown in Fig. 2A and B, expression levels of miR-182-3p were significantly increased in cells transfected with miR-182-3p mimics, while significantly decreased in cells transfected with miR-182-3p inhibitors. These results demonstrated that miR-182-3p could be overexpressed in GS cells by transfection of miR-182-3p mimics, while knockdown endogenous miR-182-3p through transfection of inhibitors. We found that mimics/inhibitors had better overexpression/knockdown efficiency at 24 h post transfection compared with at 48h, which was applied in the following experiments.

After stimulation with *V. parahaemolyticus* flagellin (1 μg/mL), overexpression of miR-182-3p reduced *Ec*TLR5M mRNA expression compared with NC group (Fig. 2C). In contrast, knockdown of endogenous miR-182-3p significantly augmented *Ec*TLR5M mRNA expression compared with NC inhibitors group (Fig. 2D). Interestingly, the effect of miR-182-3p on *Ec*TLR5M mRNA expression in the unstimulated state was consistent with that in the flagellin-stimulated state. Thus, miR-182-3p was proposed as a negative regulator on *Ec*TLR5M expression.

Table 3
Sequences of primers used for recombinant plasmids constructions.

Primer	Sequence (5′–3′)
*Ec*TLR5M-3′UTR-WT-*Xho*I-F	AATTCTAGGCGATCGCTCGAGACTCTAACTGTGTGATAACGTGTTGATAC
*Ec*TLR5M-3′UTR-WT-*Xho*I-R	AAACGAATTCCCGGGCTCGAGTTTGATATTGTGCAACAGATCACCT
*Ec*TLR5M-3′UTR-MUT-F	TAAGTGCGCAGGTTAGACTTAGAAGCTGTCTGGTCCTGA
*Ec*TLR5M-3′UTR-MUT-R	AGTCTAACCTGCGCACTTAAACATGAAGGACTTTTTGAAGA

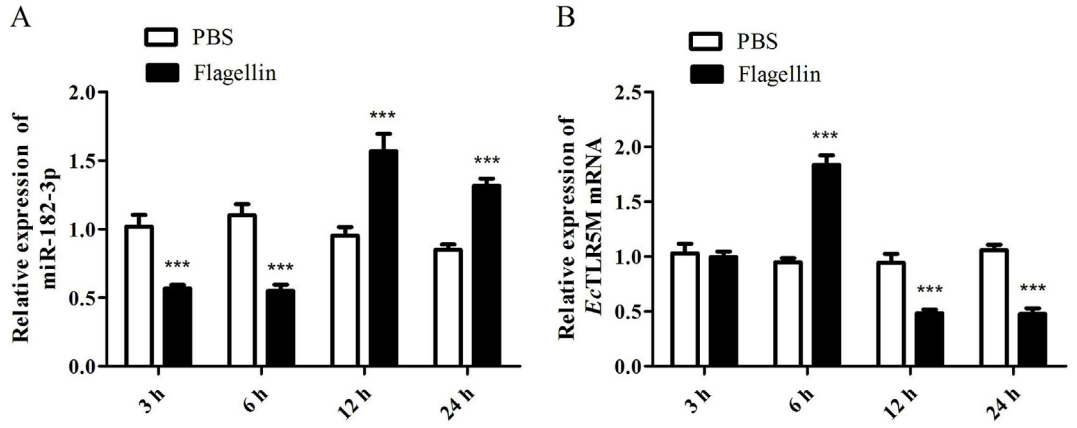

Fig. 1. The expression patterns of miR-182-3p and *Ec*TLR5M upon *V. parahaemolyticus* flagellin stimulation. GS cells were treated with *V. parahaemolyticus* flagellin for 3, 6, 12 and 24 h, and then collected for RNA purification. The expression of miR-182-3p (A) and *Ec*TLR5M (B) were measured by RT-qPCR. U6 and EF-1α served as internal control, respectively. Data indicates the mean ± SEM (n = 3). $^{***}p < 0.001$.

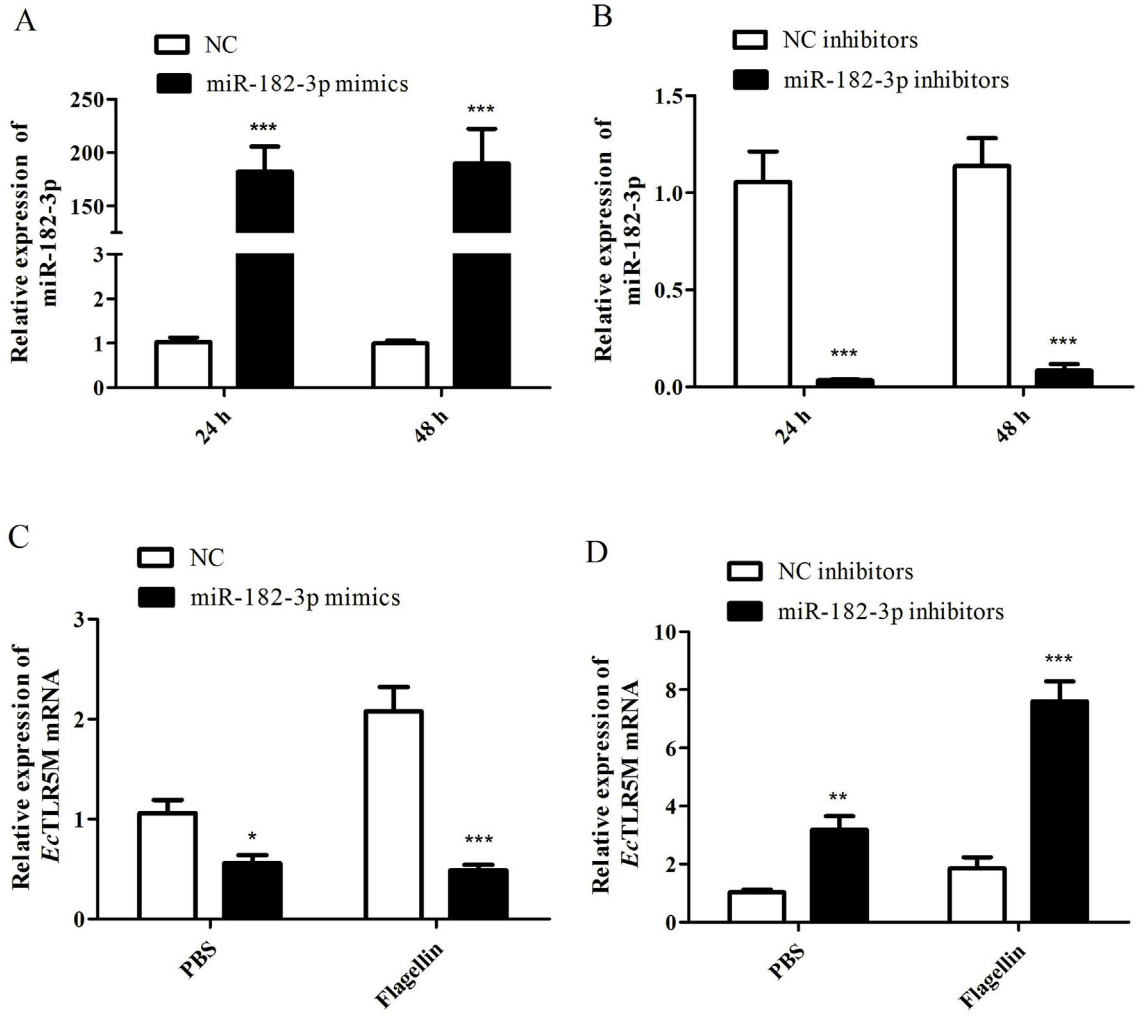

Fig. 2. MiR-182-3p negatively regulates *Ec*TLR5M expression upon *V. parahaemolyticus* flagellin stimulation. (A) The level of miR-182-3p was efficiently enhanced by transfection of mimics in GS cells. (B) The level of miR-182-3p was efficiently reduced by transfection of specific inhibitors in GS cells. U6 served as internal control. GS cells were seeded into 6-well plates and cultured overnight, then transfected with either NC, NC inhibitors, miR-182-3p mimics, or miR-182-3p inhibitors at a final concentration of 60 nM. (C–D) The effect of miR-182-3p mimics or inhibitors on *V. parahaemolyticus* flagellin-induced *Ec*TLR5M mRNA expression. GS cells were seeded into 96-well plates and cultured overnight, then transfected with either NC, NC inhibitors, miR-182-3p mimics, or miR-182-3p-inhibitors at a final concentration of 60 nM. After transfected with miR-182-3p mimics or inhibitors into GS cells at 24 h, cells were stimulated with *V. parahaemolyticus* flagellin for 6 h, and then collected for RNA purification. The expression of *Ec*TLR5M was measured by RT-qPCR, EF-1α was detected as internal control. Data indicates the mean ± SEM (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

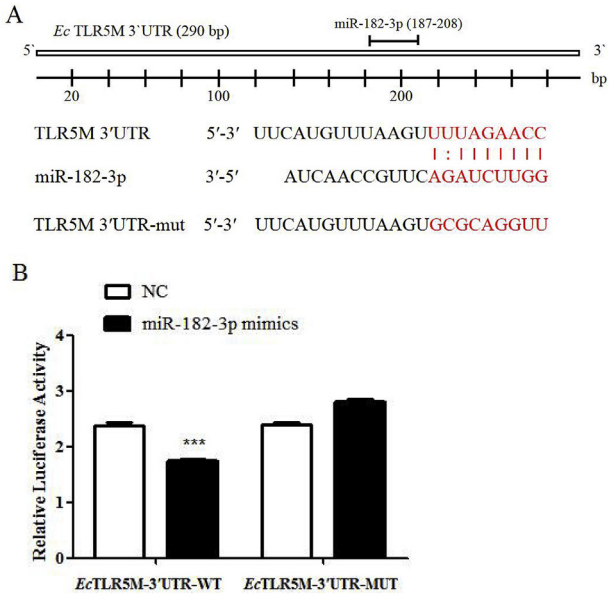

Fig. 3. MiR-182-3p directly targets 3′UTR of *Ec*TLR5M. (A) Putative binding sequences of miR-182-3p in 3′UTR of *Ec*TLR5M. (B) Luciferase reporter assay. HEK 293T cells were co-transfected with either miR-182-3p mimics or NC along with psiCHECK-2-*Ec*TLR5M-3′UTR-WT or psiCHECK-2-*Ec*TLR5M-3′UTR-MUT plasmids. At 24 h post transfection, cells were collected and then used to detect the relevant luciferase activity. Data indicates the mean ± SEM (n = 4). ***$p < 0.001$.

3.3. MiR-182-3p targeted EcTLR5M

Targetscan (http://www.targetscan.org/) and MiRanda (http://www.microrna.org/) programs revealed a potential miR-182-3p binding site in the 3′UTR of *Ec*TLR5M (Fig. 3A).

To verify that *Ec*TLR5M is the target gene for miR-182-3p, we constructed psiCHECK-2-*Ec*TLR5M-3′UTR-WT or psiCHECK-2-*Ec*TLR5M-3′UTR-MUT plasmids, and co-transfected with miR-182-3p mimics or NC into HEK 293T cells, respectively. At 24 h post transfection, dual-luciferase activity assay was performed. As shown in Fig. 3B, miR-182-3p mimics significantly reduced *Ec*TLR5M-3′UTR- WT luciferase activity compared with the NC group. In contrast, miR-182-3p mimics dose not effect in the *Ec*TLR5M-3′UTR-MUT luciferase activity. These results showed that miR-182-3p could directly target *Ec*TLR5M at 3′UTR.

3.4. MiR-182-3p suppressed V. parahaemolyticus flagellin-induced NF-κB activation

Previous study, we observed that the IκBα phosphorylation was significantly increased at 60 min treatment with flagellin in GS cells [22]. Following phosphorylation and degradation of IκBα, NF-κB was phosphorylated and then transported into the nucleus to regulate the expression of pro-inflammatory cytokines.

To further explore the potential influence of miR-182-3p on the TLR5M signaling pathway, GS cells were transfected with either NC, miR-182-3p mimics or miR-182-3p inhibitors, and then stimulated with *V. parahaemolyticus* flagellin. After 60-min stimulation, cells were collected. Their extracted total protein was used to detect the phosphorylation level of NF-κB-p65 protein by Western blot. Compared with NC group, miR-182-3p inhibitors group has increased the phosphorylation of NF-κB-p65 induced by flagellin (Fig. 4). Whereas, the miR-182-3p mimics group did not significantly reduced the phosphorylation of NF-κB-p65, which may be affected by the low baseline level of NF-κB-p65 phosphorylated protein. The miR-182-3p inhibitors group results indicated that miR-182-3p has an inhibitory effect on *V. parahaemolyticus* flagellin-induced NF-κB activation.

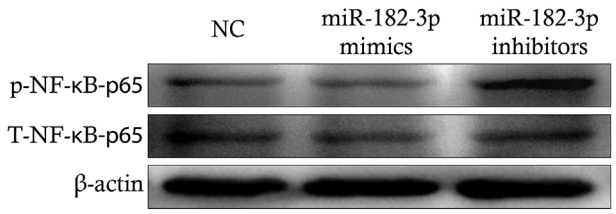

Fig. 4. Knockdown of miR-182-3p promotes NF-κB-p65 phosphorylation upon *V. parahaemolyticus* flagellin stimulation. GS cells were seeded into 12-well plates and transfected with NC, miR-182-3p mimics or miR-182-3p inhibitors at a final concentration of 48 nM. At 24 h post transfection, cells were treated with 1 μg/mL *V. parahaemolyticus* flagellin for 60 min, then total protein samples were collected and used to detect the total and phosphorylated level of NF-κB-p65 protein (T-NF-κB-p65, p-NF-κB-p65) by Western Blot. β-actin was detected as internal control.

3.5. MiR-182-3p suppressed V. parahaemolyticus flagellin-induced pro-inflammatory cytokines expression

To further explore the potential influence of miR-182-3p on TLR5M-mediated pro-inflammatory cytokines expression during *V. parahaemolyticus* flagellin stimulation, we transfected miR-182-3p mimics/inhibitors into GS cells to overexpress or knockdown miR-182-3p, and detected the expression of pro-inflammatory cytokines after flagellin stimulation. As shown in Fig. 5, miR-182-3p overexpression reduced IL-6 and TNF-α mRNA expression compared with NC group (Fig. 5A–B). In contrast, knockdown of endogenous miR-182-3p significantly augmented cytokines IL-6 and TNF-α mRNA expression compared with NC inhibitors group (Fig. 5C–D). These results showed that miR-182-3p could negatively regulate the expression of pro-inflammatory cytokines.

4. Discussion

At present, the potential modulatory mechanisms of miRNAs in the inflammatory responses associated with the immune signaling pathways in teleost fish against different pathogens or stimuli has been studies [15,16,23,24]. Like mammals, fish miRNAs mediate TLR-signaling pathways by regulating the expression of TLR proteins, TLR-associated signaling molecules, transcription factors or downstream cytokines. For example, miR-200a-3p was involved in TLR1 expression regulation in LPS and *V. anguillarum* stimulated miiuy croaker [25]; miR-21 suppressed cytokines IL-6 and TNF-α expression by negatively regulating TLR28 in poly (I:C) stimulated miiuy croaker [26]; miR-148 suppressed cytokines IL-6 and IL-1β expression by targeting MyD88 in *V. harveyi* and LPS stimulated miiuy croaker [27]; miR-146a was involved in TNF receptor-associated factor 6 (TRAF6) expression regulation in IFN-γ2 and red spotted grouper nervous necrosis virus stimulated orange-spotted grouper [28,29]. Nevertheless, fewer target miRNAs of TLR5 and flagellin-mediated signaling molecules have been reported. In fish, grass carp TLR5-3′UTR contains the binding sites of miR-115 and miR-142a-3p. Subsequently, miR-115 and miR-142a-3p were found that could directly target TLR5, leading to down-regulation of flagellin-induced downstream IL-1β, IL-8 and TNF-α expression in *Ctenopharyngodon idella* kidney cells [30]. In mammals, miR-150 alleviated neuropathic pain development by targeting TLR5 in rat chronic sciatic nerve injury model [31]. In this study, we found that miR-182-3p could directly target *Ec*TLR5M by using bioinformatic analysis and dual-luciferase reporter. *V. parahaemolyticus* flagellin-induced miR-182-3p and *Ec*TLR5M expression presented an opposite trend. Overexpression of miR-182-3p significantly suppressed the expression of *Ec*TLR5M in both unstimulated and flagellin-stimulated states. These results suggested that the expression of miR-182-3p was increased rapidly after stimulated with *V. parahaemolyticus* flagellin, and then miR-

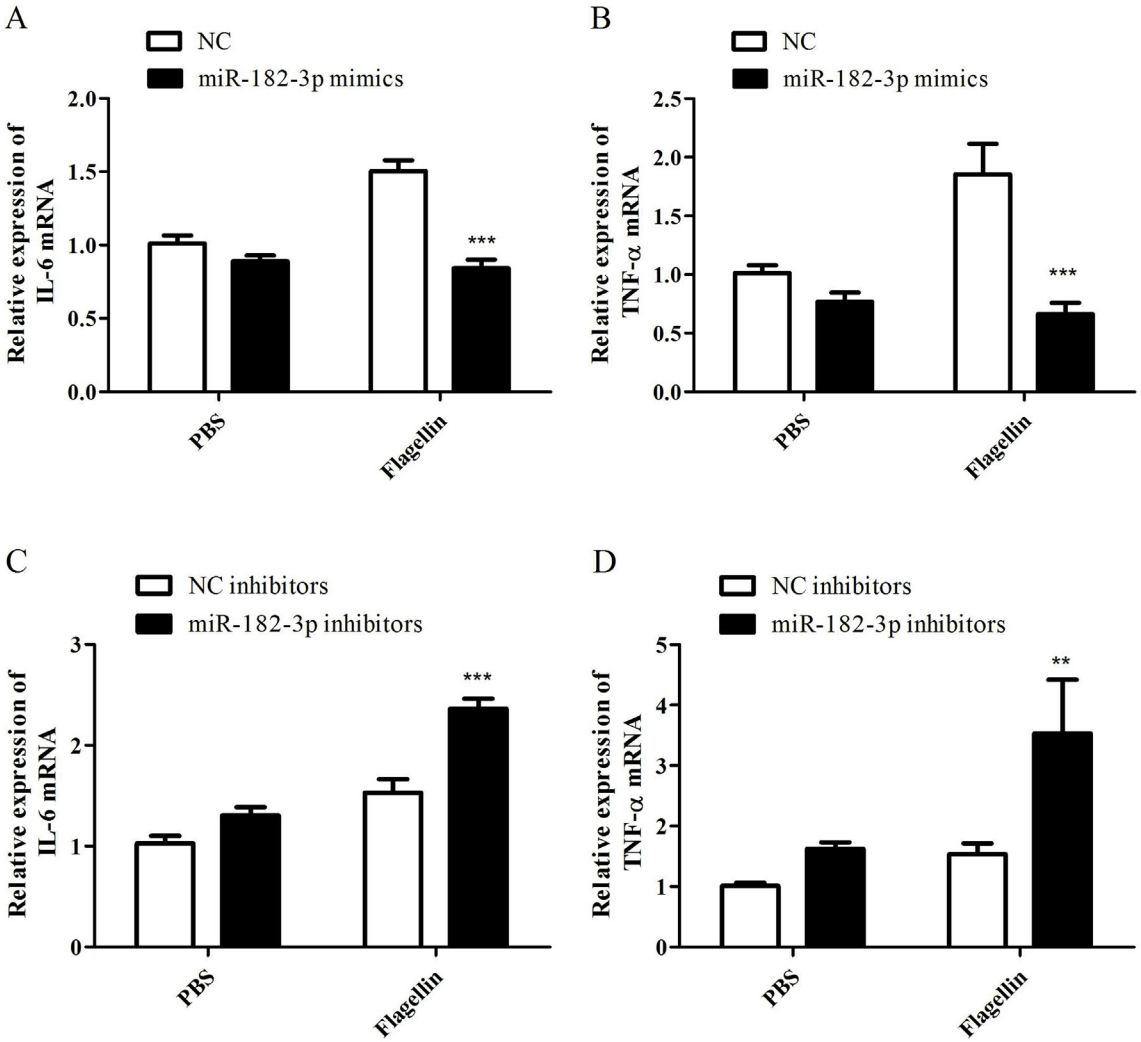

Fig. 5. MiR-182-3p suppresses the expression of pro-inflammatory cytokines IL-6 and TNF-α upon *V. parahaemolyticus* flagellin stimulation. GS cells were seeded into 96-well plates and transfected with miR-182-3p mimics or inhibitors (60 nM), then were stimulated with 1 μg/mL *V. parahaemolyticus* flagellin. At 6 h post stimulation, the mRNA expression levels of pro-inflammatory cytokines IL-6 and TNF-α were detected by RT-qPCR, and EF-1α was detected as internal control. Data indicates the mean ± SEM (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$.

182-3p was negatively regulated the expression of *Ec*TLR5M to prevent the emergence of excessive immune response via targeting *Ec*TLR5M thereby helping maintain homeostasis.

Previous study, we found that *V. parahaemolyticus* flagellin induced expression of *Ec*TLR5M and downstream cytokines, as well as the activation of mitogen-activated protein kinases (MAPKs) and NF-κB pathways in GS cells [22]. Similarly, rainbow trout TLR5M enhanced the activity of transcription factor NF-κB upon flagellin stimulation [32]. After knockdown of endogenous miR-182-3p in GS cells, the phosphorylation levels of flagellin-induced NF-κB-p65 was enhanced compared with NC group, proposing that miR-182-3p could regulate flagellin-induced *Ec*TLR5M-NF-κB signal axis by a negative feedback mechanism. We cannot explain exactly why miR-182-3p overexpression has little effect on the NF-κB-p65 phosphorylation. The NF-κB transcription factor play a key role in a number of fundamental physiological processes [33], so the body has evolved a sophisticated and complex network to monitor the activation of NF-κB to maintain homeostasis.

As a member of miR-183 family, miR-182 was regarded as a cancer-associated oncogenic miRNA [34], and its level was positively correlated with the occurrence and development of a variety of tumors [35]. In mammals, the role of miR-182 in DNA repair, disease and cancer has been reported. In skeletal muscle atrophy diseases, miR-182 suppressed atrophy-related gene expression by directly targeting forkhead box O3 (FOXO3) [36]. MiR-182 positively regulated interferon regulatory factor 7 (IRF7) by directly targeting FOXO3, leading to activation of type I IFN response to suppress human cytomegalovirus (HCMV) replication in neural cells [37]. In osteoporosis rat model, down-regulated miR-182-5p promoted adenylyl cyclase isoform 6 (ADCY6) expression and Rap1/MAPK pathway activation, thus promoted the proliferation and differentiation of osteoblasts [38]. MiR-182 was up-regulated in cells or tissues of tumors, such as lung cancer [39], liver cancer [40], colorectal cancer [41] and breast cancer [42]. In the occurrence and development of breast cancer, miR-182 reduced breast-cancer-associated gene 1 (BRCA1) gene expression by impacting DNA repair, and eventually lead to the proliferation, invasion and metastasis of cancer cells [43]. The potential regulatory role of miR-182-3p in the phenotype of smooth muscle cells has been reported. In human aortic artery smooth muscle cells, asymmetrical dimethylarginine (ADMA)-induced down-regulation of miR-182-3p increased myeloid-associated differentiation marker (MYADM) expression and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. Sun et al., deduced that ADMA down-regulated miR-182-3p expression induced the smooth muscle cells phenotype change via MYADM-mediated ERK signaling activation [44]. At the present, the role of miR-182-3p in innate immune has been little known, let alone in bacterial-induced

inflammation. According of our study, we proved for the first time that miR-182-3p suppresses the expression of bacteria-induced pro-inflammatory cytokines, such as IL-6 and TNF-α, speculating that miR-182-3p might be a negative regulator in bacteria-induced inflammation.

Our previous research showed that *V. parahaemolyticus* flagellin as a virulence factor, which mediates cytokines expression through the *Ec*TLR5M and *Ec*TLR5S pathways [22]. In this study, our results revealed that the potential role of miR-182-3p in innate immunity, and supported the hypothesis that miR-182-3p, as a novel negative regulator of orange-spotted grouper TLR5M, suppressed *Ec*TLR5M-NF-κB signaling activation and attenuated pro-inflammatory cytokines expression, thereby monitored flagellin-induced immune response development to avoid damage caused by excessive inflammation. Our findings provide a scientific basis for understanding the interaction between miRNAs and genes that defense against pathogen invasion, and provide a new targeting idea for suppressing bacterial inflammatory diseases.

Conflicts of interest

The authors declare no competing financial interests.

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