Ning et al. *Zool. Res.* 2024, 45(1): 25−35
https://doi.org/10.24272/j.issn.2095-8137.2022.520

ZR | Zoological Research

Article Open Access

LncRNA pol-lnc78 as a ceRNA regulates antibacterial responses via suppression of pol-miR-n199-3p-mediated SARM down-regulation in *Paralichthys olivaceus*

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ABSTRACT

Long non-coding RNAs (lncRNAs) function as key modulators in mammalian immunity, particularly due to their involvement in lncRNA-mediated competitive endogenous RNA (ceRNA) crosstalk. Despite their recognized significance in mammals, research on lncRNAs in lower vertebrates remains limited. In the present study, we characterized the first immune-related lncRNA (pol-lnc78) in the teleost Japanese flounder (*Paralichthys olivaceus*). Results indicated that pol-lnc78 acted as a ceRNA for pol-miR-n199-3p to target the sterile alpha and armadillo motif-containing protein (SARM), the fifth discovered member of the Toll/interleukin 1 (IL-1) receptor (TIR) adaptor family. This ceRNA network regulated the antibacterial responses of flounder via the Toll-like receptor (TLR) signaling pathway. Specifically, SARM acted as a negative regulator and exacerbated bacterial infection by inhibiting the expression of inflammatory cytokines IL-1β and tumor necrosis factor-α (TNF-α). Pol-miR-n199-3p reduced SARM expression by specifically interacting with the 3' untranslated region (UTR), thereby promoting SARM-dependent inflammatory cytokine expression and protecting the host against bacterial dissemination. Furthermore, pol-lnc78 sponged pol-miR-n199-3p to ameliorate the inhibition of SARM expression. During infection, the negative regulators pol-lnc78 and SARM were significantly down-regulated, while pol-miR-n199-3p was significantly up-regulated, thus favoring host antibacterial defense. These findings provide novel insights into the mechanisms underlying fish immunity and open new horizons to better understand ceRNA crosstalk in lower vertebrates.

Keywords: LncRNA; SARM; miRNA; ceRNA; Antibacterial response

INTRODUCTION

Sterile alpha and armadillo motif-containing protein (SARM), the last discovered Toll/interleukin 1 (IL-1) receptor (TIR) adaptor, exhibits a highly conserved sequence across evolution, from nematodes to mammals (Jault et al., 2004; Lindsay & Wasserman, 2014; Vériepe et al., 2015; Zhou et al., 2013). Indeed, phylogenetic analysis suggests a potential bacterial origin for animal SARM, likely arising from horizontal gene transfer (Malapati et al., 2017; Zhang et al., 2011). Like the other four TIR adaptors, myeloid differentiation primary response 88 (MyD88), MyD88 adaptor-like (Mal), TIR domain-containing adapter-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM), SARM features a TIR domain, which facilitates its role in Toll-like receptor (TLR) signaling and innate immunity (Panneerselvam & Ding, 2015). Functional analyses demonstrated that, across all examined species, from worms and flies to mammals, SARM significantly influences immune responses to pathogenic infections, with varied outcomes despite sequence conservation. In *Caenorhabditis elegans*, the SARM ortholog TIR-1 regulates host defense against fungal and bacterial infections, enhancing survival without relying on the sole Toll receptor (Couillault et al., 2004). In *Drosophila melanogaster*, the SARM ortholog Ect4 is vital for survival post-viral infection, possibly reducing Toll signaling to restore homeostasis (Monsanto-Hearne et al., 2017). In mammals, SARM acts as a negative regulator in immune defense against live bacterial infection by suppressing TLR signaling (Carty & Bowie, 2019). Although limited in scope, investigations conducted on teleost fish, which serve as representatives of lower vertebrates, have

Received: 27 April 2023; Accepted: 07 September 2023; Online: 08 September 2023

Foundation items: This work was supported by the National Natural Science Foundation of China (42006082), Natural Science Foundation of Jiangsu Province of China (BK20221323), "JBGS" Project of Seed Industry Revitalization in Jiangsu Province (JBGS [2021] 034), and State Key Laboratory of Developmental Biology of Freshwater Fish (2021KF009)

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indicated the involvement of SARM in the immune responses of grass carp to grass carp reovirus (GCRV) infection and mandarin fish to stimulation with poly (I:C) and lipopolysaccharide (LPS) (Wang et al., 2021a; Yan et al., 2015). Nonetheless, the precise role of SARM in bacterial infections in fish remains undetermined, and its immunological mechanism remains elusive. Recent research on worms and flies has revealed that SARM expression is mediated by non-coding RNAs (ncRNAs), specifically miR-956 and miR-71, respectively (Finger et al., 2019; Monsanto-Hearne et al., 2017), providing a crucial clue for elucidating the regulatory mechanisms of SARM.

NcRNAs, including microRNAs (miRNAs), lncRNAs, and circular RNAs (circRNAs), have attracted considerable attention in gene regulation. As highly conserved small ncRNAs (~22 nucleotides long) (Cheng et al., 2005), miRNAs play vital roles in biological processes, such as growth, development, reproduction, and immunity (Andreassen & Høyheim, 2017; Hatfield et al., 2005; Sarma et al., 2014; Vasadia et al., 2019; Wienholds & Plasterk, 2005). The regulatory mechanisms governing miRNAs are well characterized, involving the repression of gene expression by promoting mRNA degradation or suppressing mRNA translation (Bagga et al., 2005). LncRNAs, a class of ncRNAs longer than 200 nucleotides, can be distinguished by their transcriptional but non-translational nature (Kapranov et al., 2007). LncRNAs exert diverse effects on development, differentiation, metabolism, and immunity (Cesana et al., 2011; Du et al., 2017; Sarangdhar et al., 2018; Wang et al., 2017b). LncRNAs can regulate gene expression directly through *cis*- or *trans*-acting mechanisms (Ponting et al., 2009). Recently, a novel mechanism of lncRNA-mediated regulation has emerged, whereby lncRNAs function as miRNA sponges to modulate target genes, known as competitive endogenous RNA (ceRNA) activity (Wang et al., 2013). LncRNA-mediated ceRNA crosstalk has been extensively detected in mammalian immunity (Cong et al., 2019; Song et al., 2017; Wu et al., 2017). In the context of fish, it is anticipated that ceRNA networks participate in immune responses (Wu et al., 2021, 2022; Ye et al., 2021). However, given that the intricacies of the lncRNA-miRNA-mRNA regulatory mechanism remain poorly understood, with only recent studies clarifying this mechanism in miiuy croaker antipathogen infections (Chu et al., 2020, 2021b; Zheng et al., 2021a, 2021b), assessing the extent of the ceRNA mechanism across different fish species remains crucial.

Teleosts are representative of early vertebrate evolution and can serve as key models in lower vertebrate studies. However, these economically valuable aquaculture species face significant threats from bacterial diseases, amplifying their importance in immunology research. Notably, vibriosis, a prevalent aquatic disease caused primarily by *Vibrio anguillarum* (Egidius, 1987), has prompted great research efforts into fish defense mechanisms (Ning & Sun, 2020a, 2020b, 2021a, 2021b; Qi et al., 2021; Wang et al., 2021b; Xu et al., 2019). Nevertheless, in-depth investigations on fish defense responses remain severely impeded due to limitations in experimental techniques, including the lack of suitable cell lines. Furthermore, fish immune processes and regulatory cascades exhibit notable differences when contrasted with the well-characterized mammalian immunity.

In this study, we identified a lncRNA-miRNA-mRNA ceRNA network, consisting of pol-miR-n199-3p, pol-lnc78, and SARM, which exhibited the capacity to regulate the host immune response in the teleost Japanese flounder (*Paralichthys olivaceus*). SARM acted as a negative regulator, inhibiting the expression of inflammatory cytokines, and was declined upon *V. anguillarum* infection. Pol-miR-n199-3p suppressed SARM expression by targeting the 3' untranslated region (UTR), thereby contributing to host defense against pathogens. Moreover, pol-lnc78 functioned as a ceRNA, counteracting the inhibitory effects of pol-miR-n199-3p on SARM, and thus regulating SARM-mediated immune responses. Our results represent the first ceRNA regulatory mechanism identified in flounder. These findings should help advance our understanding of ceRNA crosstalk in lower vertebrates.

MATERIALS AND METHODS

Fish and bacterial infection

Clinically healthy Japanese flounder (~50 g) were purchased from a commercial fish farm in Shandong Province, China, and maintained at 20±1 °C in aquariums equipped with aerated seawater and digital temperature sensing controllers as reported previously (Ning & Sun, 2020a). Fish were acclimated in the laboratory for at least one week before any experimental manipulation. Bacterial challenge was performed as reported previously (Ning & Sun, 2020a). Briefly, flounder individuals were injected intramuscularly with 100 μL of *V. anguillarum* C312, a pathogenic strain isolated from diseased flounder (Zheng et al., 2010), at a concentration of 5×10^6 colony forming units (CFU)/mL. The same volume of phosphate-buffered saline (PBS) was injected into flounder as a control. At 12 h post-infection (hpi), fish were euthanized, and spleen tissue samples were collected for RNA extraction. All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals in China and were approved by the Nanjing Normal University Animal Ethics Committee (Permit No. SYXK2015-0028).

Bio-samples from the spleen, previously utilized in a transcriptome study examining the responses of flounder to *V. anguillarum* over various time points (Ning & Sun, 2020a), were used to evaluate SARM and pol-lnc78 expression at 6, 12, and 24 hpi and for subsequent correlation analyses.

RNA extraction and reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA from spleen samples was extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocols. The cDNA and miRNA cDNA templates were synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Japan) and miRNA First-Strand cDNA Synthesis Kit (Vazyme, China), respectively, according to the manufacturer's instructions. The RT-qPCR (mRNA and lncRNA) and stem-loop RT-qPCR (miRNA) procedures were carried out with SYBR Premix Ex TaqII (TaKaRa, China) using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA) according to the manufacturer's protocols. The expression level was determined using the $2^{-\Delta\Delta Ct}$ comparative Ct method (Ning & Sun, 2020b). β-actin and α-tubulin were used as the internal controls for samples of uninfected and *V. anguillarum*-infected flounder, respectively. 5S rRNA was used as the internal control for pol-miR-n199-3p. The primers used for RT-qPCR are listed in Supplementary Table S1. Correlation analysis was performed using the cor.test in R (v.3.5.2).

Plasmid construction

To construct the SARM expression plasmid (pCN3-SARM), the *SARM* gene sequence in flounder (Gene ID 109630267) was amplified using PCR with specific primers, with the PCR product then cloned into the expression vector pCN3 at the EcoR V site as reported previously (Jiao et al., 2009). Similarly, the pol-lnc78 expression plasmid (pCN3-lnc78) was constructed by cloning the pol-lnc78 sequence (accession number PRJNA554220) into pCN3 at the EcoR V site. To construct the SARM 3'UTR reporter plasmid (pmir-SARM-wt), the 3'UTR region of SARM was amplified by PCR, with the PCR product then inserted into the pmirGLO vector at the Sac I/Xba I sites. The mutant form of the SARM 3'UTR reporter plasmid (pmir-SARM-mut) was identical to the SARM 3'UTR reporter plasmid, except that the sequence (5'-CAGTGTT-3') complementary to the seed sequence of pol-miR-n199-3p was mutated to 5'-GTCACAA-3'. Similarly, the pol-lnc78 reporter plasmid (pmir-lnc78-wt) and mutated-pol-lnc78 reporter plasmid (pmir-lnc78-mut) were constructed by inserting the pol-lnc78 sequence and pol-lnc78 with mutated sequence (5'-CAGTGTT-3' mutated to 5'-GTCACAA-3') into the pmirGLO vector, respectively. The primers used to construct plasmids are listed in Supplementary Table S1.

MiRNA mimic and agomir

Pol-miR-n199-3p mimic and agomir, as well as respective negative control miR-NC and agomir-NC, were synthesized by GenePharma (China).

Dual-luciferase reporter assay

Human embryonic kidney epithelial (HEK293T) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 37 °C with 5% CO_2 in 24-cell plates. To investigate the interaction between pol-miR-n199-3p and SARM 3'UTR, the HEK293T cells were transfected with pmir-SARM-wt alone or co-transfected with pmir-SARM-wt or pmir-SARM-mut, along with pol-miR-n199-3p mimic or miR-NC using Lipofectamine™ 3000 (Invitrogen, USA) according to the manufacturer's instructions. After transfection for 8 h, the culture medium was renewed. At 24 h post-transfection, the cells were lysed, and luciferase activity was measured using a Dual Luciferase Reporter Assay Kit (Vazyme, China). Similarly, the interaction between pol-miR-n199-3p and pol-lnc78 was determined with HEK293T cells transfected with pmir-lnc78-wt alone or co-transfected with pmir-lnc78-wt or pmir-lnc78-mut, along with pol-miR-n199-3p mimic or miR-NC. To detect the competition between pol-lnc78 and SARM in binding to pol-miR-n199-3p, the HEK293T cells were co-transfected with pol-miR-n199-3p mimic or miR-NC, together with the pCN3 vector or pol-lnc78 expression plasmid, along with SARM 3'UTR, or transfected with SARM 3'UTR alone. All luciferase activity values were normalized against the *Renilla* luciferase control.

In vivo overexpression and effects on bacterial infection

Flounder with SARM or pol-lnc78 overexpression were constructed, with four groups of flounder used. Fish in the former two groups were injected with expression plasmid pCN3-SARM or pCN3-lnc78 with 2 μg plasmid/g fish, respectively. Fish in the latter two groups were injected with or without the same volume of empty vector pCN3 as a control. Spleen tissues were collected at 3 d post-plasmid administration, and the expression levels of SARM, pol-lnc78, and pol-miR-n199-3p were examined by RT-qPCR.

To investigate the effects of SARM or pol-lnc78 overexpression on *V. anguillarum* infection, flounder were injected with the plasmid pCN3-SARM or pCN3-lnc78, as described above. At 2.5 d post-plasmid administration, flounder were injected with *V. anguillarum* (5×10^5 CFU/fish). At 12 h post *V. anguillarum* injection, number of bacteria was determined using the plate count method, as reported previously (Zhang et al., 2020).

Flounder with pol-miR-n199-3p overexpression or pol-miR-n199-3p and pol-lnc78 co-overexpression were constructed, with a total of five groups used. For the former three groups, flounder were injected with or without pol-miR-n199-3p agomir or agomir-NC (2 μg/g fish) for 12 h. For the latter two groups, fish were injected with pCN3-lnc78 or pCN3 for 2.5 d, then injected with pol-miR-n199-3p agomir (2 μg/g fish) for another 12 h. The expression levels of pol-miR-n199-3p, SARM, and pol-lnc78 were determined by RT-qPCR.

To investigate the effects of pol-miR-n199-3p overexpression on *V. anguillarum* infection, fish were injected with pol-miR-n199-3p agomir (2 μg/g fish) together with *V. anguillarum* (5×10^5 CFU/fish). At 12 hpi, the number of *V. anguillarum* in the spleen and liver was determined by plate count.

Statistical analysis

Data are presented as means±standard deviation (SD) from three independent replicates. Student's *t*-test was used to compare the values between different groups. Statistical analyses were performed using SPSS software (v.23.0). Statistical significance was considered at $P<0.05$.

RESULTS

Flounder SARM negatively regulates bacterial infection

Symptoms in fish challenged with *V. anguillarum* were first confirmed. Notably, after infection, common pathological findings of vibriosis were observed in flounder, including ulcerative skin, fin hemorrhage, and swollen spleen (Figure 1A). To evaluate the expression profiles of flounder SARM in response to *V. anguillarum* challenge, RT-qPCR was conducted. Results showed that SARM expression was significantly reduced during *V. anguillarum* infection at 6, 12, and 24 hpi (Figure 1B). In mammals, SARM is an inhibitor of TLR signaling via the suppression of IL-1β and TNF-α expression after LPS stimulation. Accordingly, we examined whether flounder SARM could inhibit these inflammatory cytokines upon bacterial infection. Fish exhibiting SARM overexpression were established using plasmid pCN3-SARM injection. Enhanced SARM expression was validated by RT-qPCR at 3 d post-administration (Supplementary Figure S1). As depicted in Figure 1C, D, *in vivo* overexpression of SARM significantly down-regulated IL-1β and TNF-α expression in *V. anguillarum*-infected flounder. To investigate the effect of SARM on pathogen infection, bacterial counts were determined in *V. anguillarum*-infected flounder with or without SARM overexpression. Results showed that bacterial loads in fish with SARM overexpression were significantly higher than that in fish without SARM overexpression in both the spleen and liver (Figure 1E, F). These findings suggest that antibacterial immune defense is triggered via suppression of SARM expression, which activates the TLR signaling pathway and inflammatory response, resulting in the clearance of bacterial pathogens.

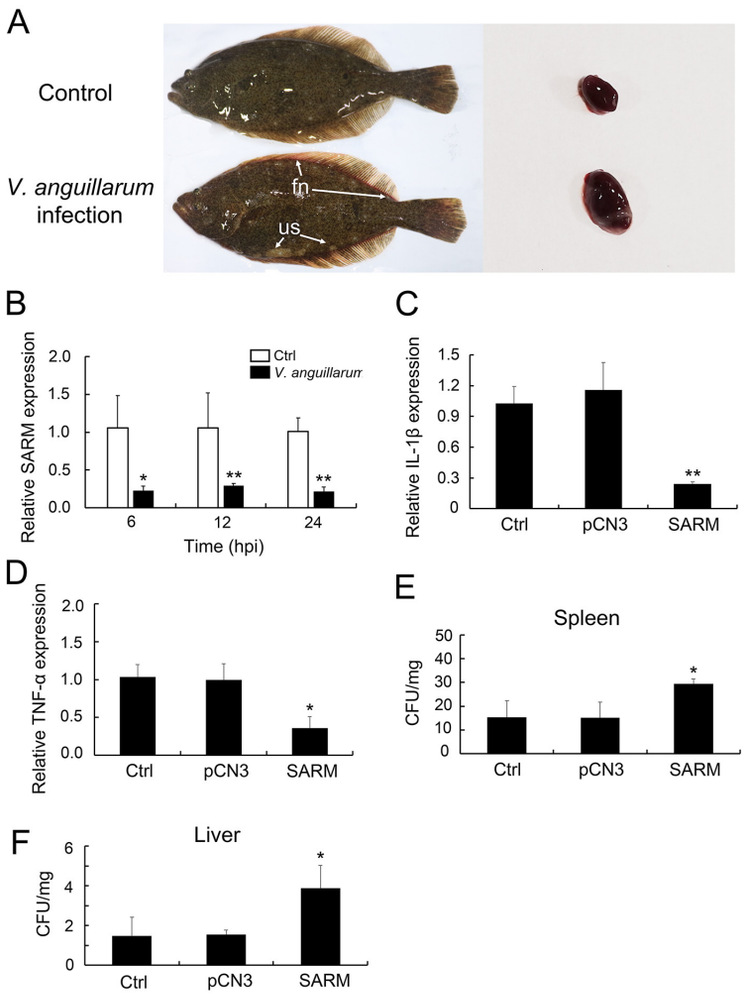

Figure 1 SARM acts as a negative regulator in host defense against *Vibrio anguillarum* infection

A: Symptoms of flounder infected with *V. anguillarum*. Compared to control fish, *V. anguillarum*-infected flounder exhibited ulcerative skin (us) and fin hemorrhage (fh). Swollen spleen was observed in *V. anguillarum*-infected flounder compared to control fish. B: SARM expression decreased in response to *V. anguillarum* infection. SARM expression in flounder infected with or without (control) *V. anguillarum* for 6, 12, and 24 h was examined. C, D: SARM inhibited expression of inflammatory cytokines. Flounder were administered with SARM expression plasmid or pCN3 vector for 2.5 d, then injected with *V. anguillarum* for another 12 h. Expression levels of IL-1β (C) and TNF-α (D) were examined. E, F: SARM exacerbated *V. anguillarum* infection. Flounder were administered with or without (control) SARM expression plasmid for 2.5 d, then injected with *V. anguillarum* for another 12 h. Bacterial loads in spleen (E) and liver (F) were determined through plate count. Ctrl: Control; hpi, hours post infection. *P*-values were calculated with Student's *t*-test. *: $P<0.05$; **: $P<0.01$. Error bars indicate SD.

SARM is a target gene of pol-miR-n199-3p

In our previous investigation on the micro-transcriptome of *V. anguillarum*-infected flounder, SARM was predicted to be a potential target of pol-miR-n199-3p (Ning & Sun, 2020b). To elucidate the mechanism underlying SARM modulating *V. anguillarum* infection, we examined the regulatory role of candidate miRNAs on SARM. The sequence features were first analyzed. As shown in Figure 2A, the sequence of SARM 3'UTR was completely complementary to the seed sequence of pol-miR-n199-3p. To confirm the specific binding site between pol-miR-n199-3p and SARM 3'UTR, a mutated form of SARM 3'UTR (SARM-mut) was constructed (Figure 2A). The dual-luciferase reporter assay was then conducted using HEK293T cells to verify the interaction between pol-miR-n199-3p and SARM 3'UTR. Results showed that pol-miR-n199-3p significantly reduced luciferase activity when co-transfected with the wild-type SARM 3'UTR but not with the mutated type (Figure 2B). Moreover, no significant decrease in luciferase activity was observed when pol-miR-n199-3p was replaced by miR-NC or wild-type SARM 3'UTR alone (Figure 2B). Finally, to determine the regulatory effect of pol-miR-n199-3p on SARM expression, a flounder model with pol-miR-n199-3p overexpression was established. Elevated pol-miR-n199-3p expression was confirmed by RT-qPCR (Supplementary Figure S2). SARM expression was significantly suppressed in fish with pol-miR-n199-3p overexpression, but not in the control fish (Figure 2C). Thus, these results indicate that pol-miR-n199-3p represses SARM expression via specific interactions with its 3'UTR.

Pol-miR-n199-3p enhances host antibacterial immunity by suppressing SARM

In our previous study on miRNA expression profiles in *V. anguillarum*-infected flounder, we found that pol-miR-n199-3p is significantly up-regulated in response to *V. anguillarum* infection at 6, 12, and 24 hpi (Ning & Sun, 2020b). In the present study, the expression levels of pol-miR-n199-3p and SARM were shown to be significantly negatively correlated

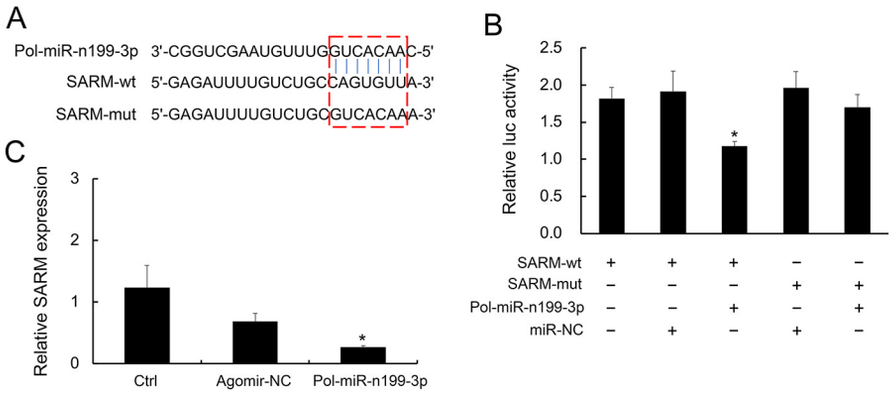

Figure 2 *SARM* is a target gene of pol-miR-n199-3p

A: SARM contained binding site for pol-miR-n199-3p. Sequence alignments between pol-miR-n199-3p seed sequence and its binding site in wild-type SARM 3'UTR (SARM-wt), as well as mutant-type with binding site mutation (SARM-mut), are shown. Red box indicates seed sequence in pol-miR-n199-3p, binding site in SARM-wt, and mutated sequence in SARM-mut. B: Pol-miR-n199-3p targeted 3'UTR of SARM. HEK293T cells were co-transfected with pol-miR-n199-3p mimic or miR-NC, along with SARM-wt or SARM-mut, or transfected with SARM-wt alone, with Luc activity then examined and normalized. NC, negative control. miR-NC, negative control of pol-miR-n199-3p mimic. Luc, luciferase. C: Pol-miR-n199-3p inhibited expression of SARM. Flounder were administered with or without (control, Ctrl) pol-miR-n199-3p agomir or administered with negative control of pol-miR-n199-3p agomir (agomir-NC) for 12 h. Expression levels of SARM were examined by RT-qPCR. *P*-values were calculated with Student's *t*-test. *: $P<0.05$. Error bars indicate SD.

($P<0.05$) (Figure 3A). Thus, we subsequently explored whether pol-miR-n199-3p had any effect on SARM expression. Results showed that pol-miR-n199-3p significantly suppressed SARM expression in flounder (Figure 3B). Given that SARM exacerbated bacterial infection by suppressing inflammatory cytokines, we wondered whether pol-miR-n199-3p could affect SARM-mediated IL-1β and TNF-α. As shown in Figure 3C, D, overexpression of pol-miR-n199-3p markedly increased the expression of IL-1β and TNF-α. To examine the direct effects of pol-miR-n199-3p on bacterial infection, bacterial counts were determined in fish with or without pol-miR-n199-3p overexpression upon *V. anguillarum* challenge. Our findings indicated that the up-regulation of pol-miR-n199-3p contributed to the inhibition of bacterial disseminations in both the spleen and liver (Figure 3E, F). These observations suggest that pol-miR-n199-3p promotes host defense against bacterial infection via increasing the expression of SARM-mediated inflammatory cytokines.

Pol-lnc78 regulates pol-miR-n199-3p expression via interaction

In our previous study on lncRNAs in flounder, we predicted that pol-lnc78 may target pol-miR-n199-3p (Ning & Sun, 2021a). To determine whether pol-lnc78 interacts with pol-miR-n199-3p, a dual-luciferase reporter assay was performed in HEK293T cells. The sequence of pol-lnc78 was first analyzed to identify the binding site for pol-miR-n199-3p (Figure 4A). Subsequently, both a wild-type pol-lnc78 reporter and mutated-type reporter with a mutated binding site were constructed. The dual-luciferase assay revealed that pol-miR-n199-3p strongly inhibited luciferase activity of the wild-type pol-lnc78 reporter but elicited no response in the mutated-type reporter (Figure 4B). To investigate the regulatory roles of pol-lnc78 on the target, a flounder model with pol-lnc78 overexpression was established using plasmid pCN3-lnc78 injection. After verification of increased pol-lnc78 expression at 3 d post-administration (Supplementary Figure S3), pol-miR-n199-3p expression was determined. As shown in Figure 4C, pol-lnc78 significantly reduced the expression of pol-miR-n199-3p *in vivo*. Together, these observations indicate that pol-lnc78 negatively regulates pol-miR-n199-3p expression through direct interaction.

Pol-lnc78 aggravates bacterial infection via suppression of pol-miR-n199-3p

In our previous research, we identified pol-lnc78 as a potential immune-related lncRNA engaged in *V. anguillarum* infection (Ning & Sun, 2021a). Here, validation of expression patterns indicated that pol-lnc78 was significantly down-regulated in the *V. anguillarum*-challenged flounder at 6, 12, and 24 h (Figure 5A). Correlation analysis showed that pol-lnc78 was significantly and negatively correlated ($P<0.05$) with pol-miR-n199-3p expression upon bacterial stimulation (Figure 5B). Moreover, *in vivo* overexpression of pol-lnc78 significantly decreased the expression of pol-miR-n199-3p in *V. anguillarum*-infected flounder (Figure 5C). Exploration of the potential regulatory effects of pol-lnc78 on inflammatory cytokines mediated by pol-miR-n199-3p during infection showed that overexpression of pol-lnc78 significantly repressed IL-1β and TNF-α expression in *V. anguillarum*-infected flounder (Figure 5D, E). An *in vivo* infection study was conducted using flounder with or without pol-lnc78 overexpression. Results indicated significantly increased bacterial loads in both the spleen and liver of fish overexpressing pol-lnc78 (Figure 5F, G). Collectively, these findings suggest that pol-lnc78 promotes bacterial dissemination by inhibiting pol-miR-n199-3p.

Pol-lnc78, as a ceRNA, sponges pol-miR-n199-3p to increase SARM expression

In our previous study on lncRNA-miRNA-mRNA networks in *V. anguillarum*-infected flounder, we predicted that the pol-lnc78-pol-miR-n199-3p-SARM axis may be involved in immune-related ceRNA networks (Ning & Sun, 2021a). In the current study, we validated the interaction between pol-lnc78 and pol-miR-n199-3p and the targeting of SARM by pol-miR-n199-3p. Subsequently, we explored whether pol-lnc78 could regulate SARM expression. *In vivo* analysis showed that overexpression of pol-lnc78 significantly up-regulated SARM expression (Figure 6A). Moreover, correlation analysis showed that the expression levels of pol-lnc78 and SARM

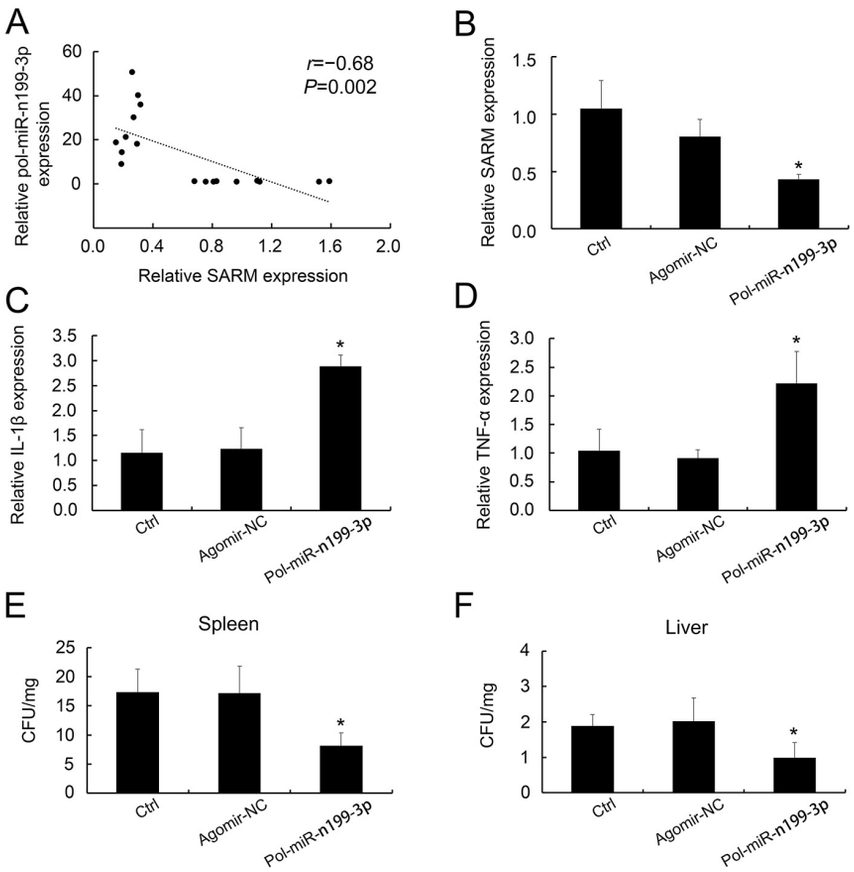

Figure 3 Pol-miR-n199-3p enhances host antibacterial defense by suppressing SARM expression

A: SARM and pol-miR-n199-3p were significantly and negatively correlated. Expression levels of SARM and pol-miR-n199-3p in flounder challenged with or without *Vibrio anguillarum* for 6, 12, and 24 h were tested, then subjected to correlation analysis. *r*, correlation coefficient. B: Pol-miR-n199-3p suppressed SARM expression upon *V. anguillarum* stimulation. Expression levels of SARM in flounder administered with pol-miR-n199-3p agomir or negative control (agomir-NC), along with *V. anguillarum* infection for 12 h, were detected. C, D: Pol-miR-n199-3p promoted expression of inflammatory cytokines that were reduced by SARM. Flounder were administered with pol-miR-n199-3p agomir or agomir-NC, along with *V. anguillarum* infection for 12 h. Expression levels of IL-1β (C) and TNF-α (D) were then examined. E, F: Pol-miR-n199-3p inhibited *V. anguillarum* infection. Flounder were administered with pol-miR-n199-3p agomir or agomir-NC, along with *V. anguillarum* infection for 12 h. Bacterial loads in spleen (E) and liver (F) were then determined by plate count. Ctrl: Control. *P*-values were calculated with Student's *t*-test. *: $P<0.05$. Error bars indicate SD.

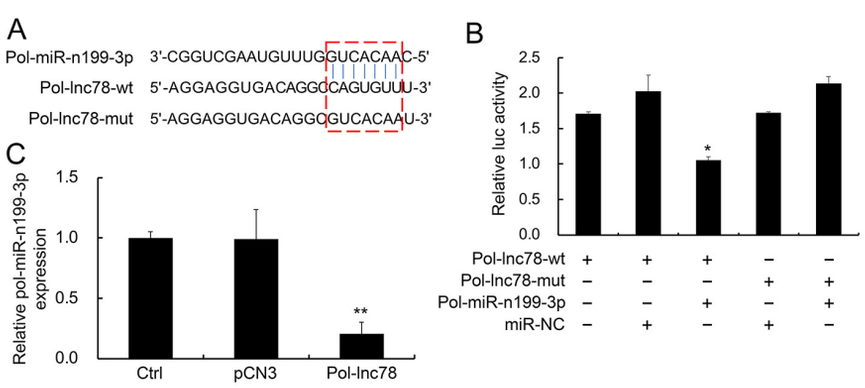

Figure 4 Pol-lnc78 regulates pol-miR-n199-3p expression by interactions

A: Pol-lnc78 contained the binding site for pol-miR-n199-3p. Sequence alignments between pol-miR-n199-3p seed sequence and its binding site in wild-type of pol-lnc78 (pol-lnc78-wt), as well as mutant-type with binding site mutated (pol-lnc78-mut), are shown. Red box indicates seed sequence in pol-miR-n199-3p, binding site in pol-lnc78-wt, and mutated sequence in pol-lnc78-mut. B: Pol-lnc78 interacted with pol-miR-n199-3p. HEK293T cells were co-transfected with pol-miR-n199-3p mimic or miR-NC, along with pol-lnc78-wt or pol-lnc78-mut, or transfected with pol-lnc78-wt alone, with Luc activity then examined and normalized. miR-NC, negative control of pol-miR-n199-3p mimic. Luc, luciferase. C: Pol-lnc78 inhibited pol-miR-n199-3p expression. Flounder were administered with or without pol-lnc78 expression plasmid or administered with pCN3 vector for 3 d. Expression of pol-miR-n199-3p was determined by RT-qPCR. Ctrl: Control. *P*-values were calculated with Student's *t*-test. *: $P<0.05$; **: $P<0.01$. Error bars indicate SD.

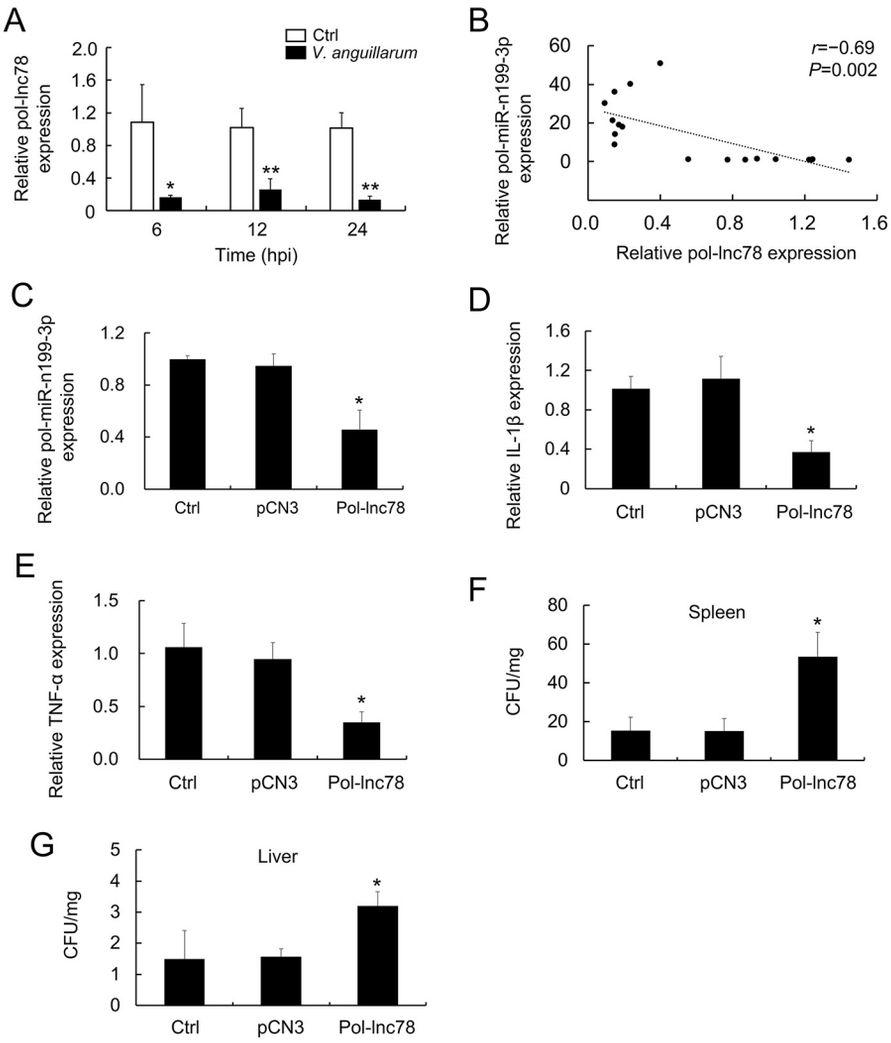

Figure 5 Pol-lnc78 suppresses anti-*Vibrio anguillarum* response by inhibiting pol-miR-n199-3p

A: Pol-lnc78 expression declined in response to *V. anguillarum* infection. Expression levels of pol-lnc78 in flounder infected with or without (control) *V. anguillarum* for 6, 12, and 24 h were examined. hpi: Hours post infection. B: Pol-lnc78 and pol-miR-n199-3p were significantly and negatively correlated. Expression levels of pol-lnc78 and pol-miR-n199-3p in flounder stimulated with or without *V. anguillarum* for 6, 12, and 24 h were tested, then subjected to correlation analysis. *r*, correlation coefficient. C–E: Pol-lnc78 suppressed expression of pol-miR-n199-3p and its mediated inflammatory cytokines upon *V. anguillarum* stimulation. Flounder were administered with or without pol-lnc78 expression plasmid or administered with pCN3 vector for 2.5 d, then injected with *V. anguillarum* for another 12 h. Expression levels of pol-miR-n199-3p (C), IL-1β (D), and TNF-α (E) were examined. F, G: Pol-lnc78 promoted *V. anguillarum* dissemination. Flounder were administered with or without pol-lnc78 expression plasmid or administered with pCN3 vector for 2.5 d, then injected with *V. anguillarum* for another 12 h. Bacterial loads in spleen (F) and liver (G) were determined by plate count. Ctrl: Control. *P*-values were calculated with Student's *t*-test. *: $P<0.05$; **: $P<0.01$. Error bars indicate SD.

were significantly and positively correlated during bacterial infection (Figure 6B), suggesting that pol-lnc78 regulates SARM expression. Thus, we next tested whether the regulatory effects of pol-lnc78 on SARM could be mediated by pol-miR-n199-3p. A dual-luciferase reporter assay was performed using HEK293T cells co-transfected with SARM 3' UTR reporter, pol-miR-n199-3p mimic, and/or pol-lnc78 expression plasmid. Results showed that pol-lnc78 counteracted the inhibitory effects of pol-miR-n199-3p on SARM 3'UTR (Figure 6C). Furthermore, comparing the expression of SARM in flounder with pol-miR-n199-3p overexpression, and with pol-miR-n199-3p and pol-lnc78 co-overexpression, we found that pol-lnc78 relieved the inhibitory effects of pol-miR-n199-3p on SARM expression (Figure 6D). Together, these observations suggest that pol-lnc78 can act as a ceRNA and sponge pol-miR-n199-3p to regulate the expression of SARM as well as SARM-mediated inflammatory cytokines, thereby modulating host antibacterial immunity (Figure 7).

DISCUSSION

Bacterial diseases greatly impact the fish farming industry, with vibriosis, one of the most prevalent aquaculture diseases globally, causing substantial economic losses (Egidius, 1987). Among the causative agents of vibriosis, *V. anguillarum* is noteworthy due to its pathogenicity and ubiquitous existence in the water environment, leading to widespread infections in various aquatic animals, including Atlantic cod (Lokesh et al., 2012), orange-spotted grouper (Huang et al., 2014), tongue sole (Zhang et al., 2015), turbot (Gao et al., 2016), miiuy croaker (Chu et al., 2017), and flounder (Gao et al., 2013). The innate immune system acts as the first and central line of defense in teleost fish, with the TLR signaling pathway playing

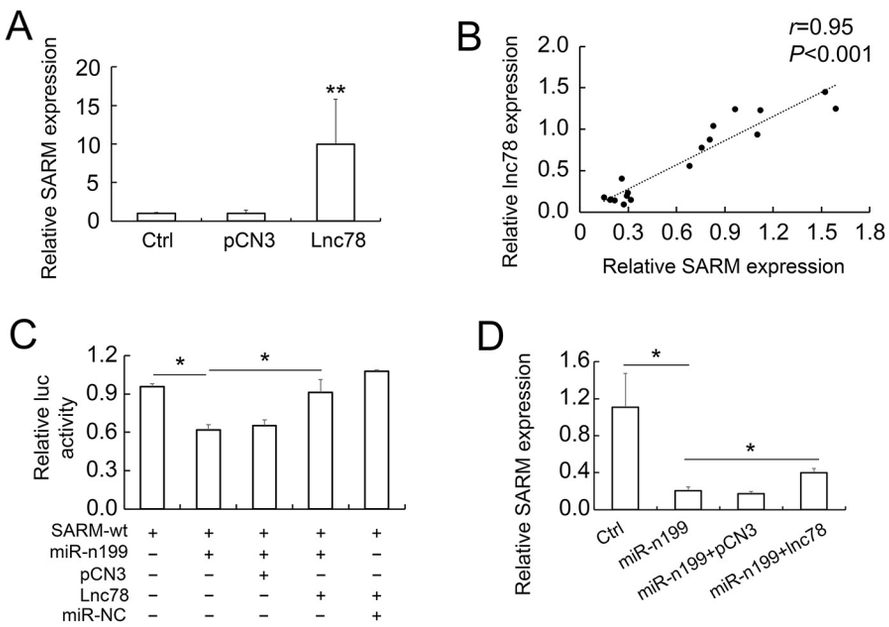

Figure 6 Pol-lnc78 as a ceRNA sponges pol-miR-n199-3p to enhance SARM expression

A: Pol-lnc78 up-regulates SARM expression. Flounder were administered with or without pol-lnc78 expression plasmid or administered with pCN3 vector for 3 d. Expression levels of SARM were determined. B: SARM and pol-lnc78 were significantly and positively correlated. Expression levels of SARM and pol-lnc78 in flounder infected with or without *Vibrio anguillarum* for 6, 12, and 24 h were examined, then subjected to correlation analysis. *r*, correlation coefficient. C: Pol-lnc78 counteracted inhibition of pol-miR-n199-3p on SARM 3'UTR. HEK293T cells were co-transfected with pol-miR-n199-3p mimic or miR-NC, together with pCN3 vector or pol-lnc78 expression plasmid, along with SARM 3'UTR, or transfected with SARM 3'UTR alone. Luc activity was examined and normalized. miR-NC, negative control of pol-miR-n199-3p mimic. Luc, luciferase. D: Pol-lnc78 counteracted inhibitory effect of pol-miR-n199-3p on SARM expression. Flounder were administered with or without pol-lnc78 expression plasmid for 2.5 d, then administered with pol-miR-n199-3p agomir for another 12 h. Expression levels of SARM were determined. Ctrl: Control. *P*-values were calculated with Student's *t*-test. *: $P<0.05$; **: $P<0.01$. Error bars indicate SD.

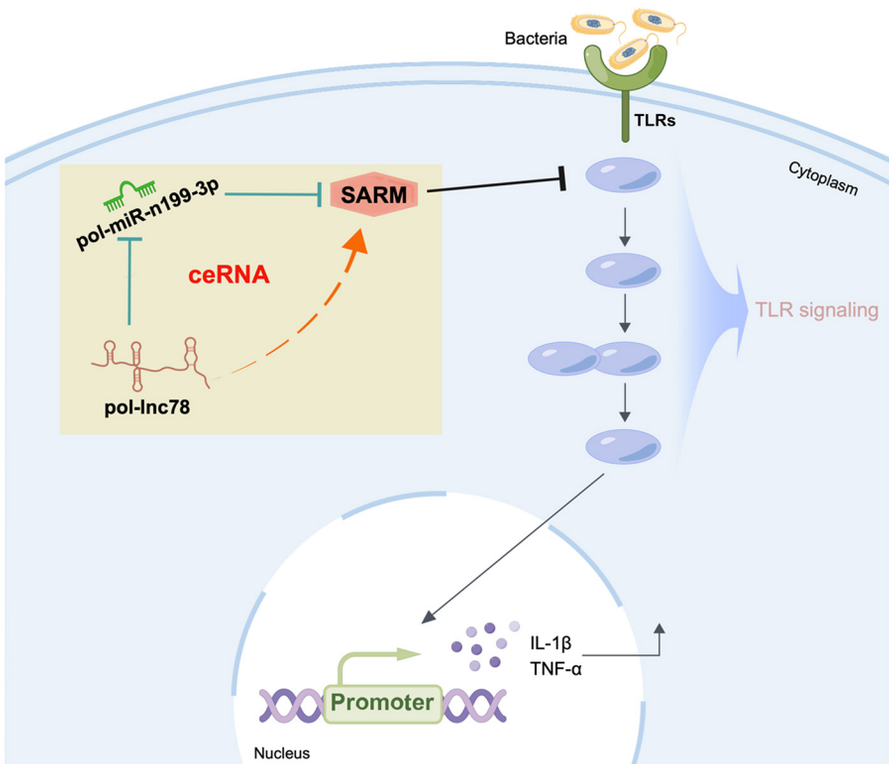

Figure 7 Mechanistic diagram of ceRNA network and antibacterial regulation of pol-lnc78, pol-miR-n199-3p, and SARM

Fish SARM negatively regulates immune response by inhibiting inflammatory cytokines IL-1β and TNF-α. Pol-miR-n199-3p targets SARM and promotes antibacterial defense by up-regulating SARM-dependent inflammatory cytokine expression. Pol-lnc78 acts as a ceRNA sponging pol-miR-n199-3p to enhance SARM, thus ensuring appropriate inflammatory and antibacterial responses for host survival.

a crucial role in antibacterial responses. Among the five TIR adaptors, SARM is uniquely inhibitory and highly conserved (O'Neill & Bowie, 2007). In the present study, we observed a significant decline in the expression of flounder SARM during *V. anguillarum* infection. The inhibitory role of SARM on the TLR pathway also appears to be conserved across mammals. For example, in human HEK293T cells, overexpression of SARM significantly represses LPS-mediated up-regulation of inflammatory cytokines, such as IL-1β and TNF-α (Carlsson et al., 2016). *Burkholderia pseudomallei* infection can increase SARM expression in mice, resulting in the suppression of TRIF and interferon β (IFNβ) production (Pudla et al., 2011). Similarly, we found that flounder SARM significantly reduced the expression of inflammatory cytokines IL-1β and TNF-α upon *V. anguillarum* stimulation. Previous studies have shown divergence in the function of SARM between invertebrates and mammals. In worms and flies, SARM orthologs contribute to host resistance against microbial pathogens (Liberati et al., 2004; Monsanto-Hearne et al., 2017), while $SARM^{-/-}$ mice exhibit higher survival from LPS-stimulated sepsis due to increased IL-1β release (Carty et al., 2019). Here, we found that overexpression of SARM in lower vertebrates such as flounder significantly exacerbated bacterial dissemination, indicating the functional resemblance of fish SARM to that of mammals.

The molecular mechanism by which SARM regulates immune function remains enigmatic, even in mammals. Studies on invertebrate model animals have suggested a potential miRNA-mediated modulation of SARM (Finger et al., 2019; Monsanto-Hearne et al., 2017). In teleost fish, miRNAs are recognized as ubiquitous and versatile regulators, critical in immune responses. For instance, in the orange-spotted grouper, miR-122 modulates the immune response to *Aeromonas hydrophila* infection by targeting IL-15 (Liu et al., 2020); in miiuy croaker, miR-144 and miR-217 act as negative modulators of the inflammatory response against *Vibrio harveyi* infection (Chu et al., 2021a). Recent studies have also suggested that miRNAs are involved in the flounder response to *V. anguillarum* challenge (Ning & Sun, 2020b). In the present study, pol-miR-n199-3p was shown to target the 3' UTR of SARM, leading to a reduction in expression. These findings are consistent with the established mechanism of miRNA modulation, whereby miRNAs interact with the 3'UTR of the target gene, thus facilitating its degradation or suppressing its translation at the post-transcriptional level (Cannell et al., 2008). Accumulating evidence suggests that fish miRNAs can affect pathogens. For example, flounder pol-miR-novel_171 promotes gram-negative bacterial dissemination (Li et al., 2020), pol-miR-150 negatively regulates gram-positive bacterial infection (Sun et al., 2021), and miR-206 increases *Mycobacterium marinum* burden in zebrafish (Wright et al., 2021). Here, we found that pol-miR-n199-3p played a protective role against pathogen infection in flounder. Of note, the inhibition of *V. anguillarum* dissemination by pol-miR-n199-3p was attributable to the increase in SARM-mediated inflammatory cytokines. Moreover, we found that SARM was linked to the regulation of lncRNA.

Despite continued research on lncRNAs since the first lncRNA (Xist) was discovered in humans (Borsani et al., 1991), identification of lncRNAs in non-model species, especially fish, has only gained momentum in recent years. In our previous study, we systematically identified flounder lncRNAs through bioinformatics analysis, highlighting pol-lnc78 as an immune-related lncRNA (Ning & Sun, 2021a). In the current study, functional experiments confirmed the importance of pol-lnc78 in the host immune response to bacterial challenge, with pol-lnc78 shown to serve as a negative regulator. Recently, lncRNA-mediated ceRNA crosstalk has become a hotspot of research. In mammals, ceRNAs have been strongly implicated in the pathogenesis of cancers, providing crucial diagnostic biomarkers and therapeutic targets (Abdollahzadeh et al., 2019; Chen et al., 2017; Wang et al., 2017a). In lower vertebrates, limited research has been conducted on lncRNA-mediated ceRNA mechanisms, except for a few studies associated with miiuy croaker immunity. Specifically, in miiuy croaker, lncRNA IRL promotes the immune response against bacterial infection by counteracting the suppressive effects of miR-27c-3p on IRAK4 (Zheng et al., 2021a), while lncRNA AANCR increases MITA expression through competitively binding to miR-210 and regulates host antiviral responses (Chu et al., 2021b). Given this context, determining whether ceRNA crosstalk occurs widely among teleost fish is important. Here, we revealed that flounder pol-lnc78 acts as a ceRNA to increase SARM expression through sponging pol-miR-n199-3p, with this ceRNA network regulating host defense responses to bacterial infection. These results highlight the essential effects of lncRNAs in host immunity and lend further support to the ubiquity of ceRNA regulation in fish.

CONCLUSIONS

Our study identified SARM, pol-miR-n199-3p, and pol-lnc78 as key regulators in the response of flounder to *V. anguillarum* infection through modulation of inflammatory processes. Pol-miR-n199-3p repressed SARM expression by targeting its 3' UTR. Concurrently, pol-lnc78 acted as a ceRNA, counteracting the inhibitory effects of pol-miR-n199-3p on SARM. These observations provide new insights into the mechanisms of SARM, as well as the roles of ceRNA in lower vertebrates.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Conceptualization, X.H.N. and S.W.Y.; Funding acquisition, X.H.N. and S.W.Y.; Methodology, X.H.N.; Investigation, B.H., Y.P., and X.H.N.; Resources, X.H.N.; Original draft, X.H.N.; Review & editing, X.H.N. and S.W.Y. All authors read and approved the final version of the manuscript.

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