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pol-miR-194a of Japanese flounder (*Paralichthys olivaceus*) suppresses type I interferon response and facilitates *Edwardsiella tarda* infection

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ABSTRACT

MicroRNAs (miRNAs) are a type of small non-coding RNAs that participate in diverse cellular processes including microbial invasion and immune defense. In a previous study, we identified a large amount of Japanese flounder (*Paralichthys olivaceus*) miRNAs responsive to megalocytivirus infection. In the present study, we examined the function of one of these miRNAs, pol-miR-194a, in association with the infectivity of *Edwardsiella tarda*, an intracellular bacterial pathogen to many fish species including flounder. We found that pol-miR-194a was induced in expression to a significant extent in the spleen, liver, and gill of Japanese flounder infected by *E. tarda*. Transfection of flounder cells with pol-miR-194a mimic significantly enhanced the intracellular replication of *E. tarda*. pol-miR-194a was able to interact specifically with the 3′UTR of IRF7 in a negative manner, resulting in inhibition of IRF7 expression. Consistently, pol-miR-194a significantly blocked the promoter activity of type I interferon. Taken together, these results indicate that pol-miR-194a plays an important role in the regulation of flounder immune response as well as microbial infection, and that pol-miR-194a probably serves as a target for *E. tarda* to manipulate and escape host immune defense.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that play vital roles in various biological systems by post-transcriptional modulation of the expression of target genes [1–3]. After being transcribed in the nucleus, the primary miRNA is modified and transported into cytoplasm, where the mature miRNA can interact with target mRNAs [4,5]. Accumulating evidences have indicated a diverse regulatory role of miRNAs in many biological processes including growth, development, and immunity [6–8]. Especially, miRNAs have been proved to be closely related to immunological defense against pathogen infection. For example, miR-23 exhibits antiviral effect against porcine reproductive and respiratory syndrome virus (PRRSV), possibly by up-regulating type I interferon [9], and miR-146a of orange spotted grouper serves as a negative-regulator of inflammatory response by targeting tumor necrosis factor receptor-associated factor 6 (TRAF6) [10]. In teleost fish, miRNAs have been found to be associated with various immune responses [11,12], for example, miRNAs are known to regulate toll-like receptor (TLR) signaling pathways by targeting multiple molecules including TLR-associated signaling proteins and TLR-induced cytokines [13]. In miiuy croaker (*Miichthys miiuy*), two miRNAs have been reported to regulate bacteria-induced immune response by targeting MyD88-mediated NF-κB signaling and IRAK4-NF-κB-mediated signaling [14,15]; in grouper (*Epinephelus coioides*), a large number of miRNAs have been demonstrated to be involved in viral infection [16–18].

Interferons (IFNs) were first discovered as an anti-virus regulator by restraining cell proliferation [19,20]. To date, large numbers of IFNs have been discovered, which can be classified into three subgroups as type I IFNs, type II IFNs, and type III IFNs [21]. Studies have shown that during pathogen invasion, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) –like receptors (RLRs), are activated by recognition of pathogen-associated molecular patterns (PAMPs) [22,23]. Then, common transcription factors including Interferon regulatory factors (IRFs) are activated through different signaling pathways, which in turn activate the expression of type I IFNs. IRFs play key roles in transcriptional regulation of IFNs and participate in signal transduction, cell proliferation and immune response [24–27].

Edwardsiella tarda is a Gram-negative bacteria and a pathogen to a wide range of host including fish and humans. In aquaculture, *E. tarda*

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is a severe fish pathogen and has caused large economic losses to the industry. *E. tarda* possesses complex virulence systems and factors, which enable the bacteria to invade various hosts [28,29]. One virulence feature of *E. tarda* is the capacity to evade host immune defense. Reports have shown that *E. tarda* is able to survive and replicate in host phagocytes, resist serum killing, and inhibit apoptosis of host cells [30–33]. However, the detailed mechanism of *E. tarda* immune evasion remains largely unknown.

Previous studies have identified a large amount of Japanese flounder (*Paralichthys olivaceus*) miRNAs regulated by megalocytivirus, which is a member of the Iridovirus family [34,35]. Bioinformatics analysis indicated that many of these flounder miRNAs potentially target key genes in immune response, such as beclin, p53 and IRFs. In this study, one megalocytivirus-induced flounder miRNA, pol-miR-194a, was found to be regulated by *E. tarda* as well. Based on this fact, we examined the regulatory function of pol-miR-194a and its effect on *E. tarda* infection. Our results support a miRNA-mediated immune-escape strategy of *E. tarda* to survive in host cells.

2. Materials and methods

2.1. Bacterial strains and cell lines

Edwardsiella tarda TX1 was cultured in Luria-Bertani broth (LB) at 28 °C as reported previously [36]. FG-9307, a Japanese flounder cell line, was cultured in L-15 (Genom, China) with 10% fetal bovine serum (Gibco, Invitrogen Corp., USA) and 1% penicillin and streptomycin (Beyotime Biotechnology, China) at 24 °C [37]. 293T cells (human embryonic kidney epithelial cells) (CBTCCCAS, Shanghai, China) were cultured in DMEM (Hyclone, USA) with 10% fetal bovine serum and 1% penicillin and streptomycin at 24 °C under the condition of 5% CO$_2$.

2.2. Fish

Clinically healthy Japanese flounder were purchased from a commercial fish farm in Shandong Province, China. Fish were maintained at 20 °C in aerated seawater and fed daily with commercial dry pellets. Before experiment, the fish were verified to be clinically healthy as reported previously [38]. Briefly, fish (5% of stock) were randomly sampled for the examination of bacteria in blood, liver, kidney and spleen; no bacteria were detected from the sampled fish. Fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) before tissue collection.

2.3. Quantitative real-time reverse transcription PCR (qRT-PCR)

E. tarda was cultured at 28 °C to an OD$_{600}$ of 0.8; the cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to 10^7 CFU/ml. Flounder were divided randomly into two groups and injected intraperitoneally with 50 μl *E. tarda* or PBS. Spleen, liver, and gill were taken from the fish (five at each time point) at 24 h and 48 h post-bacterial infection (hpi). miRNAs were extracted from the tissues and used for cDNA synthesis with specific stem-loop primer RT (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCACA -3′). pol-miR-194a expression was determined by qRT-PCR as reported previously [35] using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with α-tubulin (TUBA) (for spleen and gill) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (for gill) as internal references [39]. The experiment was performed three times, each time with five fish.

2.4. Intracellular infection assay

FG-9307 cells were cultured as above in 24-well plates. Transfection was performed as reported previously [40]. Briefly, the cells (10^5 cells/well) were transfected with 200 nM pol-miR-194a mimic or pol-miR-NC or PBS (control) for 24 h using Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The effectiveness of transfection was verified by qRT-PCR as above. The cells were then infected with *E. tarda* TX1 (multiplicity of infection, 100) at 24 °C for 2 h. The extracellular bacteria were killed by adding gentamycin (final concentration 200 ng/μl) and incubated at 24 °C for 1 h. The cells were washed three times with PBS and cultured in L15 medium containing 5% FBS and 20 ng/μl gentamycin for 2 h or 6 h; at each time point, the cells were lysed with 0.25% trypsin, and intracellular bacterial number was determined by plate count in LB plates containing 20 μg/ml tetracycline. The experiment was performed three times, each time in triplicate.

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)[a]
F1	TGATGAAAGCTGCGCACTAGTATCTCAAACTTCTTC
R1	AAAAGATCCTTTATTAAGCTTAGGGTTAAACCATTT
F2	GACAATGTCAAGTGAAAGACAA
R2	TCACTTGACATTGTCTCTCGGTAATGAAGG

[a] Underlined nucleotides are restriction sites used in this study.

2.5. Luciferase reporter assay

To construct the plasmid pmiR194a-Report, the 3′UTR of Japanese flounder IRF7 (IRF7-3′UTR) was amplified by PCR using the primers pair of F1/R1 (Table 1) and inserted into the luciferase reporter vector pMIR-REPORTER (AmBio, USA) at the Spe I/Hind III sites. The pMIR-3UTR-IRF7-mut reporter plasmid was identical to thepmiR194a-Report plasmid, except that the sequence (5′- CTGTTAC-3′) complementary to the seed sequence of pol-miR-194a was mutated to “5′-GACAATG-3” by overlapping PCR with the primer pair of F2/R2 (Table 1). To determine interaction between pol-miR-194a and IRF7-3′UTR, 293T cells were transfected with pmiR194a-Report alone or with pmiR194a-Report plus pol-miR-194a mimic, pmiR194a-Report plus pol-miR-NC, pmiR194a-Report plus pol-miR-194a-mut mimic, pmiR194a-Report-mut plus pol-miR-194a mimic or pmiR194a-Report-mut plus pol-miR-NC. At 24 h post transfection, the cells were lysed and measured for luciferase activity using a firefly luciferase reporter gene assay kit (Beyotime Biotechnology, China) with β-galactosidase as an internal control. To determine the effect of pol-miR-194a on the promoter activity of type I interferon, FG cells were transfected with pGL3-IFNp [35] plus pol-miR-194a mimic or pGL3-IFNp plus pol-miR-NC, and the control cells were transfected with pGL3-IFNp alone; luciferase activity was determined as above. All experiments were performed three times, each time in triplicate.

2.6. Western blot to determine IRF7 protein in FG cells

FG-9307 cells were transfected with pol-miR-194a mimic, pol-miR-NC, or PBS (control) as above for 24 h. The cells were lysed with RIPA lysis buffer (Beyotime Biotechnology, China) on ice for 30 min. The lysed product was centrifuged to collect the supernatant, which was mixed with 5 × loading buffer (250 mM Tris-HCL, 10% sodium dodecyl sulfate, 0.5% bromophenol blue, 50% glycerol, and 5% beta-mercaptoethanol) and boiled for ten minutes. The sample was then subjected to 12% SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membranes (Millipore, UK) and blocked with 5% skim milk, followed by incubation orderly with anti-IRF7 antibody (Proteintech, USA) and HRP-conjuncted anti-rabbit antibody (Abcam, UK). The membrane was finally incubated with ECL substrate (Beyotime Biotechnology, China) and visualized with GelDoc XR System (Bio-Rad, USA).

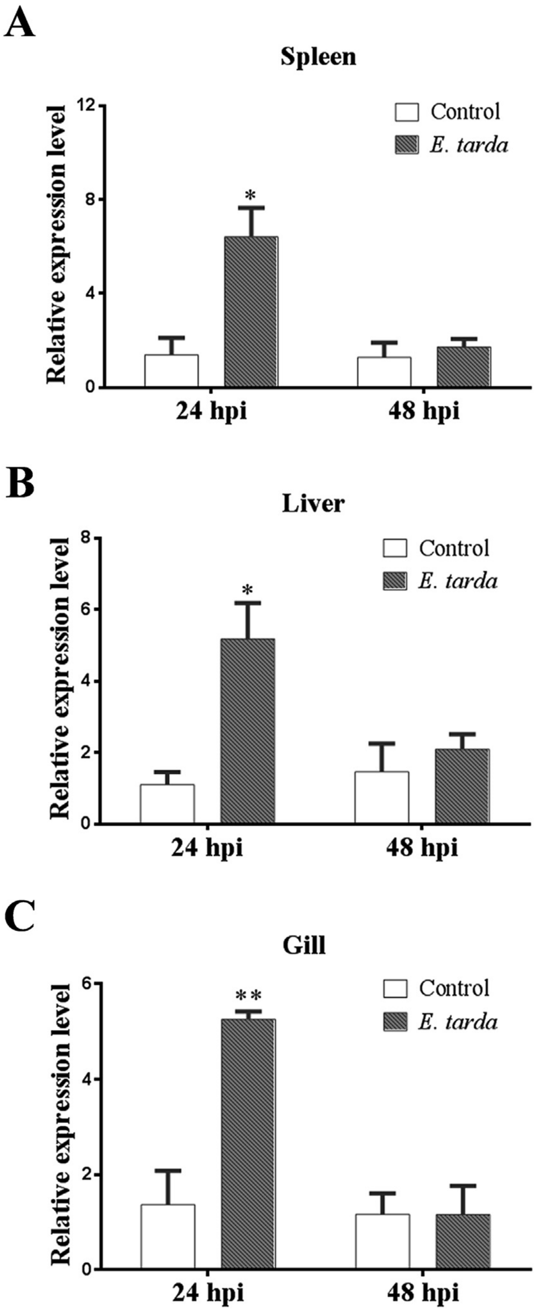

Fig. 1. Expression of pol-miR-194a in Japanese flounder after *Edwardsiella tarda* infection. Japanese flounder was infected with *E. tarda*, and pol-miR-194a expression in spleen, liver, and gill was determined by qRT-PCR at 24 h and 48 h post infection (hpi). Values are the means of three replicates and shown as means ± SEM. **, $P < 0.01$; *, $P < 0.05$.

2.7. Statistical analysis

All experiments were performed at least three times, and statistics analysis was carried out with GraphPad Prism 5 (GraphPad Software, USA).

3. Results

3.1. Regulation of pol-miR-194a expression by E. tarda

qRT-PCR analysis showed that when Japanese flounder were infected with *E. tarda*, pol-miR-194a expression in spleen, liver, and gill was significantly upregulated at 24 hpi, with a fold increase of 6.4, 5.2 and 5.3, respectively (Fig. 1). No apparent change in pol-miR-194a expression was observed at 48 hpi. These results indicate that pol-miR-194a expression was responsive to *E. tarda* at the early infection period.

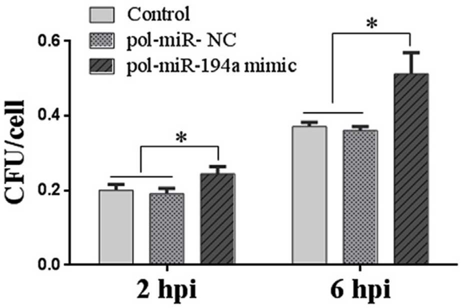

Fig. 2. Effect of pol-miR-194a on the intracellular proliferation of *Edwardsiella tarda*. FG cells transfected with pol-miR-194a mimic, pol-miR-NC, or PBS (control) were infected with *E. tarda*, and intracellular bacterial number was determined at 2 hpi and 6 hpi. Values are the means of three replicates and shown as means ± SD. *, $P < 0.05$.

3.2. Effect of pol-miR-194a overexpression on E. tarda proliferation in host cells

To examine whether pol-miR-194a affected the ability of *E. tarda* proliferation in host cells, flounder FG cells were experimented to overexpress pol-miR-194a by transfection with pol-miR-194a mimic before *E. tarda* infection. Following *E. tarda* infection for 2 h, the intracellular numbers of bacteria were comparable between pol-miR-194a mimic-transfected cells and control cells, whereas at 6 h after *E. tarda* infection, the intracellular number of bacteria in pol-miR-194a mimic-transfected cells was significantly higher than that in the control cells or in the cells transfected with negative control miRNA (pol-miR-NC) (Fig. 2).

3.3. Identification of IRF7 as the target gene of pol-miR-194a

IRF7 was predicted to be a potential target for pol-miR-194a. Potential direct interaction between pol-miR-194a and IRF7-3′UTR was examined by a luciferase reporter system, in which interaction between pol-miR-194a and IRF7-3′UTR carried in a reporter plasmid (pmiR194a-Report) would inhibit the expression of the luciferase gene. The wild type and mutant forms of the seed sequence of pol-miR-194a and its corresponding sequence in IRF7-3UTR are shown in Fig. 3A. The results showed that in 293T cells transfected with pmiR194a-Report plus pol-miR-194a mimic, luciferase expression was significantly reduced to the level of 53.2% of that in control cells transfected with pmiR194a-Report alone or in the cells transfected with pmiR194a-Report plus pol-miR-NC (Fig. 3B). In contrast, in cells co-transfected with pmiR194a-Report and pol-miR-194a-mut mimic (pol-miR-194a bearing mutation at the seed sequence), luciferase expression increased compared to that in the control cells, but the increase was not statistically significant (Fig. 3B). Similar results were observed in cells co-transfected with pol-miR-194a mimic and pmiR194a-Report-mut (pmiR194a-Report bearing mutation at IRF7-3′UTR) (Fig. 3B).

3.4. Effect of pol-miR-194a on the expression of IRF7

To examine whether pol-miR-194a had any effect on the expression of IRF7 at protein level, FG cells were transfected with pol-miR-194a mimic for 24 h, and IRF7 protein in the cells was determined. The results showed that compared to the control cells, in which the relative density of IRF7 was 1.220, FG cells transfected with pol-miR-194a mimic exhibited apparently lower level of IRF7 (relative density of

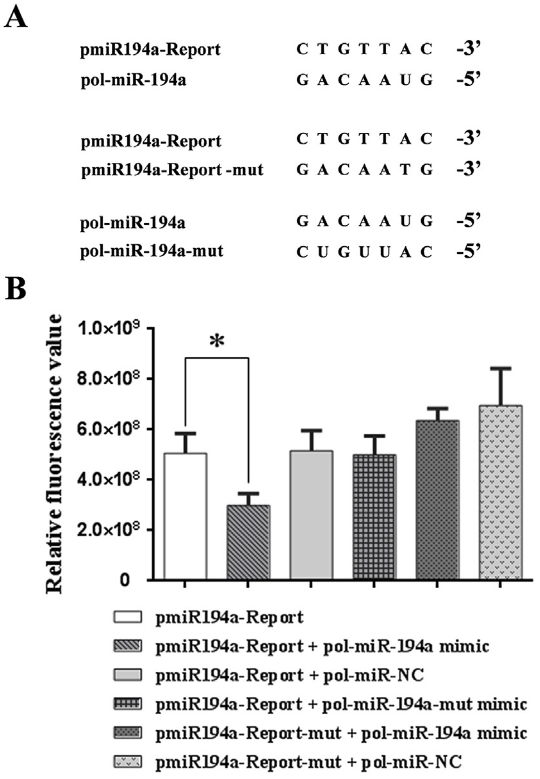

Fig. 3. Interaction between pol-miR-194a and the 3′-UTR of IRF7 (IRF7-3′UTR). (A) The seed sequence of pol-miR-194a in pmiR194a-Report and the corresponding pairing sequence of IRF7-3′UTR in pol-miR-194a (upper pair); the corresponding mutated sequences in pmiR194a-Report-mut (middle pair) and pol-miR-194a-mut (lower pair). (B) 293T cells were transfected with pmiR194a-Report alone or with pmiR194a-Report plus pol-miR-194a mimic, pmiR194a-Report plus pol-miR-NC, pmiR194a-Report plus pol-miR-194a-mut mimic, pmiR194a-Report-mut plus pol-miR-194a mimic, or pmiR194a-Report-mut plus pol-miR-NC, and luciferase activity was determined at 24 h after transfection. Values are the means of three replicates and shown as means ± SD. *, $P < 0.05$.

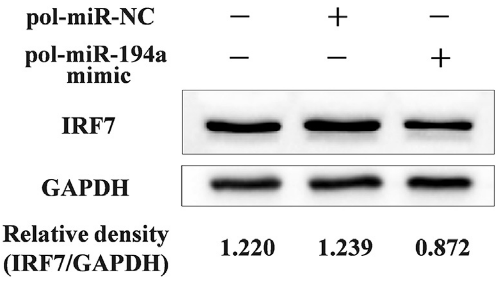

Fig. 4. Repression of the expression of IRF protein by pol-miR-194a. Western blot was conducted to examine IRF7 protein in FG cells transfected with or without pol-miR-194a mimic or pol-miR-NC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The densities of the bands were determined by densitometry analysis, and the relative densities (IRF7/GAPDH) are shown in the bottom of the figure.

0.872), whereas FG cells transfected with pol-miR-NC exhibited roughly similar level of IRF7 (relative density of 1.239) as that of the control cells (Fig. 4).

3.5. *Effect of pol-miR-194a on the promoter activity of type I interferon*

IRF7 is known to regulate type I interferon response by binding to its promoter region. To examine whether pol-miR-194a would affect the activity of type I interferon promoter, the reporter plasmid pGL3-IFNp was created, which contains a luciferase gene driven in expression by the promoter of type I interferon. When FG cells were co-transcribed with pGL3-IFNp and pol-miR-194a mimic, the luciferase activity was significantly reduced to 36% of that in the control cells transfected with pGL3-IFNp alone (Fig. 5).

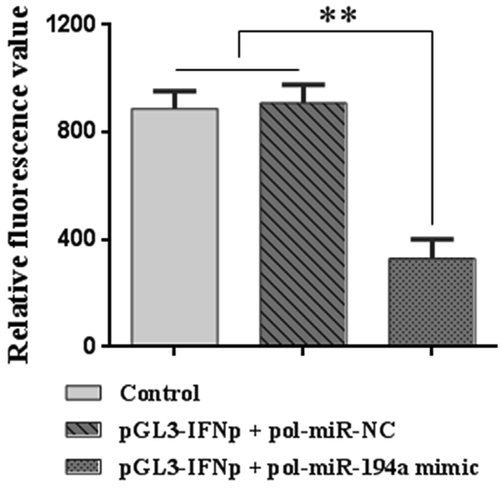

Fig. 5. Effect of pol-miR-194a on the promoter activity of type I interferon. FG cells were transfected with pGL3-IFNp plus pol-miR-194a mimic, pGL3-IFNp plus pol-miR-NC, pGL3-IFNp alone (control); luciferase activity was determined at 24 h after transfection. Values are the means of three replicates and shown as means ± SD. **, $P < 0.01$.

4. Discussion

In this study, we examined the immune function of a Japanese flounder miRNA, pol-miR-194a, and evaluated its effect on *E. tarda* infection. pol-miR-194a was identified in a previous study, in which pol-miR-194a was shown to be down-regulated in expression by megalocytivirus at the early phase of infection [34], however, the function and working mechanism of pol-miR-194 are unknown.

To date, very little study on miR-194a has been documented. In mammals, the only report on miR-194a is the observation that miR-194a in weaned piglets was down-regulated in comparison with that in suckling piglets [41]. In fish, miR-194a was only studied in zebrafish, in which, it was shown that the expression level of miR-194a was significantly changed in zebrafish liver cells (ZFL) after treatment with lipopolysaccharide, and that over-expression of miR-194a in ZFL resulted in an markedly increased expression of IL-22, lysozyme and TNF-α [42], but the underlining mechanism, including the target of miR-194a, is not clear. In the present study, we found that following *E. tarda* infection, pol-miR-194a expression was up-regulated significantly at 24 hpi, which is in contrast to the previously observed down-regulation of pol-miR-194a in megalocytivirus-infected flounder. By 48 hpi, the pol-miR-194a level in *E. tarda*-infected flounder returned to normal (similar to that in control fish), suggesting an early regulatory role of pol-miR-194a in *E. tarda* infection. This hypothesis was supported by the observation that in flounder cells transfected with pol-miR-194a mimic, *E. tarda* replication increased to significant extents in comparison to that in control cells, indicating a positive effect of pol-miR-194a on *E. tarda* infection. Previous studies have shown that during the battle of pathogen-host interaction, miRNAs can serve as a weapon for both the host and the pathogen [9,10]; from one aspect, the host can launch immune defense by miRNA-mediated regulation of gene expression, whereas from the other aspect, the pathogen can target host miRNAs to weaken host immune competence [43]. Given these findings and the observed enhancing effect of pol-miR-194a on *E. tarda* invasion, pol-miR-194a is likely a potential target of manipulation for *E. tarda*, which is known to be capable of escaping host immune killing by subverting some important host processes including apoptosis and phagocytic destruction [30,31,44,45].

In our study, dual-luciferase reporter assay identified IRF7 as the target gene of pol-miR-194a, which interacted in a negative manner with the 3′UTR of IRF7. Consistently, over-expression of pol-miR-194a in flounder cells effectively decreased the level of IRF7 protein, suggesting that pol-miR-194a-induced suppression of IRF7 mRNA expression affected the cellular level of IRF7 protein. IRF7 is a member of the IRF family, which are transcription factors that directly regulate the expression of interferon (IFN) expression and thus participate in diverse biological processes [46–48]. IRF7 is known to be a key regulator of type I IFN and IFN-stimulated genes (ISGs) and plays an important role in antiviral defense [49–51]. As a result, IRF7 is a target of manipulation for some viruses. For example, vesicular stomatitis virus induced IRF7 modification by small ubiquitin-related modifier, resulting in an attenuated activation of type I IFN [52]; enterovirus 71 could utilize its 3C protein to mediate cleavage of IRF7, thus compromising the type I IFN response [53,54]. For bacterial pathogens, reports have shown that infection of *Listeria monocytogenes* induced activation of type I IFN in mice [55,56]; pre-activation of type I IFN in human and mouse cells inhibited the infection of *Chlamydia trachomatis*, as a consequent of depletion of intracellular tryptophan and iron [57,58]. However, the function of IRF7 and type I IFN associated with *E. tarda* is unknown. A previous report showed that recombinant interferon-γ of olive flounder could activate immune responses against *E. tarda* and prevent edwardsielosis [59], suggesting an importance of interferon-γ in host clearance of *E. tarda*. In our study, we found that, in line with its ability to inhibit IRF7 expression and protein production, pol-miR-194a significantly reduced the promoter activity of type I IFN in flounder cells. This result suggests that the stimulatory effect of pol-miR-194a on *E. tarda* replication in FG cells observed in our study is likely due to depression of the type I IFN pathway by pol-miR-194a.

In conclusion, our study provides the first insight into the regulatory mechanism of miR-194a by showing flounder miR-194a as a key immune regulatory molecule that negatively regulates its target gene IRF7. pol-miR-194a is induced by *E. tarda* at the early phase of infection and inhibits the expression of IRF7, which in turn affects the activation of type I interferon, resulting in optimal intracellular infection of *E. tarda*. Thus, our study also indicates a new immune-evasion strategy of *E. tarda*, in which, pol-miR-194a serves as a host target for *E. tarda* manipulation.

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