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# Molecular characterization of troponin C (TnC) in *Scylla paramamosain* and its role in white spot syndrome virus and *Vibrio alginolyticus* infection

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ABSTRACT

Troponin C (TnC) is one member of the EF-hand superfamily. In many species, this gene had been identified and related functions had been elucidated. The TnC gene was still blank in the *Scylla paramamosain*. We obtained the TnC gene for the first time in the *S. paramamosain*. And we systematically analyzed the possible role of this gene in the innate immunity of *S. paramamosain* while infected with white spot syndrome virus (WSSV) or *Vibrio alginolyticus*. The full-length 1427 bp sequence of TnC contains a 453 bp open reading frame (ORF) for encoding a 151 amino acid protein. Detection of tissue specificity of gene expression showed that the TnC was primarily expressed in muscle tissue. The expression of TnC was successfully inhibited by RNA interference technology, and several immune genes were affected. The activity of phenoloxidase and superoxide dismutase increased, and the total hemocytes counts increased after RNAi of TnC. It was found that after infection with *V. alginolyticus* and WSSV, the expression of TnC in hemocytes decreased. Infected with *V. alginolyticus* and WSSV, the cumulative mortality and apoptotic rate of hemocytes increased after silencing the TnC gene. Our results indicate that TnC takes participate in the innate immunity of *S. paramamosain* and may plays a different role in the antiviral and antibacterial immune response.

## 1. Introduction

The immune system covers innate and adaptive response. However, vertebrates are the only phylum that can mount an adaptive immune response [1]. As is known to all, *S. paramamosain* like other invertebrates rely solely on innate immunity to defend against foreign infection [2–4], which includes the surface barrier function, the filtration function, cellular immunity and humoral immunity. As two of the severest diseases in crabs, white spot syndrome virus (WSSV) and Vibriosis have brought a destructive loss to the crab culture industry worldwide. *S. paramamosain* is as highly susceptible to WSSV as marine shrimp and is a host or carrier of WSSV [5,6]. *Vibrio alginolyticus* is a sort of conditional pathogens in oceans [7]. The disease is prone to outbreak when the environment deteriorates or the immune function decreases [8]. In general, there is no effective method to control either of these two diseases till now.

Troponin C (TnC), known as having ability of binding $Ca^{2+}$, is one member of the EF-hand superfamily. Troponin C interacts with troponin I and troponin T, forming a troponin complex which takes part in regulating muscle contraction [9,10]. In invertebrate TnC amino acid sequences, there are usually four potential EF-hand motif that can bind $Ca^{2+}$ [11]. TnC plays an important and extensive role in regulating muscle contraction which is divided into two subtypes in vertebrate: fast skeleton muscular troponin C and cardiac muscular troponin. However, the subtypes of TnC in invertebrates and insects remain mysteries [11]. Recently, it was found that TnC has a close relationship with tumor [12–14], which suggests TnC may work in non-muscle tissues. The function and mechanism of TnC has been widely studied in humans, mice, pigs and other animals, but the possible function and role of TnC has not been explored in crustaceans.

In current project, the full length sequence of TnC gene was cloned from *S. paramamosain*. On this basis, TnC in crabs was specifically knocked down by RNAi technology. Infected with bacteria and virus, comparing with the wild type group, we studied a comprehensive evaluation of immune pathway gene expression, important immune parameters, mortality, hemocytes apoptosis and phagocytosis. We aimed to reveal the inseparable relationship between TnC gene and innate immunity of *S. paramamosain* upon viral and bacterial infection.

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## 2. Methods and materials

### 2.1. Crabs and tissue preparation

The healthy adult *S. paramamosain* (approximately 100 g) were obtained from a seafood market of Hangzhou. We ordered crabs from the same breeding area in the market to ensure the single population of experimental crabs. Short-term feeding observation and pathogen detection were carried out before the experiment. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). The muscles, hepatopancreas, gills, heart, intestines and hemocytes were collected from health or challenged crabs. The samples were used immediately for RNA extraction, aiming to prevent total RNA degradation. WSSV (AF 332093.3) was purified and used in challenge experiments, as described previously [15]. *V. alginolyticus* was cultured and used to challenge the crabs according to the previous report [16].

### 2.2. Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from hemocytes of the *S. paramamosain* using PureLink™ RNA Mini Kit (Ambion, USA), following the protocol of the manufacturer. The concentration and quality of total RNA were determined by the Nanodrop Trace Spectrophotometer and 1% agarose gel electrophoresis detection, respectively. The RACE technique was utilized to clone the full-length cDNA sequence of the gene, based on the known middle fragment using SMARTer® RACE 5′/3′ Kit, following the protocol of the manufacturer. The synthesized cDNA were kept at −20 °C, used for the 3′/5′-RACE PCR with 3′ gene-specific primer (3GSP1, 3NGSP1,3 GSP2,3NGSP2) or 5′ GSP (5GSP, 5NGSP), designed on the basis of middle sequence (the primers sequences are shown in Table 1). The PCR products were purified using MiniBEST DNA Fragment Purification Kit Ver.3.0 (Takara, Japan), following the manufacturer's instruction. Amplified cDNA fragments were transferred into the pMD19-T vector (Takara, Japan). Recombinant bacteria were identified by blue/white screening and confirmed by PCR and sent to sequencing company (Sangon, China). Nucleotide sequences of the cloned cDNA were sequenced by double pass. All primers used in this experiment were designed using Primer Premier 5.0.

### 2.3. Nucleotide sequence and bioinformatics analyses

The nucleotide sequence similarities were examined by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The 5′and 3′sequences from RACEs were assembled with the partial cDNA sequences corresponding to each fragmental sequence by DNAMAN 5.0. The protein prediction was performed using the open reading frame (ORF) Finder tool. Multiple sequence alignment was created by using the DNAMAN 5.0. And the phylogenetic trees based on the amino acid sequences were performed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis, MEGA 7.1.

### 2.4. The quantitative real-time PCR

Relative TnC mRNA expression levels in various adult tissues were measured by qRT-PCR using a SYBR II® Premix Ex Taq (TliRnase Plus) (TaKaRa, Japan). Total RNA was isolated from various tissues of healthy adult crabs and hemocytes of crabs challenged by intramuscular injection of 0.2 mL of viral or bacterial suspension including WSSV ($10^5$ copies/mL) or *V. alginolyticus* ($10^5$ colony-forming units [CFU]/mL), respectively, for different times, using the EASY spin tissue/cell RNA extraction kit (Aidlab, China) according to the manufacturer's instructions. Experiments were performed in triplicate and at least three crabs were analyzed for each tissue type. cDNA synthesis

**Table 1**
Universal and specific primers used in this study.

| Name | Nucleotide Sequence (5′–3′) | purpose |
|---|---|---|
| F1 | ATCTTGAGGATGATGGGCGT | for the conservative fragment |
| R1 | CATCATCKCCATGAACTCATC | for the conservative fragment |
| 3′ race GSP1 | ACAAGGGAGGTGACGGCTACATC | first primer for 3′RACE |
| 3′ race NGSP1 | CTACGACAAGGGAGGTGACGGCTAC | second primer for 3′RACE |
| 3′ race GSP2 | GAGATGATGAGCGGTTGAAGGG | third primer for 3′RACE |
| 3′ race NGSP2 | TCAGGGCAACCAGACTCAACATC | fourth primer for 3′RACE |
| 5′ race GSP | GATGTAGCCGTCACCTCCCTTGT | first primer for 5′RACE |
| 5′ race NGSP | CTCGTCAGTCTCCGCAATAACCT | second primer for 5′RACE |
| TnC dsRNA F | AAGCTTCGTGTGGTAAACTTAGCCTGC | for TnC RNAi |
| TnC dsRNA R | GAATTCCCCTCACAAGTTTTATTCGCC | for TnC RNAi |
| TnC-realtime-F | CGCTGCCAAGTTCCTGATT | for TnC mRNA expression |
| TnC-realtime-R | CGTCCTCGTCCACCTCTT | for TnC mRNA expression |
| GAPDH-realtime-F | ACCTCACCAACTCCAACAC | for GAPDH mRNA expression |
| GAPDH-realtime-R | CATTCACAGCCACAACCT | for GAPDH mRNA expression |
| C-type-lectin-F | ACTGAGGGGAAAGTAGCC | for C-type-lectin mRNA expression |
| C-type-lectin-R | TGCCCGTGTTTATTCATC | for C-type-lectin mRNA expression |
| crustin-F | TCAGAGCACCCTGGTAAATGT | for crustin antimicrobial peptide mRNA expression |
| crustin-R | GGCAGAACTGCGAAAGAAAG | for crustin antimicrobial peptide mRNA expression |
| JAK-F | ATTGCTGAGGGGATGGATT | for JAK mRNA expression |
| JAK-R | GCCCATCACATTCCCAAA | for JAK mRNA expression |
| proPO-F | ATGAAAGAGGAGTGGAGATG | for prophenoloxidase mRNA expression |
| proPO-R | GTGATGGATGAGGAGGTG | for prophenoloxidase mRNA expression |
| TLR-F | TGTTGCCAGAGCAGAAGGT | for toll-like receptor mRNA expression |
| TLR-R | TTCCGTGAATGAACGAAGG | for toll-like receptor mRNA expression |
| myosin-F | GCCGAGATAAGTGTAGAGGAA | for myosin-II-essential-light-chain-like-proteinmRNA expression |
| myosin-R | AGTGGGGTTCTGTCCAAG | for myosin-II-essential-light-chain-like-protein mRNA expression |
| STAT-F | GACTTCACTAACTTCAGCCTCG | for STAT mRNA expression |
| STAT-R | GAGCTGAGTCTGTCTTAATGTTATCC | for STAT mRNA expression |

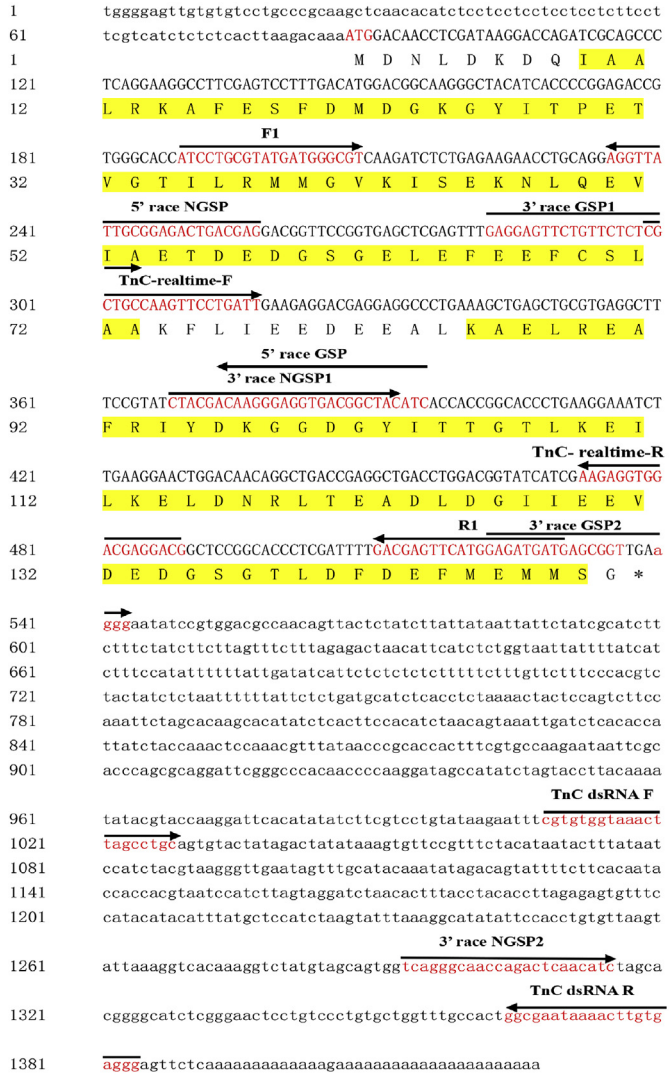


**Fig. 1.** Nucleotide and deduced amino acid sequence of TnC. The nucleotide sequence is displayed in the 5′–3′ directions and numbered at the left. The deduced amino acid sequence is shown with the single capital letter amino acid code. The 3′UTR and 5′UTR are shown with lowercase letters. Codons are numbered at the left with the methionine (ATG) initiation codon, an asterisk denotes the termination codon (TGA). Cloning the conservative fragment, RACE, real-time qPCR, and dsRNA primers are marked with arrows. Two predicted EF-hand domain amino acid sequences are highlighted in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was carried out using 200 μg of total RNA with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Code: FSQ-301; Toyobo, Japan). The synthetic cDNA was kept at −20 ˚C. RT-qPCR was carried out in Bio-Rad Two Color Real-Time PCR Detection System and the data were calculated according to the 2 –ΔΔCT [17] comparative CT method by Office Excel, with GAPDH amplification as the internal control. The design and synthesis of the RT-qPCR primers were entrusted to Generay (Shanghai, China), based on the open reading frame (ORF). Table 1 lists the primers that were designed.

### 2.5. Prokaryotic expression and purification of TnC-dsRNA

The primers (shown in Table 1) with specific restriction sites (Hind III in the forward primer and EcoR I in the reverse primer) were designed from the cloned nucleotide sequence. PCR product digested with Hind III/EcoR I was sub-cloned into LIMTUS 38i Vector (NEB, MA, USA) digested with the same enzymes to gain plasmid L38-TnC. The following process was prepared according to the previous reports [18]. The constructed L38-TnC was verified by restriction enzyme digestion and DNA sequencing. The recombinant plasmid L38-TnC was transformed into HT115 (DE3) cells with deficiency of RNase III. Single colonies of the above the engineering bacteria were separately inoculated to 5 mL of LB medium containing Amp (100 μg/mL), cultured at 37 ˚C with shaking at 200 r/min for 12–16 h, and then inoculated to LB medium containing Amp by a proportion of 1%, cultured at 37 ˚C with shaking at 200 r/min for 2–3 h (OD600 ≈ 0.6), and added with IPTG (with a final concentration of 0.8 mmol/L) to induce the expression for 4 h. After purifying with mirVana miRNA™ Isolation Kit (Ambion, USA), the dsRNAs were annealed and precipitated with 5 mol/L sodium acetate and anhydrous alcohol.

### 2.6. Knock down of TnC by RNAi and challenge experiments

Total RNA from crabs hemocytes was purified using an EASY spin tissue/cell RNA extraction kit (Aidlab, China), following the manufacturer's instructions. The total RNA solution was divided into 50 μL each tube and annealed by incubations at 95 ˚C for 2 min. During 45 min, the temperature slowly decreased from 95 ˚C to 25 ˚C and 25 ˚C for 10 min. The resulting TnC-dsRNA was ethanol-precipitated and resuspended in DEPC-treated $H_2O$. And TnC-dsRNA (100 μg/crab) was immediately injected intramuscularly into the fourth pereopodcoxa of each crab, and TnC mRNA expression levels were detected by RT-qPCR following WSSV and *V. alginolyticus* challenges. The crabs were divided into six groups: intramuscular injection with 100 μL PBS alone; intramuscular injection with 100 μL TnC-dsRNA alone; intramuscular injection with WSSV alone; injection with TnC-dsRNA for 12 h and followed by WSSV challenge; intramuscular injection with *V. alginolyticus* alone; injection with TnC-dsRNA for 12 h and followed by WSSV challenge. The mortality was monitored every 12 h after the last injection [19].

### 2.7. Kaplan–Meier survival analysis

For the pathogen challenge, healthy crab were randomly distributed into six groups (n = 9 per group, three repeat). The control group received injections of PBS alone, the TnC-dsRNA group received injections of TnC-dsRNA alone, the WSSV group received injections of WSSV in PBS, and the TnC-dsRNA + WSSV group received injections of TnC-dsRNA and WSSV, the *V. alginolyticus* group received injections of *V. alginolyticus* in PBS, and the TnC-dsRNA + *V. alginolyticus* group received injections of TnC-dsRNA and *V. alginolyticus*. The copy number of WSSV injected to the crabs was $10^4$ each crab. And the cell density of injected *V. alginolyticus* was $10^5$ each crab. The above initial infection rates were consistent in this study. Each group of crabs was cultivated under the same condition. The number of live and dead crab was counted every 12 h. The survival data was arranged and analyzed in Microsoft GraphPad 5.0.

### 2.8. Apoptosis of crab hemocytes

The hemolymph was mixed with 20 mM EDTA at a ratio of 1:1, and the mixture was centrifuged at 2000 rpm at 4 ˚C for 10 min to collect hemocytes. The hemolymph cells were then suspended in highly alkaline PBS, counted and adjusted to a density of 3–5 × $10^6$ cells/mL with PBS. The cells were stained using a BD Pharmingen™ FITC Annexin V Apoptosis Kit, and assessed by flow cytometry. The cell numbers on quadrant 4, with low PI and high annexin V staining, were considered as apoptotic. The data were presented as means ± standard deviation (SD) derived from at least three independent experiments.

### 2.9. Determination of immune parameters after RNAi

The immune parameters determined included total hemocytes

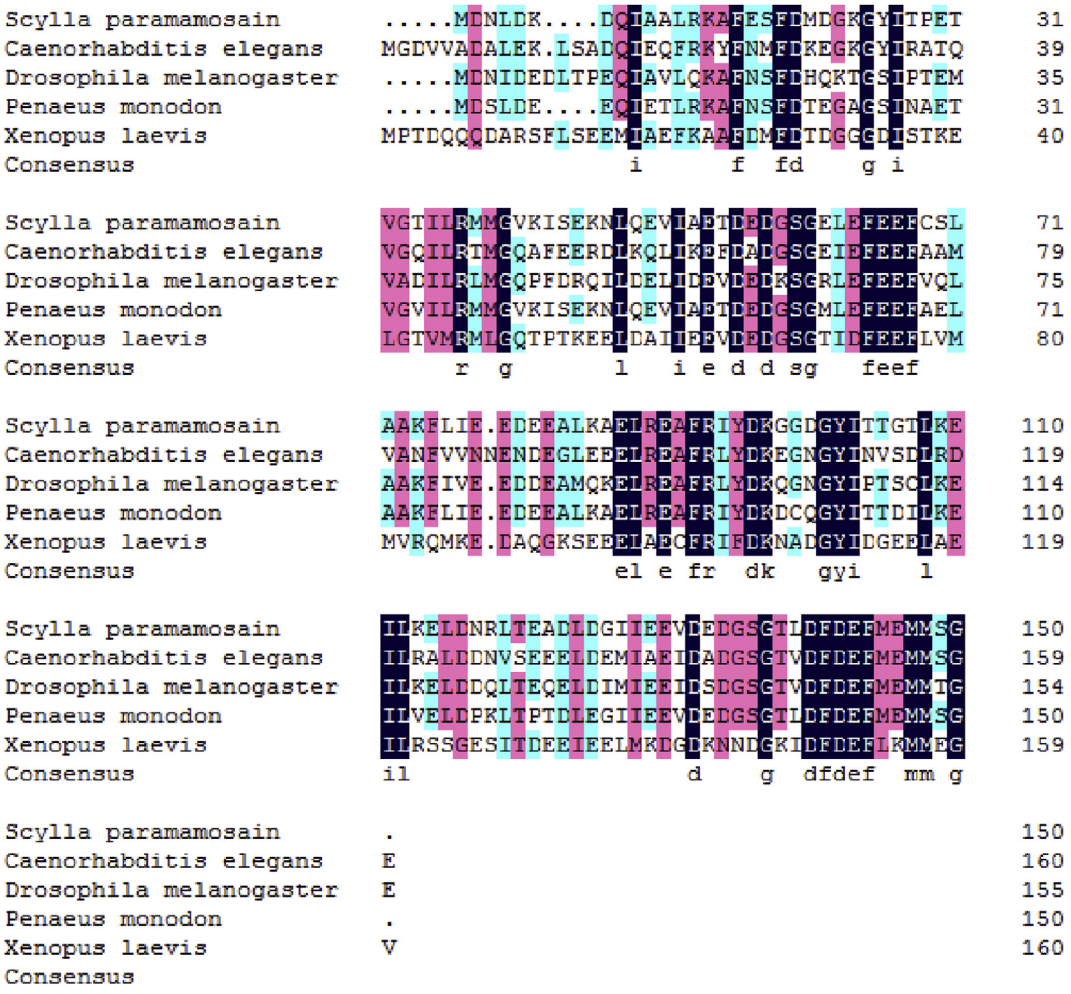


**Fig. 2.** Multiple alignments of the amino acid sequence of *S. paramamosain* TnC with other TnC sequences of common animals. *S. paramamosain* (in this study), *Caenorhabditis elegans* (NP_496251.1), *Drosophila Melanogaster* (AHN56108.1), *Penaeus Monodon* (ADV17344.1) and *Xenopus Laevis*(XP_018092657.1). Forty-two conserved amino acids are shaded and boxed.

numbers (THC), PO and SOD activities. THC was determined as described previously [19]. To determine PO and SOD activities, 500 μL of hemolymph was withdrawn into a 1 mL syringe containing 500 μL of 20 mM EDTA solution from each individual crab. PO activity was quantified in the hemolymph mixture based on the formation of dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [19]. SOD activity was quantified in hemocytes isolated from 300 μL of the hemolymph mixture, according to the improved method described by Beauchamp and Fridovich [20]. Data were presented as a percentage of the normal control.

### 2.10. Detection of WSSV copies

The crab was injected with WSSV or mixture of WSSV and TnC-dsRNA. The whole-genome was extracted from crab hemocytes collected at 12, 24 and 48 h post injection using DNA extraction Kit (Generay, China), according to the manufacturer protocol. To figure the WSSV copies, crab gDNAs of each sample were detected by WSSV vp28 probe in Two Color Real-Time PCR Detection System (Bio-Rad, CA, USA). The primer sequences are shown in Table 1.

### 2.11. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Data from three independent experiments were analyzed by oneway analysis of variance to calculate the means and standard deviations of the triplicate assays. Statistical differences were estimated using paired *t*-test followed by least-significant differences (LSD) and Duncan's multiple range test. All statistics were measured using SPSS software version 19 (IBM, USA). A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$).

## 3. Results

### 3.1. Characterization of TnCcDNA

The full-length TnC cDNA sequence was 1427 base pairs (bp), containing a 453 bp open reading frame (ORF) encoding a 151 amino acids protein. The Pfam website predicted it had two EF-hand function domains. In addition to the ORF, 86 bp and 888 bp, respectively, are untranslated regions (UTR) of the TnC 5′ and 3′ ends. The full length cDNA sequence and the amino acid sequence deduced from cDNA are shown in Fig. 1.

### 3.2. Sequence homology and phylogenetic analysis

To analysis the homology of the *S. paramamosain* TnC gene, the putataive amino acid sequence. The amino acid sequence of the *S. paramamosain* TnC was compared with multiple model organisms by using the DNAMAN version 6.0, including *Caenorhabditis elegans* (NP_496251.1), *Drosophila melanogaster* (AHN56108.1), *Penaeus monodon* (ADV17344.1) and *Xenopus laevis* (XP_018092657.1). The

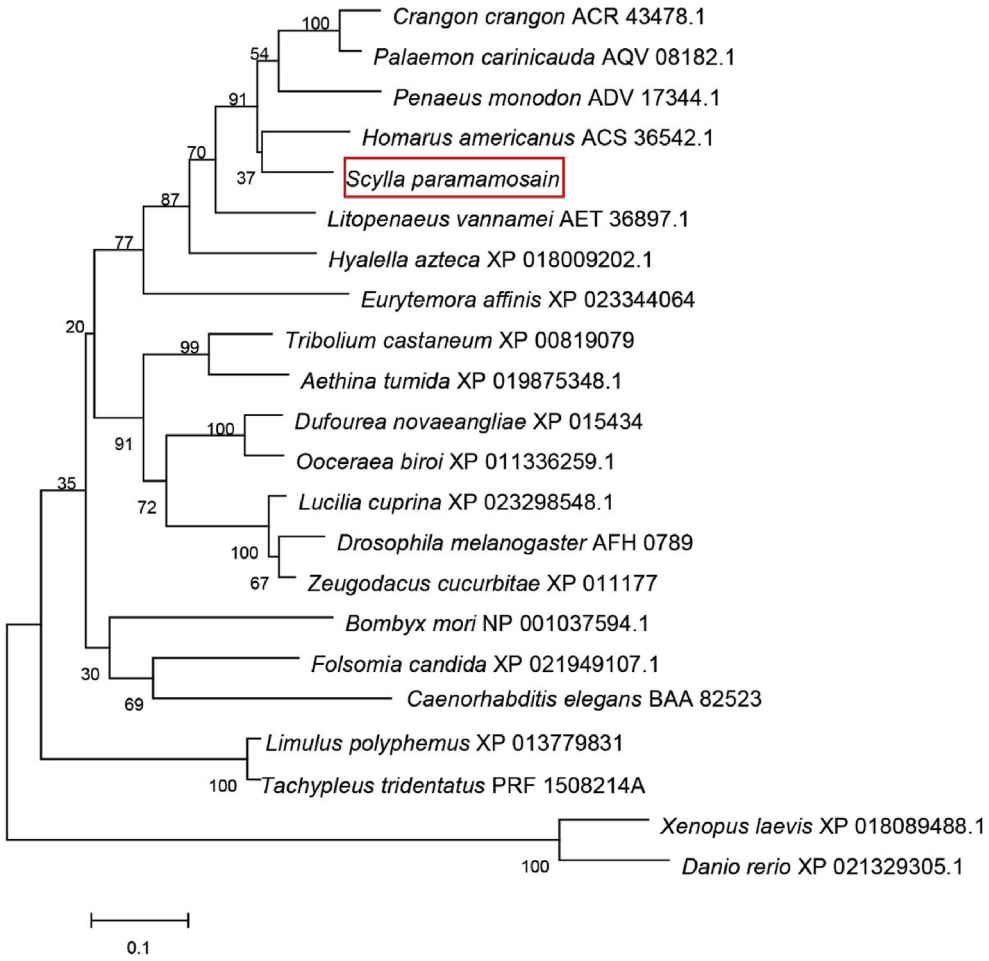


**Fig. 3.** The phylogenetic tree of TnC from different organisms based on amino acid sequence comparisons.

results showed that the homology with *C. elegans* was 51.25%, 62.58% with *D. melanogaster* and 36.02% with *X. laevis*. It has the highest homology with *P. monodon*, which is 82% (Fig. 2).

Based on the deduced amino acid sequence, a bootstrapped neighjoining tree was constructed by the neighbor-joining method using MEGA7.1 (Fig. 3). Through the phylogenetic tree we can see that the evolutionary of TnC was conserved among different species. In the arthropods, the evolution of TnC in *S. paramamosain* was backward. The amino acid sequence homology analysis revealed that the conserved domain contains multiple conserved sites. The *S. paramamosain* TnC had the highest affinity with *Homarus americanus*.

### 3.3. Tissue distribution of TnC expression

Expression profiling of TnC in different tissues of *S. paramamosain* was examined by reverse transcription-quantitative PCR (RT-qPCR) (Fig. 4-A). It was more highly expressed in the muscle tissue compared with other tissues and the lowest expression was in heart. Expression levels of TnC in muscle tissue were greater than that in the intestines, hemocytes, hepatopancreas, gills and heart, respectively. The expression level of TnC in muscle is 45 times that of intestine, 285 times that of hemocyte, and 9933 times that of hepatopancreas. This difference is actually 23,500 times compared to myocardial tissue. The expression of TnC in the muscle tissue was thus significant higher ($P < 0.01$) than in any other tissues.

### 3.4. Time course of TnC expression after WSSV or V. alginolyticus infection

We investigated the variation trend of TnC expression in crabs after infection with WSSV or *V. alginolyticus*. TnC expression changed after the infection in a time-dependent manner. TnC expression was significant down-regulated ($P < 0.01$) from 12 to 72 h post-infection in crabs with WSSV infection. The lowest expression levels occurred at 12 h, after which TnC expression gradually recovered but still not to the pre-infection levels after 72 h post-challenge (Fig. 4-B). Similarly, TnC expression was significant down-regulated ($P < 0.01$) from 12 to 48 h following infection with *V. alginolyticus* (Fig. 4-C). The following results showed that the presence of TnC was not conducive to anti-infection. The decrease of TnC expression after pathogen infection was beneficial to enhance the anti-infection ability of crabs. These results suggested that TnC may be used by pathogens after infection.

### 3.5. Effects of TnC knockdown on expression of immune genes

We tested the effect of TnC double-stranded RNA (TnC-dsRNA) on its mRNA expression using RT-qPCR. TnC mRNA expression in hemocytes was significantly knocked down by TnC-dsRNA ($P < 0.01$) (Fig. 5-A). We detected the effect of TnC-dsRNA on TnC gene expression in hemocytes of *S. paramamosain* at different times post-treatment using RT-qPCR as well. TnC-dsRNA significantly inhibited the expression of TnC mRNA in hemocytes from 24 to 72 h post-treatment ($P < 0.01$). Among them, the 24-h knockdown effect was the most significant, and the subsequent 36 h and 48 h gradually increased (Fig. 5-B). We also examined the relationship between the expression of TnC and the other

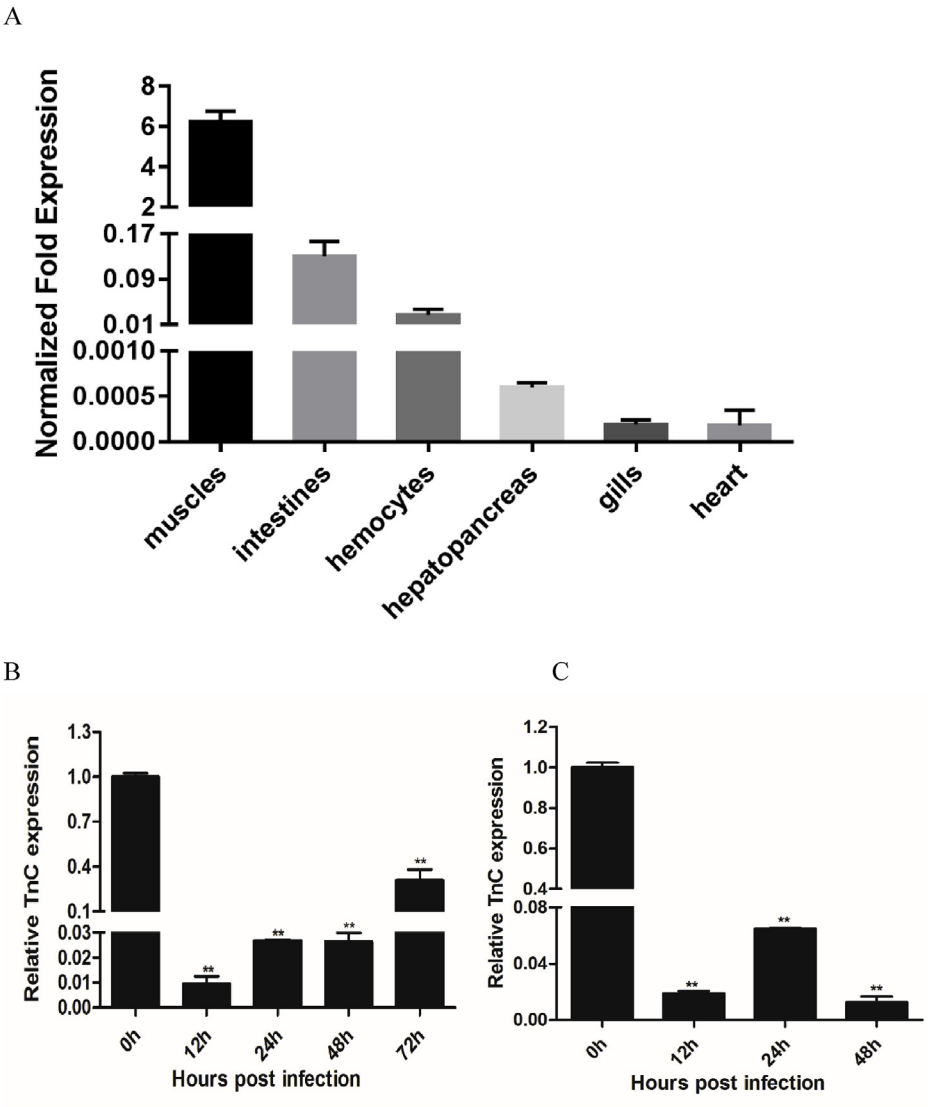


**Fig. 4.** (A) Expression characterization of TnC in various tissues from healthy *S. paramamosain* revealed by quantitative real-time PCR. And real-time RT-PCR analysis of TnC expression challenged with WSSV or *V. alginolyticus*. (B) Real-time RT-PCR analysis of TnC expression in the hemocytes of *S. paramamosain* challenged with WSSV. (C) Realtime RT-PCR analysis of TnC expression in the hemocytes of *S. paramamosain* challenged with *V. alginolyticus*. The amount of TnC mRNA was normalized to the GAPDH transcript level. Data are shown as means ± standard deviation of the tissues of three separate individuals. Capital letters indicate expression of TnC in different adult tissues. Double asterisks indicate a significant difference ($P < 0.01$) between two samples.

important immune genes in the hemocytes of crabs. Among the seven immune genes, crustin antimicrobial peptide (CAP) and prophenoloxidase (proPO) were down-regulated ($P < 0.01$), while Toll-like receptor (TLRs) was significantly up-regulated ($P < 0.01$) following TnC-dsRNA treatment (Fig. 5-C).

### 3.6. Effects of TnC knockdown on immune parameters

The total hemocyte count (THC) was significantly increased in the TnC-dsRNA group compared with PBS group ($P < 0.01$), and the THC of the TnC-dsRNA group reached the highest value at 24 h (Fig. 6-A). The THC in *V. alginolyticus* group, *V. alginolyticus* + TnC-dsRNA group and WSSV group were significantly lower than that in the PBS group at 24 h and 48 h ($P < 0.01$), but WSSV + TnC-dsRNA group was on the opposite (Fig. 6-B,C). The results indicated that the THC decreased in crabs after infection with WSSV or *V. alginolyticus* and hemocyte proliferated after the inhibition of TnC expression.

Phenoloxidase (PO) activity in crab hemolymph was significantly enhanced at 24 h and 48 h, after TnC-dsRNA treatment compared with the PBS group ($P < 0.01$), and PO activity of the TnC-dsRNA group reached the highest value at 24 h (Fig. 6-D).

Crabs treated with TnC-dsRNA showed a significantly higher SOD activity than the PBS group at 24 h and 48 h ($P < 0.01$). The SOD activity of the TnC-dsRNA group reached the highest value at 24 h (Fig. 6-E).

### 3.7. Effects of TnC knockdown on the survival of challenged crab

We evaluated the effects of TnC on the mortality of pathogen-challenged crabs by first injecting them with TnC-dsRNA and then challenging them with *V. alginolyticus* or WSSV. The negative control group had a cumulative mortality of zero which showed a similar mortality to the TnC-dsRNA group, indicating that TnC-dsRNA itself was non-toxic in crabs (Fig. 7). There was no significant difference in the cumulative mortality between *V. alginolyticus*-treated group and *V. alginolyticus* + TnC-dsRNA group within 180 h after injection. However, the cumulative mortality in crabs first treated with TnC-dsRNA then infected with *V. alginolyticus* was significantly higher than that infected with *V. alginolyticus* ($P < 0.01$) starting from 180 h post infection (Fig. 7-B). The WSSV + TnC-dsRNA group show higher mortality than the WSSV-treated group between 12 and 96 h, but thereafter, from 96 to 156 h post challenge, it had a lower mortality (Fig. 7-A). We measured the replication of WSSV in crab in three time periods (24 h, 48 h, and 72 h). The results showed that after knocking down TnC, the WSSV copy number of WSSV + TnC-dsRNA group was significantly lower at 48 and 72 h post challenge than WSSV alone group (Fig. 7-C). Overall, these results demonstrated that TnC knockdown decreased the copy number of WSSV and the mortality of crabs following WSSV

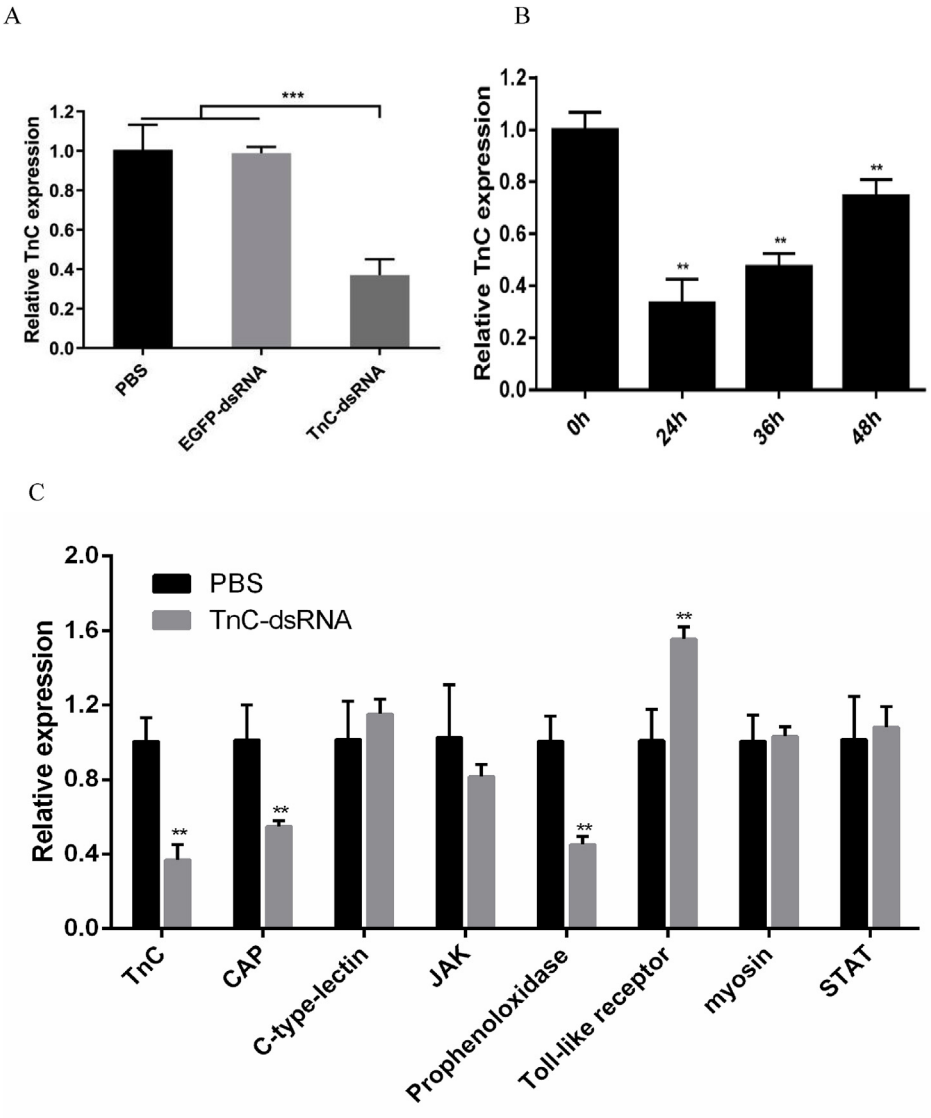


**Fig. 5.** Real-time RT-PCR analysis of TnC and immune gene expression. (A) Real-time PCR analysis of TnC expression in the hemocytes of *S. paramamosain* treated with EPGF dsRNA (EGFP-dsRNA) and TnC dsRNA (TnC-dsRNA) at 24 h post-treatment. The amount of TnC mRNA was normalized to the GAPDH transcript level. (B) Real-time RT-PCR analysis of TnC expression of hemocytes of *S. paramamosain* treated with TnC-dsRNA at different times post-treatment. (C) Real-time RT-PCR analysis of seven immune genes [crustin antimicrobial peptide (CAP), C-type-lectin (CTL), JAK, prophenoloxidase (proPO), toll-like receptor (TLR), myosin-II-essential-light-chain-like-protein (MELCLP) and STAT] expression in the hemocytes of *S. paramamosain* treated with TnC-dsRNA. The amount of TnC mRNA was normalized to the GAPDH transcript level. Data are shown as means ± standard deviation of tissues in three separate individuals. Double asterisks indicate a significant difference between two samples ($P < 0.01$).

infection. In contrast, TnC knockdown increased the mortality of crabs following *V. alginolyticus* infection.

### 3.8. Effects of TnC knockdown on hemocyte apoptosis

We investigated the role of TnC in apoptosis of crab hemocytes using flow cytometry. The apoptosis rate of TnC-dsRNA group was significantly higher than that of the PBS group (Fig. 8-G). Similarly, the *V. alginolyticus* + TnC-dsRNA group showed a higher apoptosis rate than the *V. alginolyticus* group and TnC-dsRNA group (Fig. 8-G). The rate of apoptosis was higher in the WSSV + TnC-dsRNA group compared with the WSSV group (Fig. 8-H). And the difference in the WSSV group with the WSSV + TnC-dsRNA group is slightly smaller than in the *V. alginolyticus* group with the *V. alginolyticus* + TnC-dsRNA (Fig. 8). These results indicated that TnC had an inhibitory effect on hemocyte apoptosis in crabs treated with *V. alginolyticus*, and a stimulatory effect on hemocyte apoptosis in crabs treated with WSSV.

### 3.9. Effects of TnC knockdown on phagocytosis

The effect of TnC knockdown on phagocytosis in WSSV-treated group was measured, the phagocytosis rate was small increased (Fig. 9-F). In the phagocytic assay of *V. alginolyticus*, knockdown of TnC was slightly increased compared to the PBS group, though the differences were not considerable significant (Fig. 9-E). This indicates that the presence of TnC has an inhibitory effect on phagocytosis of WSSV and *V. alginolyticus* during infection.

## 4. Discussion

Troponin is an important regulatory protein that is primarily present in muscle tissue cells. In vertebrates, it is known that troponin contains two subtypes, namely fast response type, slow response type or cardiac troponin. A troponin complex consists of three subunits, TnI, TnT and TnC, respectively. As a molecular switch, TnC is a sensor of $Ca^{2+}$, which can detect the rapid changes of $Ca^{2+}$ in cells, and has completed intracellular signal transduction and corresponding cellular responses [21]. As a member of the EF hand protein domain family, TnC typically has four $Ca^{2+}$ binding sites. A small number of subtypes have less than four $Ca^{2+}$ binding sites [11]. In past studies, research on TnC has focused on vertebrates. In recent years, the acquisition of invertebrate TnC gene clones such as sandworm, mussel, and barnacle provides the basis of gene sequences for the study of TnC in invertebrates [22].

In this study, we obtained the full-length TnC cDNA sequence of the *S. paramamosain*. Through amino acid alignment analysis and phylogenetic tree construction, the TnC gene of *S. paramamosain* is highly conserved, and has the height of the homology with other invertebrates,

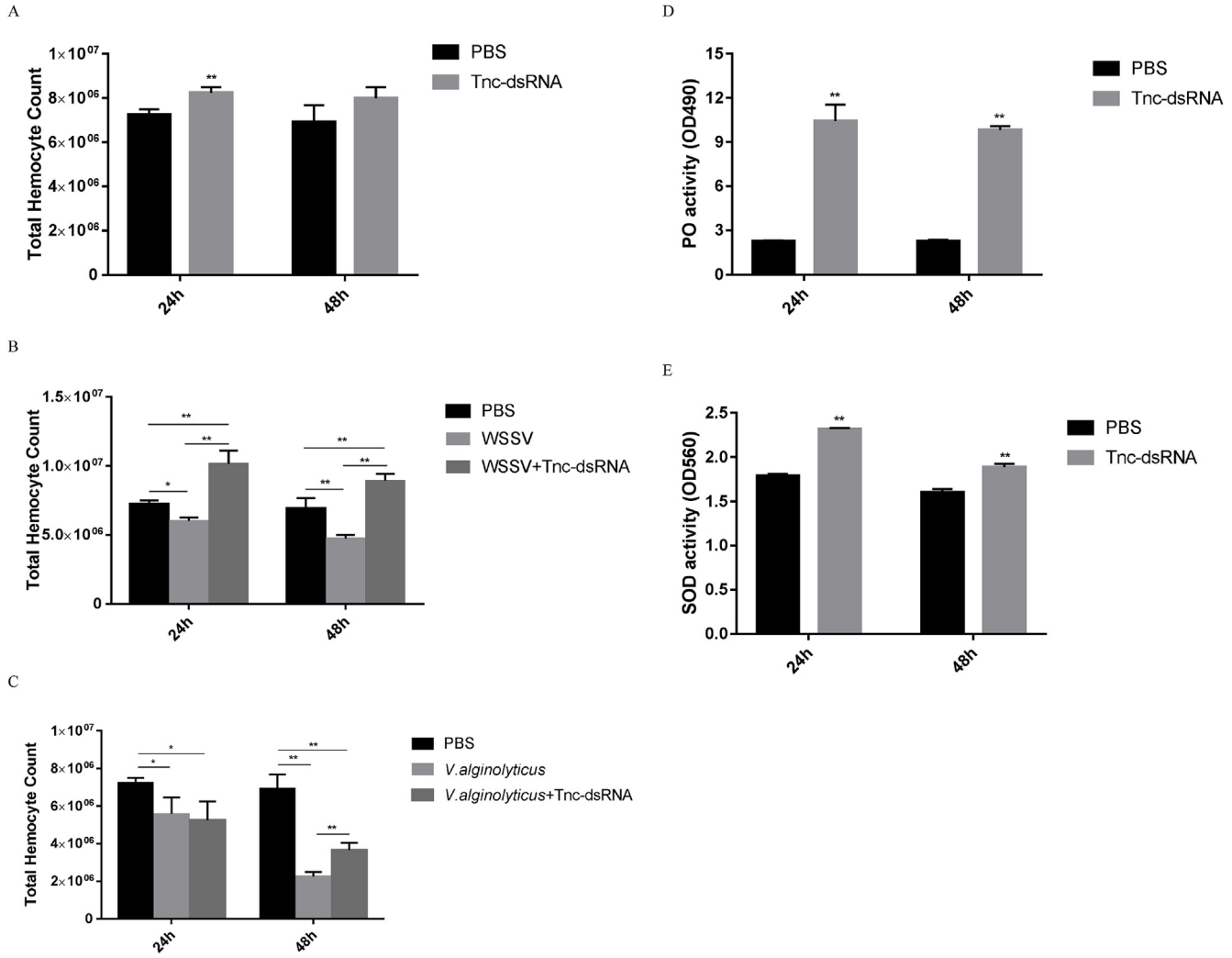


**Fig. 6.** Effects of TnC knockdown on *S. paramamosain* immune parameters, including THC and the activity of PO and SOD, as determined in healthy, WSSV-treated, or *V. alginolyticus*-treated crabs. (A) THC after PBS or TnC-dsRNA treatment; (B) THC after WSSV or WSSV + TnC-dsRNA treatment; (C) THC after *V. alginolyticus* or *V. alginolyticus* + TnC-dsRNA treatment; (D) the PO activity after PBS or TnC-dsRNA treatment; (E) Relative SOD activity after PBS or TnC-dsRNA treatment. All treatments, at each time point, included at least three individual crabs, and all experiments were repeated three times. Each column represents the mean value of triplicate assays.

especially *P. monodon*. TnC expression levels of *S. paramamosain* vary in different tissues, with extremely high levels in the muscle tissue, second highest in the intestine and with relatively low levels in the hemocytes, hepatopancreas, gills and heart. There is no doubt that TnC is highly expressed in muscle tissue because of its function to muscles contract. However, there is such a large difference in the expression between muscle tissue and myocardium. Based on this, we suspect that in *S. paramamosain*, there may be two or three types of TnC in muscle tissue and myocardium. In known vertebrates, it has been demonstrated that two common subtypes sTnC and cTnC are located in muscle tissue and myocardium, respectively [23,24]. Since there are no reports about other TnC subtypes present in the *S. paramamosain*, this inference remains to be confirmed. Relatively high expression of TnC in the intestine, a known immune organ in crustaceans, indicates that the various expressions of TnC in different tissues may reflect various roles in the crab. It was reported that Troponin I is present in human cartilage and inhibits angiogenesis [25]. It also provides some reference ideas for our research on immune-related research in the *S. paramamosain*.

RNA interference (RNAi) is a phenomenon of post transcriptional gene silencing specifically mediated by double-strand RNA (dsRNA) sequences in vivo [26]. RNAi has been widely applied in studies of an immune response in many invertebrate models to investigate the antibacterial and antiviral mechanisms of certain target proteins [27,28]. In the present study, we successfully inhibited TnC expression by injecting specific dsRNA into *S. paramamosain*, thus providing a practical method to characterize the function of TnC in the innate immune system.

We then identified the effects of TnC-dsRNA on the expression of several genes crucial to the innate immune system of crabs. Among the seven immune genes, knockdown of TnC mRNA resulted in significant down-regulation of CAP and proPO, while TLRs was significantly up-regulated. The proPO system is recognized as an important invertebrate defense cascade against parasites, bacteria and fungi [29–31]. TLRs are type I transmembrane proteins involved in innate immunity by recognizing microbial conserved structures [32]. Changes in the expression levels of these genes reflect changes in the immune process. Our study suggests that TnC may be involved in innate immunity and have obvious antibacterial and antiviral activities.

We also found that the crabs treated with TnC-dsRNA then challenged with *V. alginolyticus* had a higher mortality count than the crabs only injected with *V. alginolyticus*, while the reverse was true in the WSSV-treated group and the WSSV + TnC-dsRNA group. To explore whether TnC has other effects on innate immunity in crabs, we investigated multiple functional parameters to assess immune potential.

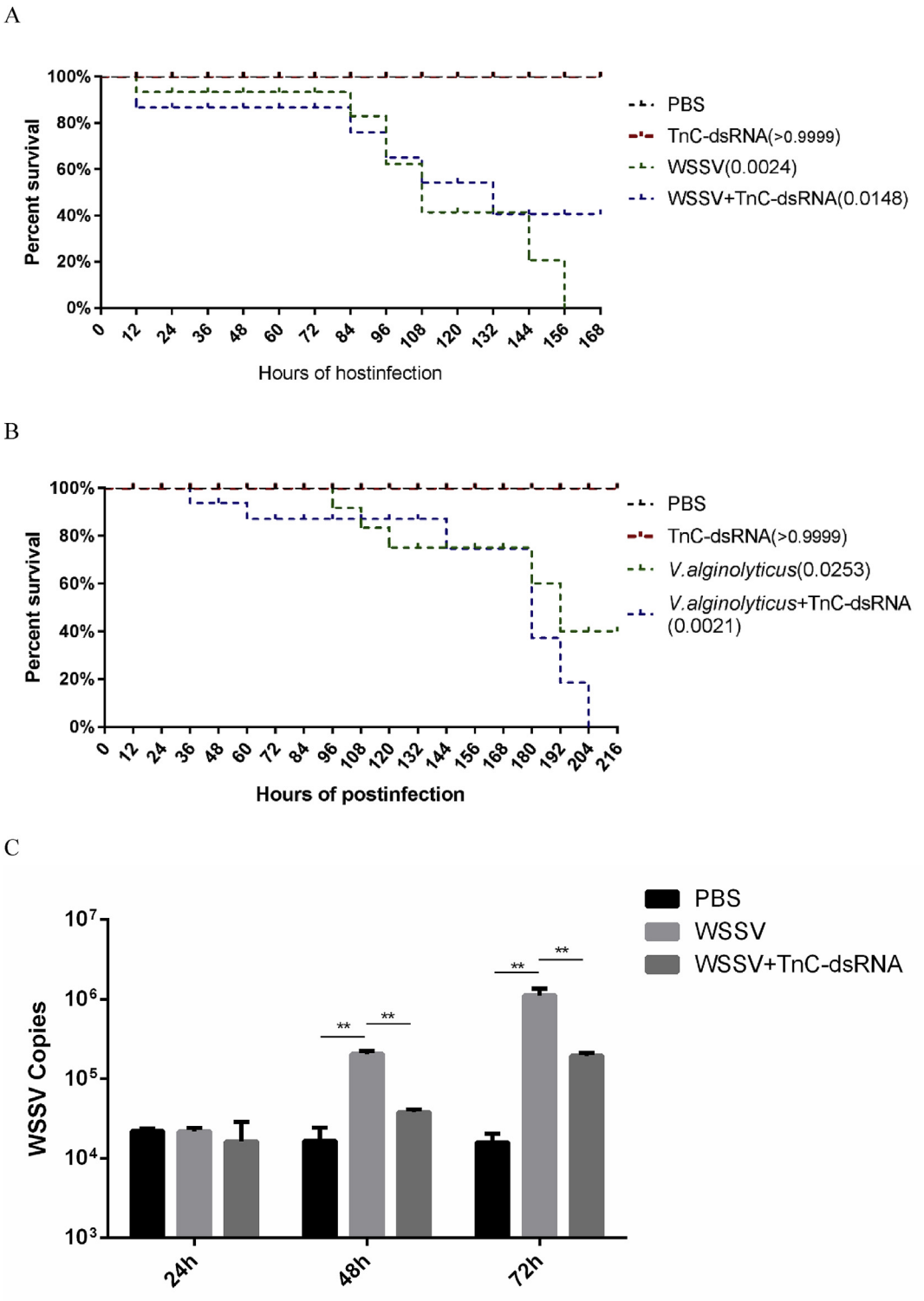


**Fig. 7.** The survival analysis of challenged crabs treated with TnC-dsRNA. (A) The survival analysis from Kaplan–Meier of WSSV challenged crabs treated with TnC-dsRNA. (B) The survival analysis from Kaplan–Meier of *V. alginolyticus* challenged crabs treated with TnC-dsRNA. The numbers in brackets indicate the significance of difference from PBS group. (C) WSSV copies detected by a TaqMan VP28 probe at different times post-infection. The solutions used for injection are shown on the right. There were eight individuals in each group.

The THC and the activity of PO and SOD were significant increased by knock-down TnC, which indicated that TnC can regulate hemocyte proliferation and the activity of PO and SOD. In our study, apoptosis was increased in WSSV-treated crabs which pretreated with TnC-dsRNA compared with the WSSV group. At the same time, the crab infected with *V. alginolyticus* and pretreated with TnC-dsRNA also increased apoptosis compared with the *V. alginolyticus* group. Apoptosis is highly regulated, programmed cell death process which plays a critical role in limiting virus infection [33]. The troponin C had been found to be involved in apoptosis of mammals [34]. The apoptosis data counts the number of caspase 3/7 activated cells, and many hemocytes with complete shape are also included in apoptosis. But the total hemocyte

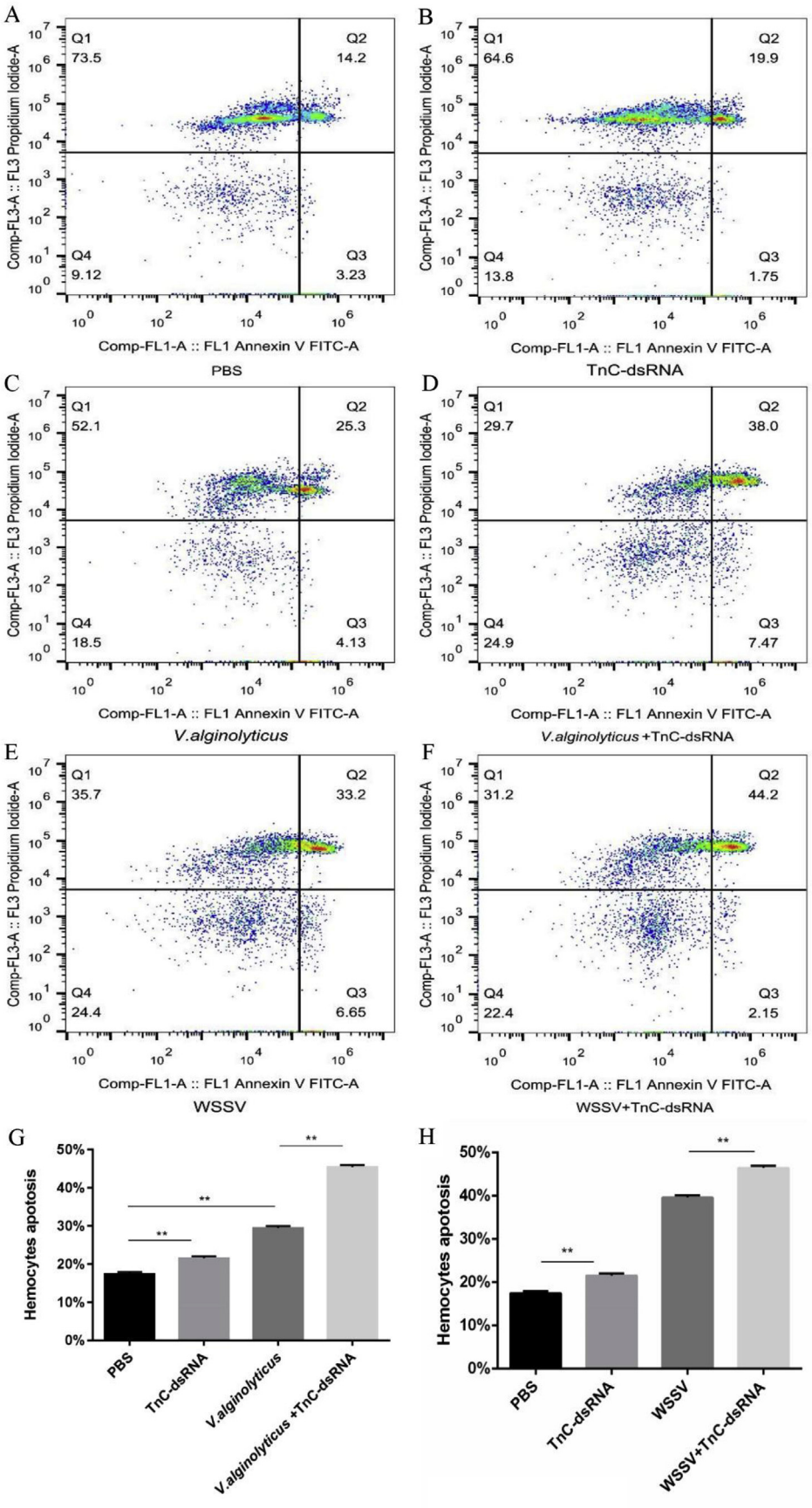


**Fig. 8.** Flow cytometry assay of apoptosis. (A) PBS; (B) TnC-dsRNA; (C) *V. alginolyticus*; (D) *V. alginolyticus* + TnC-dsRNA; (E) WSSV; (F) WSSV + TnC-dsRNA; (G) Bar graph of phagocytosis of *V. alginolyticus*; (H) Bar graph of phagocytosis of WSSV. Double asterisks indicate a significant difference ($P < 0.01$) between the sample and the challenge only.

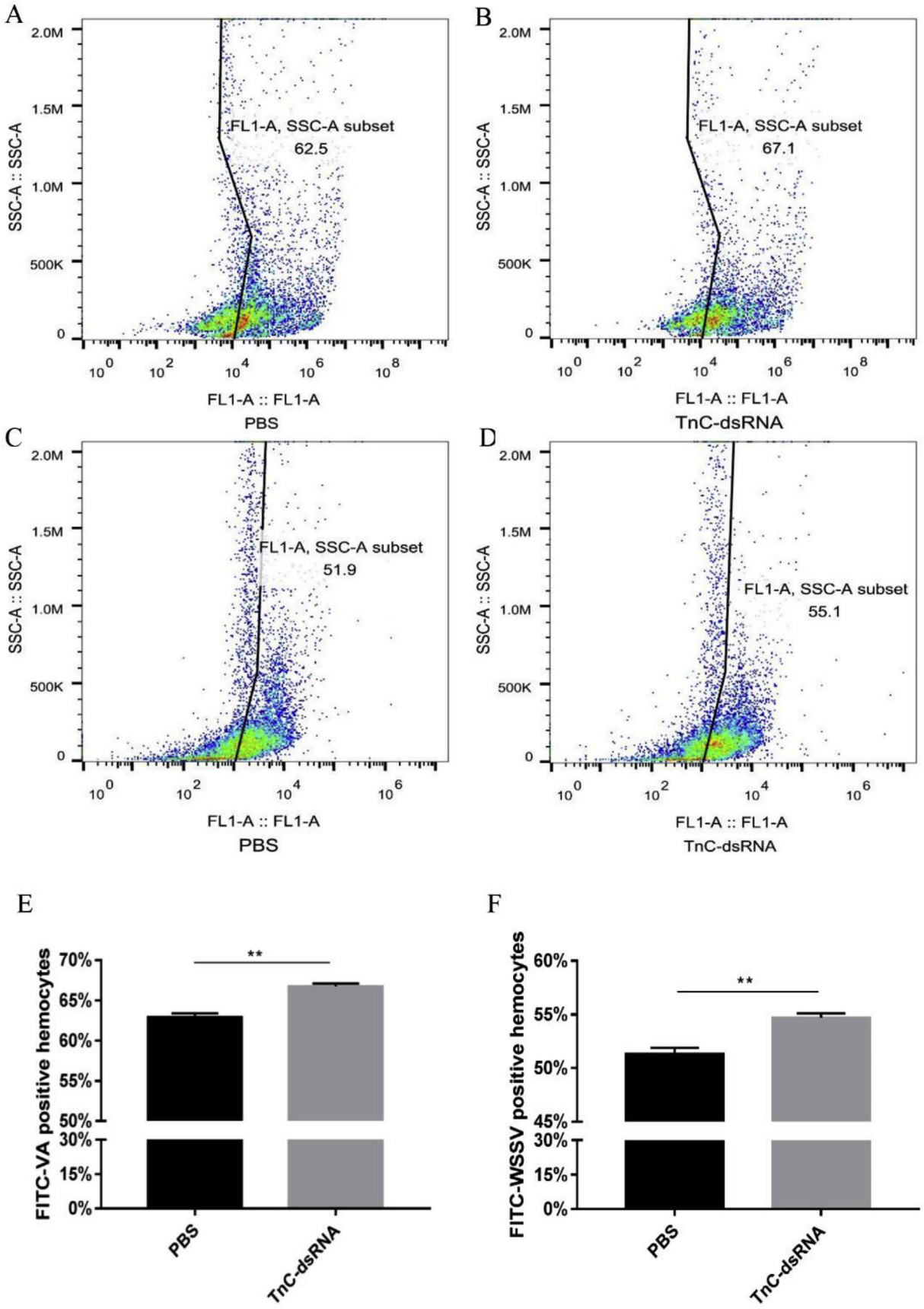


**Fig. 9.** Flow cytometry assay of phagocytosis. Inactivated WSSV and *V. alginolyticus* virions were labeled with FITC. (A) PBS (*V. alginolyticus*); (B) TnC-dsRNA (*V. alginolyticus*), (E) Bar graph of phagocytosis of *V. alginolyticus*. (C) PBS (WSSV); (D) TnC-dsRNA (WSSV), (F) Bar graph of phagocytosis of WSSV. Double asterisks indicate a significant difference ($P < 0.01$) between the sample and the challenge only.

count (THC) only counts the number of hemocytes with complete shape. So the data from hemocyte apoptosis and hemocyte count were both increased following the WSSV + TnC-dsRNA treatment.

These results indicate that TnC not only regulates THC and the activity of PO and SOD in crabs but also influences the apoptosis in hemocytes. Virus invasion and bacterial infection are two different modes and therefore can trigger different immune mechanisms. The mortality rate showed different changes after WSSV or *V. alginolyticus* challenged. However, in this study, in the hemocyte apoptosis, the infection against viruses and bacteria showed consistency.

In conclusion, our present study suggests that TnC plays a key role on the innate immune system of crabs against bacterial and viral infection. The finding reveals a more potential function of TnC and provides an initial basis for further research into the role of TnC in the innate immunity of invertebrates.

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