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microRNA-34 mediates a negative feedback loop in the JAK-STAT pathway to attenuate immune overactivation in an invertebrate

Hongliang Zuo[1,2], Alian Liao[1], Sihui Shen[1], Youxi Wang[1], Xiya Yang[1], Shaoping Weng[1,2], Jianguo He[1,2*] and Xiaopeng Xu[1,2*]

Abstract

The JAK-STAT pathway is an essential signaling mechanism that initiates immune responses against pathogen infections. The intrinsic homeostatic regulation of JAK-STAT signaling is critical for maintaining immune homeostasis. Previous studies have shown that aberrant activation of the invertebrate JAK-STAT pathway leads to inflammation-like symptoms. Understanding the homeostatic mechanisms of this pathway in invertebrates is of significant interest. Pacific white shrimp (*Penaeus vannamei*) is one of the most extensively studied invertebrates in immune system research. In this study, we demonstrate that the shrimp microRNA-34 (miR-34) attenuates JAK-STAT pathway activation by targeting JAK, thereby inhibiting STAT phosphorylation and nuclear translocation. Interestingly, miR-34 expression is directly regulated by STAT, forming a negative feedback regulatory loop in the JAK-STAT pathway. Disrupting this loop results in excessive JAK-STAT pathway activation and immune overactivation, exacerbating inflammation caused by *Vibrio parahaemolyticus* infection in shrimp. This study provides new insights into the regulatory mechanism of the JAK-STAT pathway and its roles in maintaining immune homeostasis in invertebrates.

Introduction

The JAK-STAT signaling pathway, composed of two core intracellular cytoplasmic components, Janus kinase (JAK) and signal transducer and activator of transcription (STAT), is a highly conserved cellular signal transduction mechanism involved in various physiological processes [1]. This pathway efficiently transmits signals, such as those from extracellular cytokines or growth factors recognized by cell surface receptors, into the nucleus, where STAT regulates a set of target genes that are involved in regulation of cell proliferation, differentiation, migration, apoptosis, and survival [2, 3]. Activation of JAK-STAT signaling, characterized by tyrosine phosphorylation, dimerization, and nuclear translocation of the transcription factor STAT by phosphorylated JAK, is essential for immune responses against viral, bacterial,

*Correspondence:
Jianguo He
lsshjg@mail.sysu.edu.cn
Xiaopeng Xu
xuxpeng@mail.sysu.edu.cn
[1]State Key Laboratory of Biocontrol, School of Life Sciences/ Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai)/ China-ASEAN Belt and Road Joint Laboratory on Mariculture Technology, Sun Yat-sen University, Guangzhou, P. R. China
[2]Institute of Aquatic Economic Animals and Guangdong Province Key Laboratory for Aquatic Economic Animals, Sun Yat-sen University, Guangzhou, P. R. China

and fungal infections. However, excessive activation of JAK-STAT signaling can lead to immune overactivation, resulting in inflammation and tissue damage [4–7]. Negative regulators of the JAK-STAT pathway, including protein inhibitor of activated STATs (PIAS) [8], suppressor of cytokine signaling (SOCS/CIS) [9], and protein tyrosine phosphatases (PTPs) [10], suppress JAK-STAT signaling activation and play an important role in maintaining immune homeostasis [11, 12].

JAK-STAT signaling is essential for initiating immune defense against pathogen infections in invertebrates [13–15]. However, the mechanisms that maintain balance in the invertebrate JAK-STAT pathway remain unclear, limiting the exploration of immune homeostasis regulation from an evolutionary perspective. Pacific white shrimp (*Penaeus vannamei*), the most widely farmed shrimp species globally, is one of the most extensively studied invertebrates in immunity research. The JAK-STAT pathway in *P. vannamei* consists of the cellular receptor Domeless, a JAK, a STAT, three inhibitors SOCS2, PIAS, and dual specificity phosphatase 14 (DUSP14), and an activator PTPN6 [16–21]. Accumulating evidence suggests that JAK-STAT signaling regulates immune responses in shrimp by modulating antimicrobial peptides (AMPs) expression and hemocyte phagocytosis [14, 15, 22]. However, a recent study has revealed that upon acute infection with *Vibrio parahaemolyticus*, a common bacterial pathogen in aquatic animals, shrimp can also exhibit inflammation-like symptoms that lead to mortality [23]. Overactivation of the JAK-STAT pathway, rather than the NF-κB (Dorsal and Relish) pathways, drives excessive immune responses and is a major cause of this pathological phenomenon. Therefore, elucidating the mechanisms that maintain JAK-STAT pathway homeostasis is crucial for the preventing and managing shrimp diseases.

MicroRNAs (miRNAss), typically about 20–24 nucleotides (nt) in length, are a class of conserved endogenous non-coding single-stranded RNA molecules that play crucial roles in post-transcriptional gene regulation [24, 25]. miRNAs originate from long primary transcripts (pri-miRNAs), which undergo nuclear processing to generate hairpin-shaped precursor miRNAs (pre-miRNAs) [26]. These pre-miRNAs are then exported to the cytoplasm and further processed by the Dicer enzyme to produce mature miRNA duplexes [27]. One strand of the miRNA duplex is incorporated into the RNA-induced silencing complex (RISC), where it binds to target mRNAs through imperfect complementary base pairing, leading to mRNA degradation or translational repression [28].

In recent years, numerous miRNAs have been identified in shrimp, where they play critical roles in regulating immune signaling pathways [29–31]. Previous studies have showed that miR-34 expression is upregulated after white spot syndrome virus (WSSV) infection in shrimp [32]. miR-34 regulates shrimp defense against WSSV infection and inhibits human breast cancer growth and metastasis in a cross-phylum manner. However, the mechanism underlying the role of miR-34 in shrimp immunity remains unclear. In the present study, miR-34 was found to target JAK and inhibit the JAK-STAT pathway in *P. vannamei*. The expression of miR-34 was regulated by STAT, forming a negative feedback loop. Notably, this regulatory loop serves as an intrinsic mechanism to prevent excessive immune activation during acute bacterial infection. These findings provide a foundation for further exploration of invertebrate immune function and shrimp disease resistance mechanisms.

Materials and methods

Animal and pathogens

Healthy *P. vannamei* (~ 10 g) from a shrimp farm in Zhuhai, China, were acclimated at ~ 27 ℃ in recirculating air-pumped seawater with a salinity of 5.0‰. *V. parahaemolyticus* was cultured to logarithmic phase (OD_{600} = 0.4 to 0.6) and prepared as stock solution at 3×10^9 colony-forming units (CFU)/mL in PBS. The WSSV stock was freshly prepared from the muscle of moribund WSSV-infected shrimp which stored at −80 °C, as previously described [33].

Immune challenge

Shrimps were divided into five groups ($n = 50$) and injected with 50 μL of PBS containing 1×10^6 copies of WSSV, 1×10^5 CFU of *V. parahaemolyticus*, 5 μg of lipopolysaccharide (LPS), 5 μg of Poly(I: C), or PBS alone as control. Hemocytes and gills were sampled from nine randomly selected shrimp in each treatment group at 0, 4, 12, 24, 48, 72, and 96 h post-injection for miRNA transcription analysis. For mortality analysis, dsRNAs specific to *JAK*, *STAT*, *SOCS2*, and *GFP* (as control) were prepared using the T7 RiboMax express RNAi system (Promega, USA) and each injected into shrimp at 10 μg/50 μL PBS. At 48 h post injection, shrimp were infected with 1.5×10^6 or 5×10^5 CFU *V. parahaemolyticus* to establish excessive and normal infections (EVC and NVC), respectively, as previously described [23]. For miRNA functional analysis, 5'-end cholesterol-modified miRNA mimic (agomiR-34) and inhibitor (antagomiR-34), alone with their scrambled controls (agomiR-NC and antagomiR-NC), were synthesized by GenePharma (China). At 48 h post-injection of 10 μg of miR-34 mimic, inhibitor, or their controls in 50 μL PBS, shrimp ($n = 40$) were challenged with 5×10^5 or 1.5×10^6 CFU *V. parahaemolyticus*. Shrimp mortality in each group was recorded. In a parallel experiment, gills were randomly sampled from six live shrimp per group at 8 and 16 h post-bacterial challenge to quantify *V. parahaemolyticus* content.

Total DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany). The *V. parahaemolyticus* load in gills was determined by relative qPCR using primers specific to the $pirA^{vp}$ toxin gene (*Genbank accession NO.* KP324996), with the *P. vannamei EF1-α* gene (GU136229) as an internal control.

Sequence analysis

The miR-34 sequence was retrieved from an smRNA-Seq library and verified using stem-loop real-time RT-PCR and northern-blot with specific primers and probes (Table S1), as previously reported [31]. The pre-miR-34 was analyzed using an online RNA folding prediction tool (http://www.unafold.org/mfold/applications/rna-folding-form-v2.php) and cloned into the pAc5.1/V5-His A vector (Invitrogen, USA) for transfection into *Drosophila* S2 cells. Stem-loop real-time RT-PCR and northern blot analyses were performed to validate miR-34 expression. The miR-34 promoter sequence was retrieved from the reported *P. vannamei* complete genome [34] and analyzed using Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and Jaspar (http://jaspar.genereg.net/).

qPCR

For miRNA and mRNA expression analysis, total RNA was extracted from the gills and hemocytes of 9 shrimp using the RNeasy Plus Mini Kit (QIAGEN, Germany) and reverse-transcribed into cDNA using the PrimeScript Reverse Transcription Kit (Takara, Japan) with random hexamer primers. For miRNA expression analysis, total RNA was reverse-transcribed into cDNA with a stem-loop primer specific for miR-34 (miR-34-RT). Real-time PCR was performed in a 10 μL reaction mixture containing 1 μL cDNA, 5 μL 2 × SYBR Premix Ex Taq™ II (Takara, Japan), and 500 nM of each primer (Table S1) on a LightCycle 480 System (Roche, Switzerland). The optimized thermal cycling conditions were as follows of 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Melting curves were generated by increasing the temperature from 65 °C to 95 °C at a rate of 0.5 °C/s to denature the DNA fragments. The expression levels of miRNA and mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the dual internal controls *U6* and *EF-1α*.

Northern-blot

Northern-blot for microRNA was performed as previously described [29]. Briefly, total RNA (10 μg) extracted from the gills of 9 shrimp was electrophoretically separated on a 12% polyacrylamide gel and transferred onto a positively charged nylon membrane (Roche, Swiss) for UV cross-linking and pre-hybridization. After hybridization with miR-34 and U6 locked nucleic acid (LNA) probes (Exiqon, Denmark, Table S1) at a final concentration of 0.1 nM at 65 ℃, the membrane was washed, blocked, incubated with an anti-Digoxigenin-AP antibody (Roche, USA), and further detected using CDP-Star chemiluminescent substrate (Roche, Swiss) for analysis with Amersham Imager 600 (GE, USA).

Plasmid construction

The wild-type promoter of miR-34 and its STAT-binding site mutant, generated by overlapping PCR, were cloned into the pGL3-Basic vector (Promega, USA) to construct pGL3-miR-34-wt and pGL3-miR-34-mut (as control) using the pro-miR-34-KpnIF/mutR and pro-miR-34-mutF/XhoIR primers, respectively. The promoters of immune effector genes were also cloned into pGL3-Basic. Expression vectors for STAT (pAc5.1-STAT), JAK (pAc5.1-JAK), and SOCS2 (pAc5.1-SOCS2) were constructed using primers listed in Table S1. For miRNA target identification, the JAK 3'UTR and its miR-34 target site mutant were cloned into pGL3-249 vector to generate pGL3-JAK-wt and pGL3-JAK-mut (as control).

Dual-luciferase reporter assay

To analyze miR-34 promoter activity, 100 ng of the protein expression vector was co-transfected with 50 ng of pGL3-miR-34-wt or pGL3-miR-34-mut into S2 cells in a 96-well plate using FuGENE HD transfection reagent (Promega, USA). To assess the regulatory effect of miR-34 on the JAK 3'UTR, 100 nM miR-34 or NC mimics (GenePharma, China) was co-transfected with 50 ng of pGL3-JAK-wt or pGL3-JAK-mut into S2 cells. The pRL-TK (50 ng) (Promega, USA) plasmid was co-transfected as an internal control. Each treatment was performed in six to eight replicates. At 48 h post-transfection, luciferase activity was measured using a dual-luciferase reporter assay system (Promega, USA).

EMSA

EMSA was conducted using a LightShift Chemiluminescent EMSA kit (Thermo, USA), as previously described [29], with a 5' biotin-labeled probe of miR-34 promoter (bio-pro-miR-34) containing the STAT binding site (CTTTCCGGAAATGCA) (Invitrogen, USA) and the nuclear proteins extracted from S2 cells transfected with pAc5.1-STAT or the control pAc5.1-GFP plasmid. The STAT site-deleted probe (bio-pro-miR-34-mut) and unlabeled probes (unbio-pro-miR-34/unbio-pro-miR-34-mut) were used as control, and a rabbit antibody against shrimp STAT (GL Biochem, China) was used for super-shift assays.

Western-blot

For protein level analysis, each shrimp was injected with 50 μL PBS containing 10 μg of miR-34 mimic

(agomiR-34)/inhibitor (antagomiR-34) or their controls. For each group, hemocytes and gills were sampled and pooled from 30 shrimp at 48 h post-injection. Total nuclear and cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo, USA). A 30 μg total protein per lane was detected using western blot analysis with a rabbit anti-shrimp STAT antibody (1:1,000, GL Biochem, China). Antibodies against Histone H3 (1:3000, CST, USA) and β-actin (1:3000, MBL, Japan) were used to detect the nuclear and cytoplasmic internal control proteins, respectively. The signals were detected using anti-rabbit IgG (H + L)-HRP Conjugate (1:10,000, Promega, USA), developed with SuperSignal West Femto (Thermo, USA), and captured using the Amersham Imager 600 (GE, USA). For dimmer protein analysis, hemocytes were treated with immunoprecipitation lysis buffer (Thermo, USA), separated by native-PAGE, and translocated onto a NC membrane for Western blot analysis. The gray values of the specific protein bands were calculated using Quantity one 4.6.2 software (Bio-Rad, USA) via the Gaussian model and normalized to the internal control protein levels.

Immunofluorescence

Hemolymph smears on siliconized slides were fixed with 4% paraformaldehyde for 10 min and permeabilized with 1% Triton X-100 for 20 min. Samples were then sequentially incubated with a rabbit anti-shrimp STAT antibody (1:1000, GL Biochem, China), a mouse anti-β-actin (1:3000, MBL, Japan), an Alexa fluor 488-conjugated goat anti-rabbit antibody (1:5000, Abcam, USA), and an Alexa fluor 594-conjugated goat anti-mouse antibody (1:5000, CST, USA). Nuclei were stained with Hoechst 33342 (Invitrogen, USA), and slides were visualized using a Leica LSM 410 confocal microscope (Germany).

Statistical analysis

Experiments were performed in triplicate using different batches of shrimp. Statistical analyses were conducted using two-tailed unpaired Student's *t*-test or one-way ANOVA followed by Dunnett's post hoc test. Mortality data were analyzed using Kaplan-Meier log-rank χ^2 tests.

Results

Expression profiles of shrimp miR-34

The sequence of *P. vannamei* miR-34 is identical to that of *Drosophila melanogaster* miR-34c-5p (Dme-miR-34-5p) and *Daphnia pulex* miR-34 (Dpu-miR-34) but differs from *Capitella teleta* miR-34 (Cte-miR-34), human/mouse miR-34c-5p (Mmu/Hsa-miR-34c-5p), and zebrafish miR-34b (Dre-miR-34b) by several nucleotides (Fig. 1A). The miR-34 precursor harbors the mature miR-34 in the 5'-stem (Fig. 1B). Stem-loop qPCR and northern-blot analyses demonstrated that miR-34 was widely expressed across all examined tissues, with the highest levels in muscle and nerve, and the lowest level in hepatopancreas (Fig. 1C, D). The mature miR-34 was detected in *Drosophila* S2 cells following transfection with a pre-miR-34 expression vector (Fig. 1E, F and G), confirming the sequence of pre-miR-34. The expression of miR-34 in hemocyte and gill was significantly up-regulated upon stimulation with *V. parahaemolyticus*, WSSV, LPS and poly (I: C) (Fig. 1H, I), suggesting its involvement in shrimp immune responses.

Regulation of miR-34 expression by STAT

The miR-34 promoter contains a putative STAT-binding site (Fig. 2A, Fig. S1). The dual-luciferase reporter assays performed in *Drosophila* S2 cells demonstrated that the miR-34 promoter could be activated by STAT but not by JAK or the control GFP (Fig. 2B). As JAK is the upstream kinase of STAT, this activation was further enhanced by co-expression with JAK in the dual-luciferase reporter assays (Fig. 2C). However, the STAT alone or co-expressed with JAK had little to no effect on the miR-34 promoter when the STAT-binding site was mutated, indicating that this site mediated the regulatory effect of STAT on miR-34 expression.

The interaction between STAT and the miR-34 promoter was investigated using EMSA. A retarded shift band was observed when STAT bound to the biotin-labeled miR-34 promoter probe, which was further shifted upon addition of the anti-STAT antibody and abolished by competition with a 200 × excess of unlabeled probe (Fig. 2D). In contrast, no retarded bands were detected when the labeled STAT-binding site-mutated miR-34 promoter probe was used (Fig. 2E). In vivo experiments further revealed that STAT silencing (Fig. 2F) significantly downregulated miR-34 expression, whereas silencing of the JAK-STAT pathway inhibitor SOCS2 (Fig. 2G) had the opposite effect (Fig. 2H and I).

Regulation of JAK-STAT pathway by miR-34

The miR-34 was incompletely complementary to the 3'UTR of *JAK* (Fig. 3A). Dual-luciferase reporter assays demonstrated that miR-34 reduced luciferase expression when the reporter contained the wild-type *JAK* 3'UTR but not when the miR-34 seed-binding site was mutated (Fig. 3B). Furthermore, miR-34 exhibited a dose-dependent regulatory effect on *JAK* 3'UTR (Fig. 3C). In vivo analyses revealed that the miR-34 mimic (agomiR-34) significantly decreased JAK protein levels in hemocytes and gills, whereas the miR-34 inhibitor (antagomiR-34) increased them (Fig. 3D and E). Consistently, STAT protein dimerization and nuclear translocation in hemocytes were enhanced following antagomiR-34 treatment but reduced after agomiR-34 treatment compared with

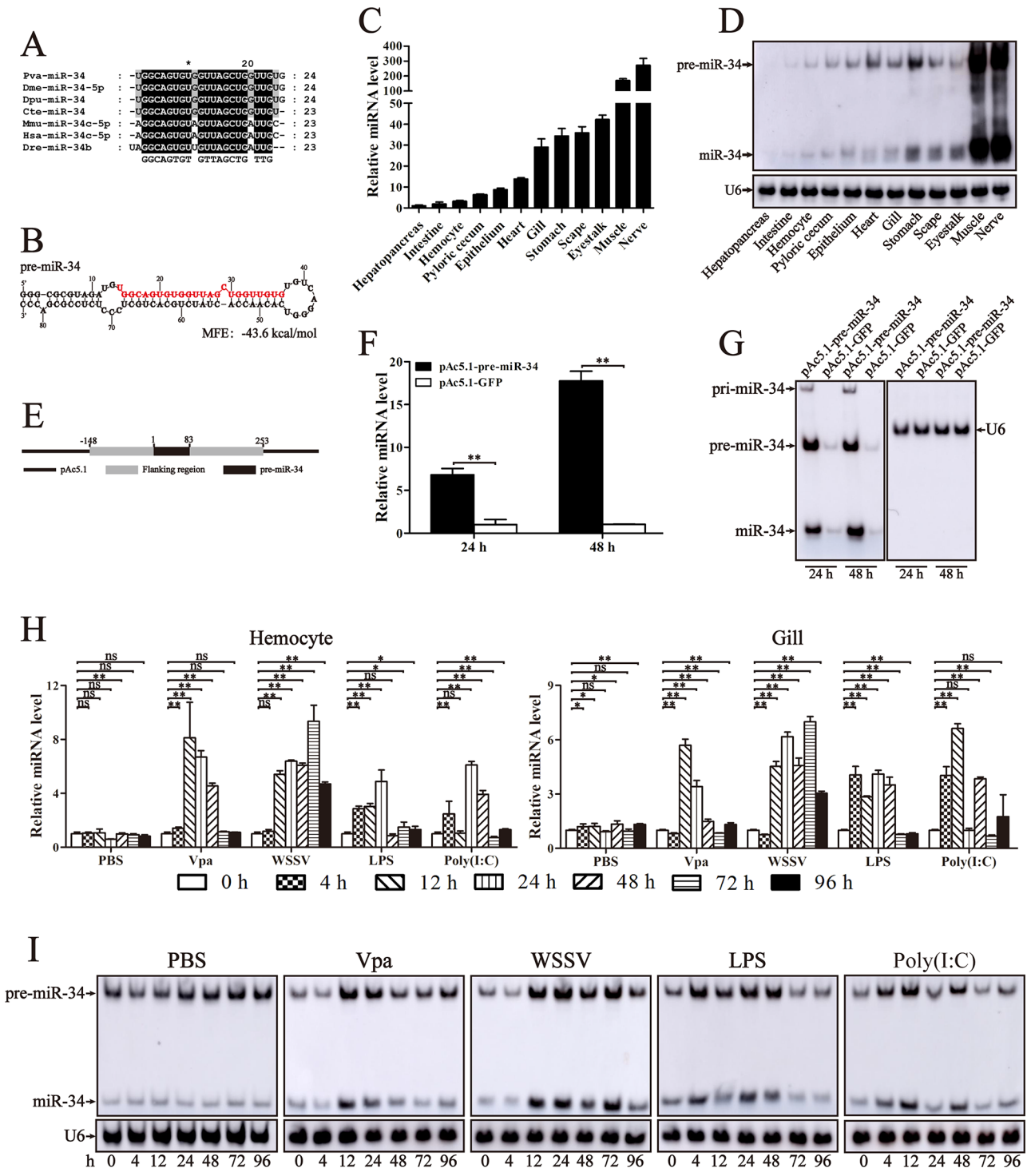

Fig. 1 Expression of miR-34 in *P. vannamei*. (**A**) Sequence comparison of mature miR-34 from *P. vannamei* (Pva-miR-34, Genbank accession NO. MT270231), *Drosophila melanogaster* (Dme-miR-34-5p, miRBase accession NO. MI0000371), *Daphnia pulex* (Dpu, MI0012244), *Capitella teleta* (Cte-miR-34, MI0010060), *Mus musculus* (Mus-miR-34c-5p, MI0000403), *Homo sapiens* (Has-miR-34c-5p, MI0000743) and *Danio rerio* (Dre-miR-34b, MI0003690). (**B**) The secondary structure of the miR-34 precursor containing the mature miR-34 (red). (**C**, **D**) Stem-loop qPCR and northern-blot analyses of miR-34 tissue distribution. Each tissue was from 9 shrimp. The U6 RNA was used as internal control. (**E**) The schematic diagram of the cloning region of pre-miR-34 expression vector. (**F**, **G**) The miR-34 expression in S2 cells at 24 and 48 h post pre-miR-34/GFP (as control) transfection analyzed by stem-loop qPCR and northern-blot. The blots are representative of three independent experiments. (**H**) The miR-34 expression in hemocytes and gill of WSSV-, *V. parahaemolyticus* (Vpa)-, LPS-, poly (I: C)-, and PBS (mock)-stimulated shrimp was analyzed by stem-loop qPCR. The values at 0 h were set as 1.0. (**I**) The miR-34 expression in gills from immune stimulated shrimp analyzed by northern-blot. Blots are representative of three experiments. For qPCR, Data are representative of three experiments and presented as means ± SD of four detections. *ns*, $p > 0.05$; *, $p < 0.05$; and **, $p < 0.01$ by Student's *t*-test

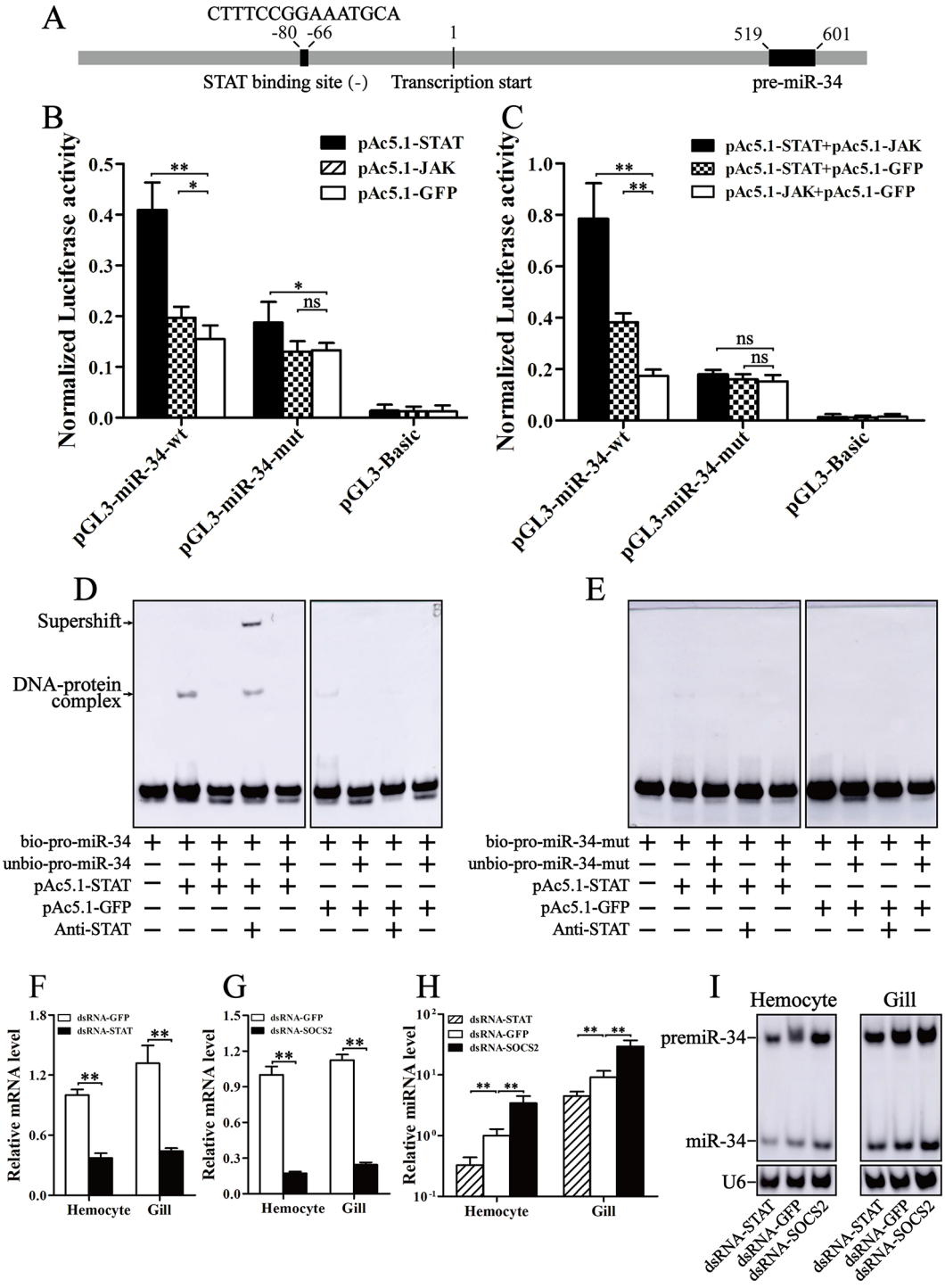

Fig. 2 Regulation of miR-34 by the JAK-STAT pathway. (**A**) Scheme of the miR-34 promoter (pro-miR-34) structure. The numbers indicate the sequence positions coordinated to the transcription initiation site (position 1). (**B**, **C**) Dual-luciferase report assays of regulatory effects of JAK, STAT and GFP (as control) or their combinations on pro-miR-34 (pGL3-miR-34-wt) and its STAT-binding site-deleted mutant (pGL3-miR-34-mut). Data are means ± SD ($n = 6$). (**D**) EMSA analysis of the interaction of STAT with miR-34 promoter. The biotin-labeled (bio-) or unlabeled (unbio-) miR-34 promoter probes and nuclear proteins extracted from S2 cells expressing STAT or GFP (as control) were used. The anti-STAT antibody was added to perform the super-shift assay. (**E**) The probes for the STAT-binding site-mutated promoter (-mut) were used as control. Results are representative of three independent experiments. (**F**, **G**) qPCR evaluation of the RNAi efficiencies of STAT and SOCS2 in vivo. The expression level of (**H**) Stem-loop qPCR analysis of miR-34 expression in STAT- and SOCS2-silenced shrimp. The values in the dsRNA-GFP-treated control group were set as 1.0. (**I**) Northern-blot analysis of the miR-34 expression in STAT- and SOCS2-silenced shrimp. *ns*, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$

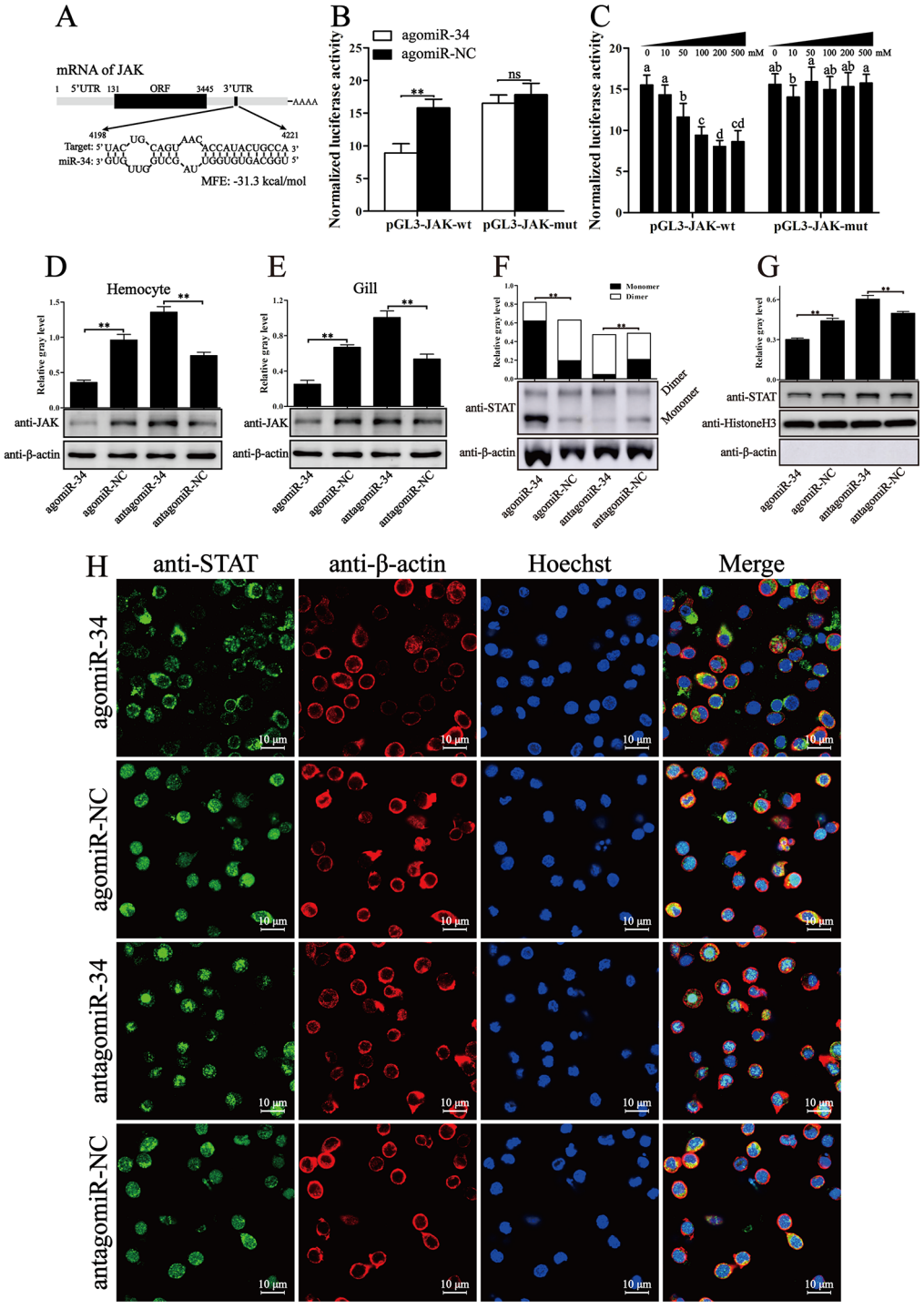

Fig. 3 Target identification of miR-34. (**A**) Scheme of the complementarity between miR-34 and the 3′-UTR of *JAK*. (**B**) Dual-luciferase reporter assays of the effects of miR-34 mimic (agomiR-34)/control (agomiR-NC) on expression of the luciferase gene with the wild-type (pGL3-JAK-wt) or miR-34 seed complementary site-mutated (pGL3-JAK-mut) JAK 3′-UTR. Data are means ± SD ($n = 8$). (**C**) The effects of gradient doses of miR-34 mimic on wild-type and mutated JAK 3′-UTR. Values with different letters indicate significantly difference by one-way ANOVA followed by Dunnett's post hoc test ($p < 0.05$). (**D**, **E**) Western-blot analysis of the effects of miR-34 mimic/control and miR-34 inhibitor (antagomiR-34)/control (antagomiR-NC) on JAK expression in hemocytes and gill. (**F**, **G**) Western-blot analyses of the effects of miR-34 mimic/inhibitor on dimerization and nuclear translocation of STAT in hemocytes. Blots are representative of three independent experiments. Gray values were analyzed by Gaussian model using three different rolling disk sizes (5, 10, and 15) and normalized to those of internal controls of β-actin or Histone H3. (**H**) Immunofluorescence assays of the effect of miR-34 on nuclear translocation of STAT in hemocytes. *ns*, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$

the controls (Fig. 3F and G). Immunofluorescence assays further confirmed that antagomiR-34 promoted STAT nuclear translocation, whereas agomiR-34 inhibited it (Fig. 3H). These findings suggest that miR-34 functions as an inhibitor of the JAK-STAT pathway in shrimp.

Dual-luciferase reporter assays showed that STAT regulated the promoters of immune effector genes, including anti-lipopolysaccharide factor (*ALF*) 2, 3 and 5, C-type lectin (*CTL*) 2 and 5, lysozyme (*Lys*) 1, invertebrate Lys (*LysIT*) 1 and 2, and penaeidin (*PEN*) 2 (Fig. 4A). qPCR assays further demonstrated that the expression of most of these genes was upregulated following miR-34 inhibitor treatment but downregulated after miR-34 mimic treatment in hemocytes and gills (Fig. 4B), confirming that miR-34 suppresses the JAK-STAT pathway in shrimp.

The miR-34 feedback loop and its role in Vibrio infection

The targeting effect of miR-34 on *JAK* and the regulation of miR-34 by STAT suggest a regulatory link from STAT to JAK. In vivo experiments showed that the *JAK* protein levels in hemocytes and gills were significantly increased after STAT silencing but decreased after SOCS2 silencing (Fig. 5A, B). However, the regulatory effects of STAT and SOCS2 on *JAK* silencing in hemocytes were attenuated following miR-34 mimic and inhibitor treatments, respectively (Fig. 5C, D). These findings indicated that STAT modulates JAK expression by regulating the production of miR-34, forming a feedback loop within the JAK-STAT pathway.

In vivo experiments showed that treatment with the miR-34 mimic increased the mortality of shrimp infected with a low-dose (5.0×10^5 CFU) of *V. parahaemolyticus*, although the difference compared with the control was not statistically significant (Fig. 5E). Consistently, the bacterial load of *V. parahaemolyticus* in shrimp tissues was significantly increased following miR-34 mimic treatment (Fig. 5F). Interestingly, in the miR-34 inhibitor treatment group, infected shrimp died significantly earlier than those in the control group, and the moribund shrimp exhibited symptoms highly similar to those of acutely infected shrimp (Fig. 5E). The *V. parahaemolyticus*-injected area appeared opaque and whitish (Fig. 5E, Inset), resembling the symptoms observed in shrimp challenged with a high-dose of *V. parahaemolyticus* [23]. The bacterial load in tissues from miR-34 inhibitor-treated shrimp was also increased (Fig. 5F). These findings indicated that excessive activation of the JAK-STAT pathway due to miR-34 inhibition could transform a normal *V. parahaemolyticus* infection into an acute infection in shrimp. In the high-dose (1.5×10^6 CFU) infected group, miR-34 inhibitor treatment significantly increased the mortality and further accelerated shrimp death (Fig. 5G). In contrast, treatment with miR-34 mimic delayed shrimp mortality compared with the control. However, bacterial loads in tissue did not change significantly after miR-34 mimic or inhibitor treatment (Fig. 5H).

Discussion

Immune regulation generally maintains a balance between activating and inhibitory signaling pathways, which is crucial for defending against pathogen invasion and maintaining immune homeostasis [35, 36]. Uncontrolled activation of immune signaling pathways leads to sustained inflammation, causing damage to the organism [37]. As a key node in immune response initiation, the JAK-STAT pathway is tightly regulated by multiple mechanisms to ensure proper function and prevent excessive or prolonged activation. In mammals, PIAS family proteins interact with STATs and block their DNA-binding activity [8, 38]. SOCS1 and SOCS3 directly bind to JAKs and suppress their activity through the kinase inhibitory region (KIR) [39]. A recently study shows that DUSP14, a member of the PTP superfamily, can bind and dephosphorylate both JAK and STAT in shrimp [21]. These inhibitory mechanisms primarily regulate JAK-STAT signaling at the protein level by modulating the activity or degradation of pathway components. In contrast, miRNAs regulate pathway components at the post-transcriptional level. In this study, miR-34 was identified as a modulator JAK expression, thereby inhibiting STAT activation in shrimp. This represents a newly discovered inhibitory mechanism of the JAK-STAT pathway.

Several miRNAs are involved in modulation of JAK-STAT signaling in mammals, such as miR-17 targeting JAK1 and STAT3 [40], miR-495 and miR-181b-5p targeting STAT3 [41, 42], miR-221 targeting STAT5 [43], and miR-196a/b targeting SOCS2 [44]. Studies have primarily focused on their roles in tumorigenesis, cancer progression, and inflammation. In invertebrates, several miRNAs are also known to target the components of the JAK-STAT pathway. For example, miR-9041 and miR-9850 target STAT to attenuate the antiviral immune response in *Macrobrachium rosenbergii* [45], while miR-279 suppresses the expression of the JAK/STAT extracellular ligand Upd to regulate circadian rhythm in *Drosophila* [46]. In this study, miR-34 was found to bind the 3'-UTR of *JAK* and inhibit its expression in shrimp. Similar to mammals and *Drosophila*, shrimp *JAK* is a tyrosine kinase that initiates intracellular signal transduction in the JAK-STAT pathway by phosphorylating STAT [17]. Inhibition of miR-34 mimicked JAK gain-of-function, enhancing STAT phosphorylation and nuclear translocation, opposite to the effects observed with miR-34 mimic treatment. These findings suggest that miR-34 functions as an inhibitor of the JAK-STAT pathway in shrimp. Interestingly, the miR-34 promoter contains

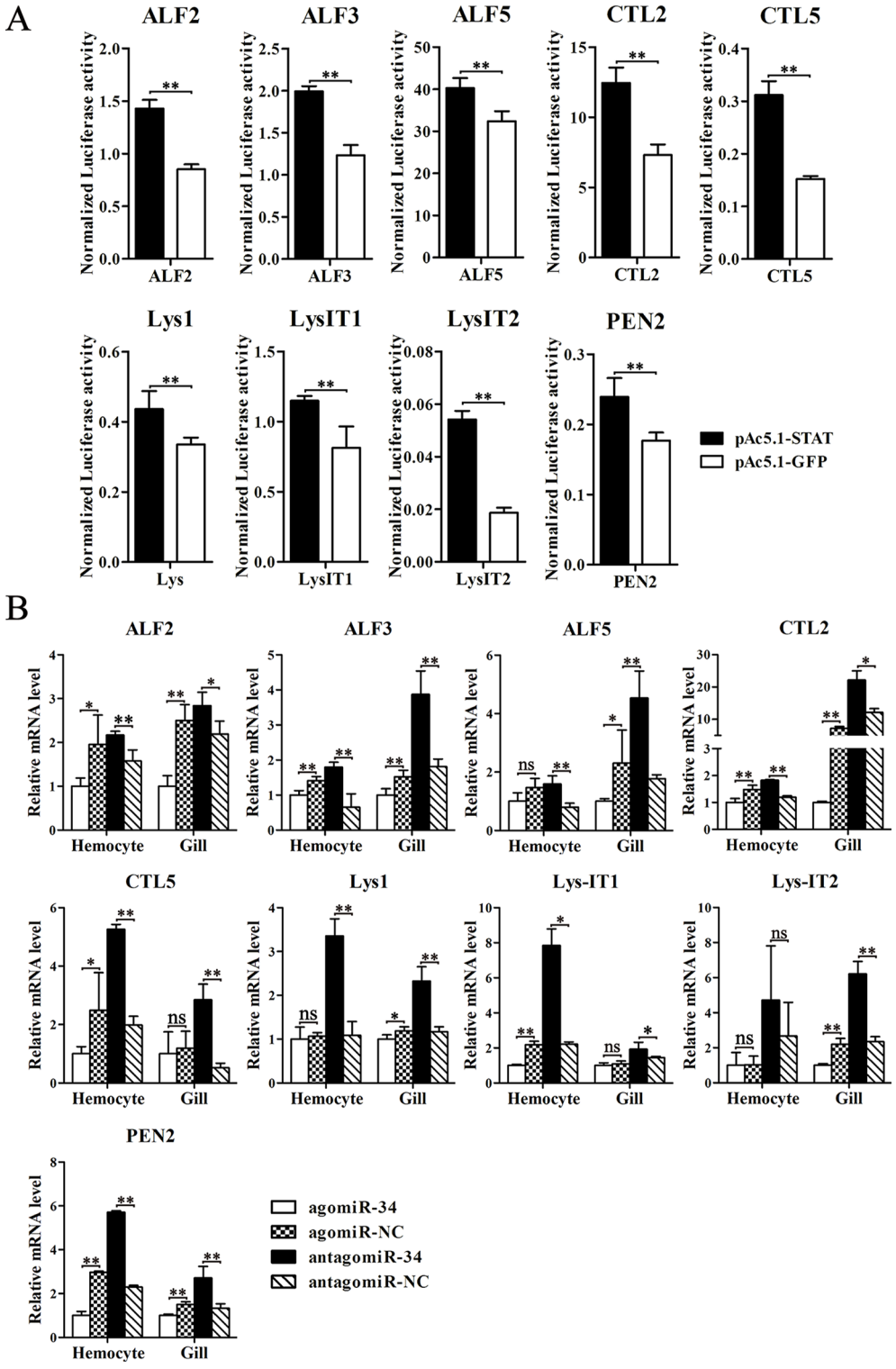

Fig. 4 Effect of miR-34 on JAK-STAT downstream genes. (**A**) Dual-luciferase reporter assays of the regulation of gene promoters by STAT. Data are means ± SD ($n = 8$). (**B**) Expression of genes in hemocytes and gill at 48 h post miR-34 mimic (agomiR-34)/inhibitor (antagomiR-34) treatment analyzed by qPCR. Results are representative of three independent experiments with data presented as means ± SD of four detections. *ns*, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$

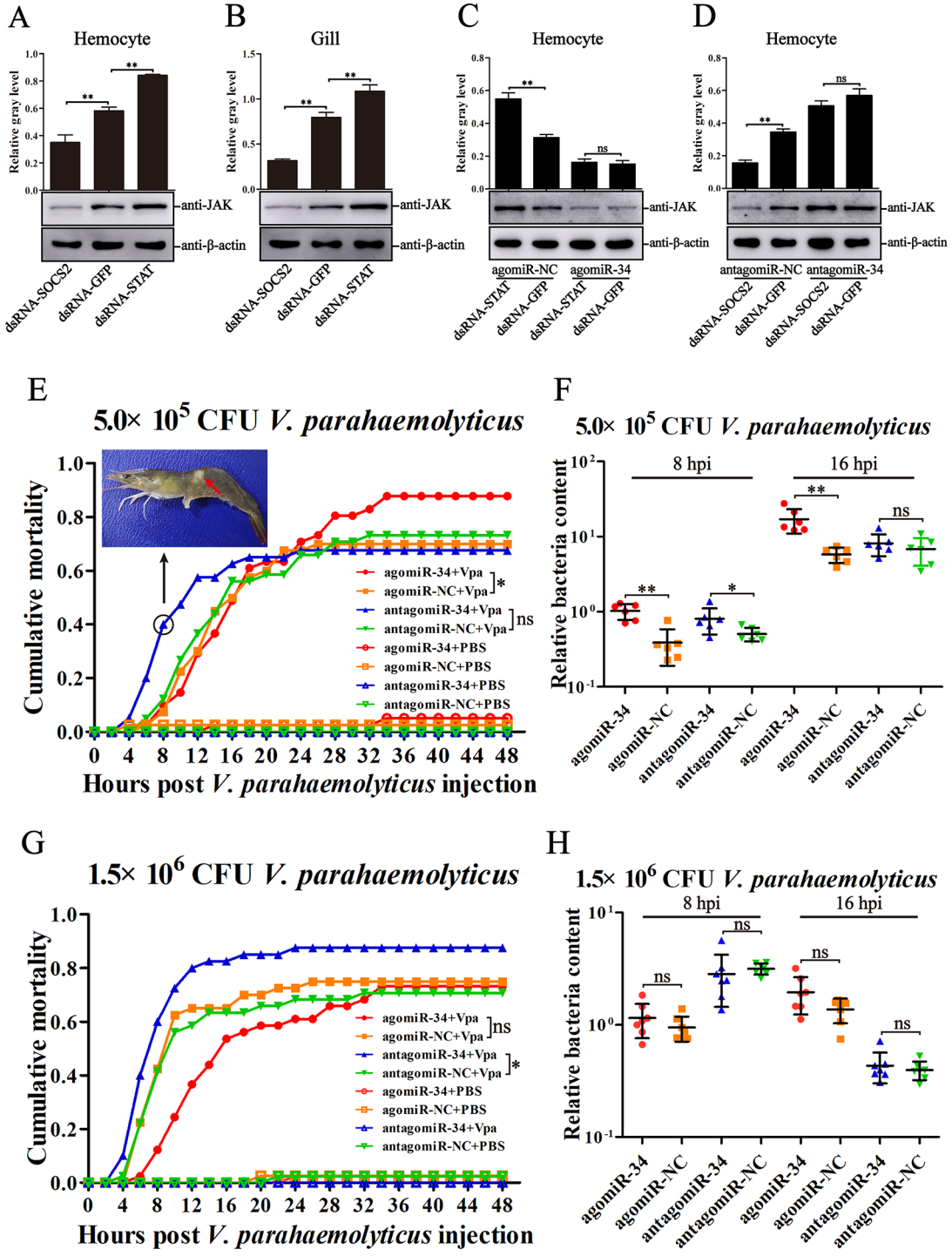

Fig. 5 Roles of miR-34 in regulation of immune response. (**A**, **B**) Western-blot analysis of the effects of STAT- and SOCS2-silencing on expression of JAK in hemocytes and gill. (**C**, **D**) Western-blot analysis of the combined effects of miR-34 mimic (agomiR-34) (**C**)/inhibitor (antagomiR-34) (**D**) and dsRNA-STAT/-SOCS2/-GFP on JAK. (**E**, **G**) The mortality of miR-34 mimic-/inhibitor-treated shrimp after infection with low-dose (5.0×10^5 CFU) and high-dose (1.5×10^6 CFU) *V. parahaemolyticus*. The inset showed the typical pathological sign of miR-34 inhibitor-treated shrimp with red arrow indicating the infected area. (**F**, **H**) The bacterial load in gill was analyzed by qPCR. *ns*, $p>0.05$; *, $p<0.05$; **, $p<0.01$

a STAT-binding motif and is directly regulated by the JAK-STAT pathway. Therefore, miR-34 mediates a negative feedback loop in the JAK-STAT pathway in shrimp, wherein activated STAT promotes miR-34 expression, which in turn suppresses STAT activation by inhibiting JAK activity upstream.

Previous studies in *Drosophila melanogaster* and *Daphnia pulex* have demonstrated that miR-34 was involved in regulating ageing, neurodegeneration, and stress responses [47–49]. Although miR-34 is known to serve as a node in the intricate interplay between ecdysone signaling and innate immunity in *Drosophila* [50], its specific role in invertebrate immune regulation remains unclear. Our study reveals a novel immune-regulatory function of miR-34 in shrimp, directly linking it to JAK-STAT pathway modulation. This highlights evolutionary divergence in miRNA functions. Moreover, the JAK-STAT and NF-κB signaling pathways are closely linked in regulatory mechanisms. For example, STAT can inhibit the activation of Dorsal by regulating the expression of miR-1, which targets the upstream adaptor MyD88 of the Toll/Dorsal signaling pathway in shrimp [31]. Given that miR-34 acts as an inhibitor of the JAK-STAT pathway, its impact on the NF-κB pathway warrants further investigation.

A recent study highlighted that, compared with normal infection caused by a low dose of *V. parahaemolyticus*, shrimp acutely infected with high-dose of *V. parahaemolyticus* develop inflammatory syndrome [23]. In this case, shrimp die rapidly while the bacterial load in tissues remains unchanged, suggesting that death resulting from acute *Vibrio* infection is primarily attributed to overactivated immune responses rather than bacterial replication. Overactivation of the JAK-STAT pathway, rather than the NF-κB signaling pathway mediated by Dorsal and Relish, is the major cause of immune overactivation during acute infection. Attenuating JAK-STAT signaling by silencing *JAK* or *STAT* using an RNAi strategy reduced both symptoms and mortality in acutely infected shrimp. Conversely, enhancing JAK-STAT signaling by silencing *SOCS2* converted a normal infection caused by a low dose of *V. parahaemolyticus* into an acute one [23]. In this study, treatment with miR-34 mimic in vivo down-regulated the expression of a set of JAK-STAT signaling-targeted genes, whereas treatment with miR-34 inhibitor had the opposite effects. Following low-dose *Vibrio* infection, miR-34 mimic increased shrimp mortality and tissue bacterial load, opposite to the results observed in the miR-34 inhibitor treated group. These findings indicate that the miR-34 feedback loop antagonizes the positive effect of the JAK-STAT pathway on the shrimp immune response against low-dose infection. However, after acute infection with high-dose *Vibrio*, miR-34 mimic treatment postponed shrimp death, contrary to the result of miR-34 inhibitor treatment. Neither the mimic nor the inhibitor treatment affected the bacterial load in tissues, which is also consistent with previous studies showing that the pathogenicity of acute *Vibrio* infection in shrimp is related to the immune status of the host rather than to the replication of *Vibrio* [23]. These results also suggest that the role of the miR-34 feedback loop in acute *Vibrio* infection is opposite to its role in low-dose infection.

Taken together, the miR-34-mediated feedback loop is an intrinsic mechanism that prevents overactivation of the JAK-STAT pathway, thereby maintaining immune homeostasis. However, when the responses to acute infection exceed the regulatory capacity of this mechanism, immune overactivation may occur, leading to pathological changes in shrimp that resemble acute inflammatory responses in mammals. Therefore, the study suggests that the pathogenicity of *Vibrio* bacteria in crustaceans is influenced not only by its inherent virulence, but also by its ability to hyperactivate the host immune system. This factor should be considered in the prevention and treatment of aquatic vibriosis.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Author contributions

HZ and XX conceptualized and designed the research. JH and XX supervised the research. XX and HZ wrote the manuscript. HZ performed the experiments and analyzed the data, with contributions from AL, SS, YW, and XY. SW contributed to preparation and resources of the research. All authors have read and approved the final version.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable. This study was carried out on an invertebrate.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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