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Nucleus-translocated matrix metalloprotease 1 regulates innate immune response in Pacific abalone (*Haliotis discus hannai*)

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ABSTRACT

As an important economical shellfish in coastal area of China, abalone is susceptible to bacterial infection, especially *Vibiro parahemolyticus* (*V. parahemolyticus*). Matrix metalloproteinases (MMPs) have been extensively investigated in the immune response of mammals. However, little is known about the involvement of MMP in abalone innate immune system against pathogen infection. In this study, the role of MMP-1 in the immune response of Pacific abalone (*Haliotis discus hannai*) was explored. The results showed that *V. parahemolyticus* infection induced significantly elevated expression of MMP-1 as well as immune related genes including allograft inflammatory factor 1 (AIF-1), macrophage expressed gene 1 (MPEG-1) and TPA-inducible sequence 11 family protein (Tis11FP). Notably, silencing of MMP-1 reduced the expression of these genes, suggesting that MMP-1 was an upstream regulatory factor in *V. parahemolyticus* infection. Further analysis showed that MMP-1 was engaged in the regulation of cellular (phagocytosis, apoptosis) and humoral [superoxide dismutase (SOD), alkaline phosphatase (ALP), acid phosphatase (ACP)] immunity. Interestingly, the extracellularly distributed MMP-1 could be translocated to the nuclei of hemocytes, thereby functioning as a transcriptional regulator or by selectively activating or inactivating other components through proteolysis. Hence, our study established an important role of MMP-1 in abalone innate immunity against *V. parahemolyticus* infection and it represented the first report on the investigation of MMP in abalone.

1. Introduction

Abalone, a kind of mollusc, inhabits the tidal-to-subtidal coastlines of tropic to temperate regions. The largest producers of wild abalone are Australia, South Africa, Japan, New Zealand, Mexico and the United States, while the largest producers of farmed abalone are China, Korea, South Africa, Australia and Chile [1]. Currently, the abalone production in China has increased from 56, 511 to 139, 697 metric tons (MT) from 2010 to 2016 [2,3], which is attributed to a shift in using land-based farming methods to more efficient offshore sea-cage farming methods. The Pacific abalone, *Haliotis discus hannai*, known for its delicious flavor, unique texture and high market value, is the most important cultivated abalone in China. To date, *H. discus hannai* occupies more than 80% of all abalone production in China [4]. The southern Chinese provinces including Fujian and Guangdong are the favored locations of most farms while the majority of abalone seed production occurs between Dalian and Shandong Peninsula in the northern China [5]. In 2016, Fujian Province accounted for over 80% of all abalone production in China [2].

The Vibrionaceae is a large family of Gram-negative γ-proteobacteria. They live in a variety of aquatic environments and even in extreme habitats [6]. Members of the genus *Vibrio* have gained attention because several species are associated with human and animal diseases, such as *V. harveyi*, *V. alginolyticus* and *V. parahemolyticus*. They have been identified as opportunistic pathogens, causing disease when the host is stressed or immune suppressed [7]. In particular, *V. parahemolyticus* occurs in marine coastal waters and is commonly found in estuarine environments worldwide. In addition to its status as a human pathogen, *V. parahemolyticus* has also been associated with the mass mortality of molluscs, such as abalone [8]. Studies have reported the

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Table 1
Primers used in the qPCR assay.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
MMP-1	CACGACATCGCCTTGGTGAAACTCT	CCTGCTTGGTCCATCGGTATGAGTG
AIF-1	CGGTTGTCAGTGAGCCAGAG	TTCCATCTCCTCCATTAGTTTCCC
MPEG-1	CTTATATGGTCCAGCTAAAGGCGAT	TTCCAACCGTGTTTGACAGTATG
Tis11FP	GTGTAACGCTAAGCCAATCAG	TCTAGTATCCGAACCACGACT
TLR	TGGAGGCTGTTTTCTAAGGAC	CCCATTTTCTAGTTTGGGAGT
NF-κB	AGGTGTGAGTGAACGAGGAAAT	GGTGTTGGTGAAGGAGTATCTGG
β-actin	AGATGTTGCTGCGTTGGTTAT	GATGGGGTACTTGAGGGTGAG

outbreaks of mass mortality caused by *V. parahemolyticus* on the south coast of China [9]. During *V. parahemolyticus* infection, several tissues of the abalone are colonized, such as the hemolymphatic sinuses and gill epithelial cells [10]. A report with green fluorescent protein (GFP)-labeled *V. parahemolyticus* also indicates that the gills are the ideal tissue of adhesion and entrance of the bacteria [11].

The matrix metalloproteinases (MMPs) are a widespread family of zinc-dependent metallopeptidases, which currently comprise twelve structurally characterized families with common features [12,13]. They are often co-expressed or co-repressed in response to glucocorticoids, retinoids, pathogens infection and stress [14]. The activity of MMPs is tightly controlled at the level of transcription, pro-peptide activation and inhibition by tissue inhibitors of MMPs (TIMPs) [15]. In mammals, MMPs are involved in multiple physiological and pathological processes, including embryogenesis, tissue remodeling, immune response and many diseases such as cancer, arthritis, atherosclerosis, ulceration and encephalomyelitis [16–21]. In addition to vertebrates, MMPs are also widely present in invertebrates, where they participate in dendritic remodeling, tracheal growth, histolysis, regeneration and matrix degradation during hatching [22].

Despite of some previous studies on the transcriptional regulation of immune related genes after pathogens infection, such as interleukin-1 receptor-associated kinase 4 (IRAK4), interleukin 17 (IL-17) and I-κB [23], caspase [24], galectins [25] and clip-domain serine protease (cSP) [26], the knowledge about its immune response is still limited. MMP-1, also known as interstitial collagenase, specifically breaks down the types I, II, and III collagens. Although the full-length of MMP-1 cDNA has been cloned from Pacific abalone in our previous study [27], the involvement of MMP in the innate immunity of abalone has not been extensively characterized. In this study, the role of MMP-1 in the innate immunity of Pacific abalone was explored. The results showed that MMP-1 played a positive role in anti-*Vibrio* immunity of abalone by regulating the cellular and humoral immune responses of abalone, as well as the expressions of well-known immune related genes. Interestingly, MMP-1 could be translocated into the nuclei of hemocytes, where it might function as a transcription factor or activate/inactivate other proteins through proteolysis. Therefore, our study revealed an important function of MMP in innate immunity of molluscs.

2. Materials and methods

2.1. Abalone culture and V. parahemolyticus infection

Abalones (*H. discus hannai*), with approximately 50 g in body weight and 6.0–6.5 cm in body length, were purchased from Xiamen Seashine International Seafood Trading Center (Xiamen, China) and cultured in groups of 20 individuals in 25-L tanks with filtered aerated seawater (salinity 30‰) at 20 °C. The abalones were starved for 4 days prior to experimental manipulation and received no food during the trails.

Bacteria grown in LB medium at 37 °C overnight were resuspended in phosphate buffered saline (PBS) and adjusted to the concentration of $OD_{600} = 0.4$ (assuming 1 OD measurement has equivalent of 5×10^8 CFU/mL) [28]. For pathogenic challenge, abalones were injected intramuscularly with 100 μL of *V. parahemolyticus* suspension (2×10^8 CFU/mL). Healthy abalone were injected with 100 μL PBS and then kept separately as a control group. Tissue samples (hemocytes, hepatopancreas, gill, gonad, mantle and muscle) of the abalones (three individuals per time point) were collected at 0, 12, 24, 48 and 72 h after infection, snap frozen in liquid nitrogen and stored at −80 °C for further experiments.

2.2. Quantitative real-time PCR

TransStart® Top Green qPCR SuperMix (TransGen, Beijing, China) was used to determine the transcript levels of genes according to the manufacturer's instructions in an ABI Prism 7300 System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). β-actin was used as a reference gene. Briefly, the total RNA was extracted from cells or tissues using an RNAsimple Total RNA Kit (Tiangen, Shanghai, China). The first-strand cDNA was synthesized from the total RNA using TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (TransGen). Then, quantitative real-time PCR (qPCR) was conducted using gene-specific primers to determine the transcript levels of the MMP-1, allograft inflammatory factor 1 (AIF-1), macrophage expressed gene 1 (MPEG-1), TPA-inducible sequence 11 family protein (Tis11FP), toll-like receptor (TLR) and nuclear factor-κB (NF-κB). Gene-specific primers used in this assay were listed in Table 1. Following initial denaturation at 95 °C for 30 s, 45 cycles of PCR amplification were performed at 95 °C for 5 s and 60 °C for 31 s, with a dissociation curve at the end of the amplification reaction. The relative amount of the target gene mRNA was normalized to that of β-actin.

2.3. Western blot analysis

Abalone hemocytes or tissues were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 pH 8.0) containing 2 mM phenylmethanesulfonyl fluoride (PMSF) on ice. Twenty micrograms of proteins in each sample were separated on a 12% SDS-polyacrylamide gel (SDS-PAGE) and then electrotransferred to a nitrocellulose membrane (GE Healthcare, Waukesha, WI, USA) in transferring buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol). The membrane was then blocked with 5% non-fat milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 pH 8.0) for 2 h at room temperature, followed by washing with TBST for three times. Afterwards, the membrane was incubated with a primary antibody in TBST buffer containing 1% non-fat milk at 4 °C overnight. After extensive washing in TBST buffer, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse secondary antibody (Beyotime Biotechnology, Shanghai, China) for 2 h at room temperature. The membrane was developed using enhanced chemiluminescence (ECL) as substrate and the results were recorded using a chemiluminescence, fluorescence and visible light detection gel imaging system (Alpha Innotech, San Leandro, CA, USA). To obtain anti-MMP-1 IgG, the catalytic domain of MMP-1 fused with a His-tag was expressed in *Escherichia. coli* and purified from inclusion body. The rabbit polyclonal antibody against MMP-1 was prepared in Laboratory Animal Center of Xiamen University (Xiamen, China) according to standard

Table 2
siRNAs used in the RNA interference assay.

Name	sense (5'-3')	antisense (5'-3')
MMP1-siRNA1	GCTACCTCAACACCGACTA	TAGTCGGTGTTGAGGTAGC
MMP1-siRNA2	GGTTGCTGCTCATGAATTT	AAATTCATGAGCAGCAACC
MMP1-siRNA3	GCAAGAGCCGCATCTTCAT	ATGAAGATGCGGCTCTTGC
scrambled siRNA	TTCTCCGAACGTGTCACGT	ACGTGACACGTTCGGAGAA

procedure. In brief, a New Zealand white rabbit was immunized with 1 mg purified recombinant MMP-1 in complete Freund's adjuvant (FA). Four weeks and six weeks later, the rabbit was boosted with MMP-1 in incomplete FA, respectively. The rabbit was sacrificed one week after the final boost to collect serum. MMP-1-specific IgG was obtained after Protein A-Sepharose affinity column purification. The mouse monoclonal antibody against β-actin (sc-69879, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was raised against a slightly modified synthetic peptide corresponding to β-cytoplasmic actin. It was recommended for detection of β-actin of broad species.

2.4. RNA interference of MMP-1

RNA interference (RNAi) was conducted through injection of MMP-1-specific siRNAs to suppress the expression of MMP-1 *in vivo*. The MMP-1-specific siRNAs and scrambled siRNA (Table 2) were designed online (https://rnaidesigner.thermo-fisher.com/rnaiexpress) and synthesized using *in vitro* transcription T7 kit (TaKaRa, Japan) according to the manufacturer's instructions. The synthesized siRNAs were dissolved in siRNA solution (50 mM Tris-HCl, 100 mM NaCl pH 7.5) and quantified by spectrophotometry. *V. parahemolyticus* suspension and 30 μg MMP-1-specific siRNAs were co-injected into the abalone muscle. As controls, PBS, *V. parahemolyticus* alone and scrambled siRNA were included. At different time points post injection, the abalones were collected for further analysis.

2.5. Detection of V. parahemolyticus copies by qPCR

At different time points post *Vibrio* infection and MMP-1 knockdown, the abalone hemolymph was collected and centrifuged at 500 × *g* for 10 min. Afterwards, total DNA was extracted from the collected samples using a tissue DNA extraction kit (Tiangen), and the *Vibrio* copies were detected by qPCR with toxR-specific primers (5′-AAACGA GGCTATCAACTCATTTG-3′ and 5'-GTCGCTAAAGACGGCTCT- AC-3') and a toxR-specific TaqMan probe (5'-FAM-ACTGTTGAACGCCTAA GCC-CGC-BHQ1-3'). As toxR is a single-copy gene in *V. parahemolyticus* genome (GeneBank ID: NC_004603.1 and NC_004605.1), it could serve as a good specific target to quantify *Vibrio* genome copies. A linearized plasmid containing a 200 bp *V. parahemolyticus* toxR DNA fragment was quantified according to the following formula: genome copies/μL = $(C \times N_A \times 10^{-9})$/(genome length (bp) × Mw), wherein C is a measured concentration of linearized plasmid (ng/μL), N_A is Avogadro's constant (6.02×10^{23} molecule/mole) and Mw is molecular weight of 1 bp which is 660 Da in double-strand DNA. Afterwards, the linearized plasmid was diluted 10-fold as an internal standard. The 10 μL PCR solution contained 5 μL of Premix Ex Taq (Perfect Real Time) (TaKaRa), 0.2 μL of 10 μM forward and reverse primers, respectively, 0.15 μL of 10 μM TaqMan probe, 200 ng of DNA template, and distilled water up to 10 μL. The qPCR conditions were 95 °C for 1 min followed by 45 cycles of 30 s at 95 °C, 30 s at 52 °C, and 30 s at 72 °C.

2.6. Phagocytosis assay

V. parahemolyticus bacteria were collected by centrifugation at 12,000 × *g* for 5 min. After being washed with PBS, the bacteria were incubated in PBS containing 1 mg/mL fluorescein isothiocyanate (FITC) (Sigma Aldrich, St. Louis, MO, USA) for 1 h at room temperature with gentle stirring.

A total of 2×10^5 hemocytes were resuspended in PBS and mixed with FITC-labeled bacteria at a ratio of 1:10 (cells: bacteria). Then the cells were incubated at room temperature for 30 min, followed by washes for three times with cold PBS to remove unbound FITC-labeled bacteria. The cells were resuspended with 1% paraformaldehyde (Sigma Aldrich) and subjected to flow cytometry (Guava easyCyte 6-2L, Millipore, Hayward, CA, USA) to evaluate the phagocytic rate. For each sample of flow cytometry, 5000 cells were counted.

2.7. Evaluation of apoptosis

Apoptosis of hemocytes was carried out using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, New Jersey, USA) according to the manufacturer's instructions. For Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) staining, the collected hemocytes (1×10^5 cells from each group) were resuspended in 100 μL 1 × binding buffer and added with 5 μL Annexin V-FITC and 5 μL PI. Then, the uniformly mixed cells were incubated at room temperature for 15 min in the dark, followed by the addition of 400 μL 1 × binding buffer to each tube. The cells were subjected to flow cytometry (Guava easyCyte 6-2L, Millipore) within 1 h and apoptosis ratio was determined as percentage of Annexin V-positive cells.

2.8. Superoxide dismutase activity

Twenty microliters of cell-free hemolymph were sampled for superoxide dismutase (SOD) activity detection with SOD assay kit (WST-1 method, Nanjing Jiancheng Bioengineering Institute, China) as described previously [29]. One unit of SOD activity was defined as the amount of enzyme required for inhibiting half of superoxide-induced oxidation. The specific SOD activity was expressed as SOD unit per milliliter sample.

2.9. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity in abalone cell-free hemolymph was measured with ALP assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Five microliters of sample were used in this experiment. One unit of ALP activity was defined as the amount of enzyme in 100 mL sample necessary to produce 1 mg nitrophenol for 15 min at 37 °C.

2.10. Acid phosphatase activity

Acid phosphatase (ACP) activity in abalone cell-free hemolymph was measured with ACP assay kit (Nanjing Jiancheng Bioengineering Institute) by calculating the optical density using *p*-nitrophenylphosphate as substrate. Five microliters of sample were used in this experiment. One unit of ACP activity was defined as the amount of enzyme in 100 mL sample necessary to produce 1 mg nitrophenol for 30 min at 37 °C.

2.11. Immunofluorescence assay

At different time points post bacterial infection, the abalone hemocytes were applied onto glass slide precoated with poly-lysine (Sigma Aldrich) and incubated at 20 °C for 30 min. After fixation with 4% paraformaldehyde, the hemocytes were permeabilized with PBST (PBS, 0.25% Triton X-100) for 10 min at room temperature and further blocked with PBST containing 1% BSA for 30 min. Then, the cells were incubated with rabbit anti-MMP-1 IgG overnight at 4 °C, after which they were incubated with Alexa 488-conjugated goat anti-rabbit IgG (Sangon Biotech, China) for 1 h at room temperature. After extensive wash with PBS, the hemocytes were incubated with

tetramethylrhodamine-conjugated phalloidin (AAT Bioquest, Sunnyvale, CA, USA) for 1 h at room temperature to label actin filaments. Subsequently, the cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and viewed under a confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY, USA).

2.12. Statistical analysis

All biological experiments were repeated three times independently. Numerical data were analyzed using a one-way analysis of variance. The statistical significance between treatments was analyzed using Student's *t*-test.

3. Results

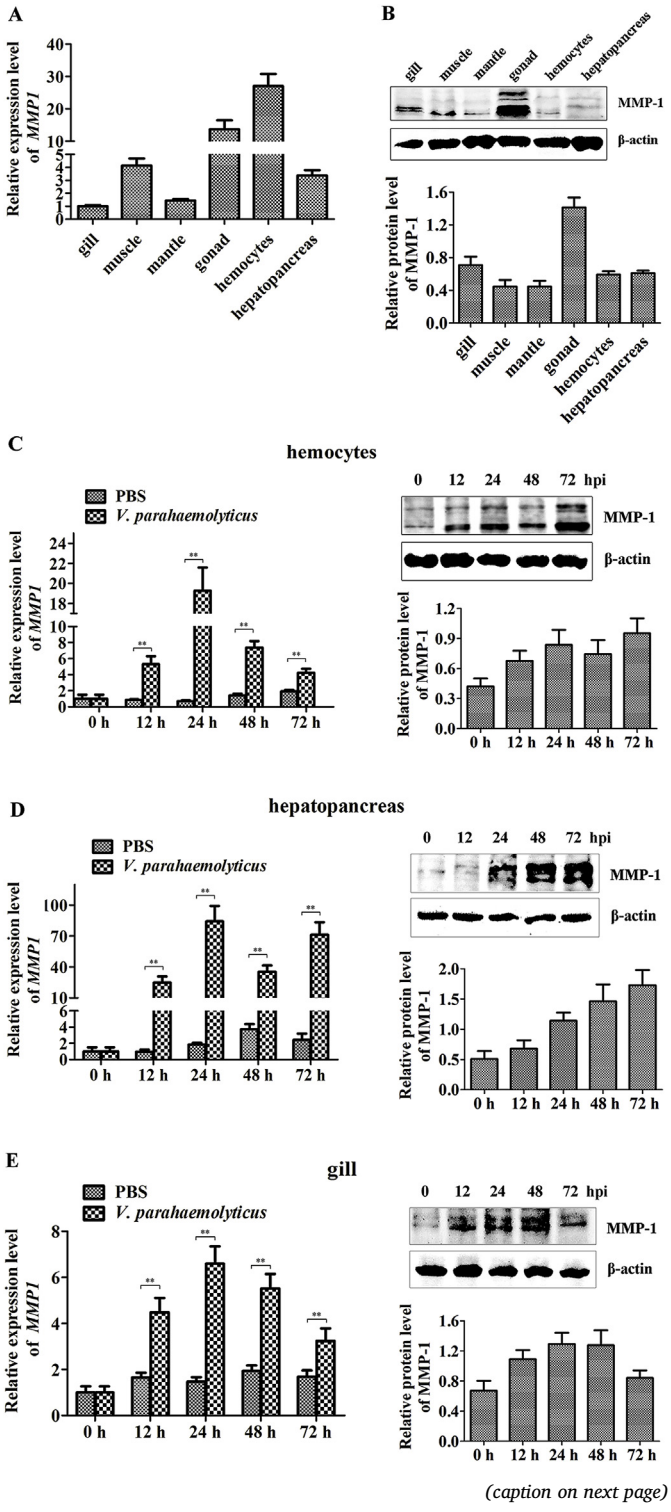
3.1. Expression analysis of MMP-1 in different tissues and in response to pathogenic infection

To investigate the tissue-specific expression profile of MMP-1, qPCR and Western blot analysis were used to determine the transcript and protein levels of MMP-1 in different tissues of abalone. The results showed that the MMP-1 gene was broadly expressed in all tissues selected. As shown in Fig. 1A, MMP-1 mRNA was highly expressed in hemocytes and gonad, moderately expressed in muscle, hepatopancreas, while mRNA level was lower in mantle and gill. Western blot analysis revealed that MMP-1 protein was predominantly present in gonad, while the protein level was lower in muscle, gill, mantle, hemocytes and hepatopancreas (Fig. 1B).

Abalone hemocytes, hepatopancreas and gill are the most important immune-related organs. Thus, the temporal expressions of MMP-1 in hemocytes, hepatopancreas and gill of abalone were monitored after *V. parahemolyticus* challenge. The results showed that MMP-1 mRNA levels were significantly upregulated post infection, with the peak values at 24 h, which were approximately 19-, 80- and 4-fold increment compared with the control group in the hemocytes, hepatopancreas, and gill, respectively (Fig. 1C, D and 1E, left). Similar results were observed at protein levels in hemocytes and hepatopancreas. The MMP-1 protein levels in these two tissues were significantly upregulated from 12 h to 72 h post *V. parahemolyticus* infection (Fig. 1C and D, right). However, the protein level in gill increased gradually from 12 h to 48 h and dropped back to the initial level at 72 h (Fig. 1E, right). Taken together, these results showed that *V. parahemolyticus* infection induced MMP-1 expression in various tissues of abalone. Hence, MMP-1 might play a critical role in the immune defense against pathogenic bacterial infection in abalone.

3.2. Engagement of MMP-1 in the regulation of Vibrio infection in abalone

RNA interference (RNAi) mediates gene silencing in a majority of eukaryotes and has been widely used to explore gene functions [30]. By introducing gene specific siRNAs, the expression of target genes would drop to low levels. In order to elucidate the effects of MMP-1 on the bacteria infection in abalone, the MMP-1 expression level was knocked down by MMP-1-specific siRNA. The results showed that MMP-1 expression was significantly reduced at both transcript and protein levels after MMP-1-specific siRNAs injection when compared with that of the scrambled siRNA-injection group (Fig. 2A). In spite of no significance in MMP-1 expression between scrambled siRNA and bacteria suspension co-injection and bacteria alone groups, scrambled siRNA injection upregulated the expression level of MMP-1 to some extent, which might be attributed to the stress response of siRNA injection in abalone. Fig. 2B showed that the *Vibrio* copies increased gradually as the infection time prolonged. Besides, MMP-1 silencing induced significant augment of bacteria copies from 24 h to 72 h post-infection when compared with control groups (Fig. 2B). These results indicated that MMP-1 is engaged in the anti-*Vibrio* defense in abalone.



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3.3. Involvement of MMP-1 in the regulation of abalone cellular immunity

Molluscs are known to protect against environmental stress and pathogenic infections by innate immunity, consisting of cellular and humoral mechanisms [31]. Cellular immunity is mediated primarily by the hemocytes which are distributed in the hemolymph and tissues [32]. Flow cytometry has been applied for the analysis of mollusc hemocytes activities under normal and stressful conditions [33]. To characterize the role of MMP-1 in the regulation of phagocytosis, the phagocytic activity of abalone hemocytes was evaluated with FITC-

Fig. 1. Expression analysis of MMP-1 in different tissues of abalone and in response against pathogenic infection. (A) The qPCR detection of MMP-1 transcript levels in different tissues of abalone. Lane headings indicated the specific tissue. β-actin was used as the reference gene for internal control. Columns represented the mean of triplicate assays. (B) The detection of MMP-1 protein levels in different tissues of abalone by Western blot assay using the MMP-1-specific antibody, β-actin was used as the loading control. Values represented fold changes of MMP-1 proteins normalized against β-actin protein. (C, D, E) At different time points post *V. parahemolyticus* infection, the transcript (left) and protein (right) levels of MMP-1 in hemocytes (C), hepatopancreas (D) and gill (E) were detected by qPCR and Western blot analysis, respectively. Samples challenged with PBS were adopted as control group. β-actin was served as the reference gene for transcript level detection. In Western blot assay, β-actin was used as a loading control. Values represented fold changes of MMP-1 proteins normalized against β-actin protein. Lane headings indicated the time points post infection (hpi). In all panels, the significant differences are indicated by asterisks (**$p < 0.01$).

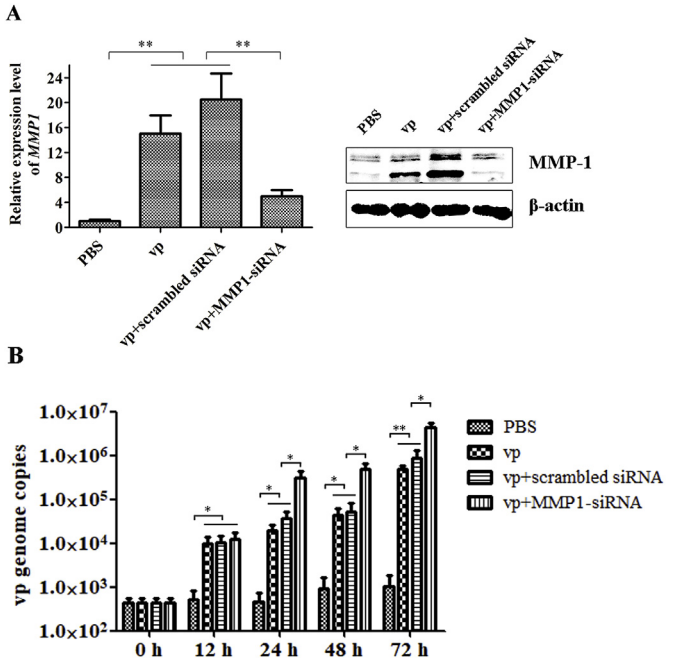

Fig. 2. Effects of MMP-1 on *V. parahemolyticus* infection in abalone. *V. parahemolyticus* suspension and MMP-1-specific siRNAs were co-injected into abalone. As controls, PBS, *V. parahemolyticus* alone and scrambled siRNA were included. (A) At 24 h post injection, hemocytes were collected and subjected to determination of mRNA (left) and protein (right) levels of MMP-1. β-actin was served as the reference gene for transcript level detection and a loading control for protein level detection. (B) The effects of MMP-1 on *Vibrio* infection in abalone. At different time points post *Vibrio* infection and MMP-1 knock-down, the hemolymph was collected and centrifuged. Total DNA was extracted and the genome copies of *Vibrio* were determined by qPCR. vp represented *V. parahemolyticus*. In all panels, the significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

labeled *V. parahemolyticus*. The results showed that bacterial infection led to a significant decrease in the phagocytic activity of abalone hemocytes when compared with uninfected group, while MMP-1 silencing further suppressed the activity (Fig. 3A). Annexin V can bind with phosphatidylserine transferred from the inner to the outer membrane of cells with high affinity. PI, which selectively enters the membrane of dead cells and stains the double-stranded DNA in nuclei, is an ideal fluorescent dye that can be used to mark the dead hemocytes. Hence, the effects of MMP-1 on apoptosis of abalone hemocytes were carried out by double staining of hemocytes with Annexin V-FITC and PI. Fig. 3B indicated that *V. parahemolyticus* infection induced an increment of apoptosis ratio, indicating that bacterial infection triggered host

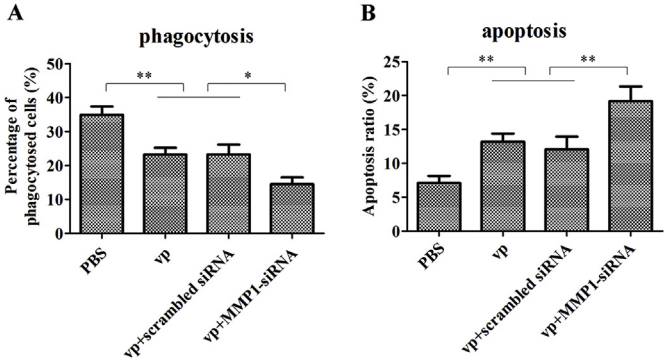

Fig. 3. The influence of MMP-1 on cellular immunity of abalone. *V. parahemolyticus* suspension and MMP-1-specific siRNAs were co-injected into abalone. As controls, PBS, *V. parahemolyticus* alone and scrambled siRNA were included. (A) The influence of MMP-1 on phagocytosis of abalone hemocytes against FITC-labeled *V. parahemolyticus*. The MMP-1 gene in abalone was silenced, followed by evaluation of phagocytosis against FITC-labeled bacteria at 24 h post infection. (B) The effects of MMP-1 on apoptosis of abalone hemocytes. At 24 h post injection, hemocytes were collected and subjected to flow cytometry by double staining with Annexin V-FITC and PI. Columns represented the mean of triplicate assays. vp represented *V. parahemolyticus*. In all panels, the significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

apoptosis, while knockdown of MMP-1 in *V. parahemolyticus*-infected abalone further increased the mortality of hemocytes to some extent. These data revealed that MMP-1 was involved in the regulation of abalone cellular immunity.

3.4. MMP-1 mediated the regulation of humoral immunity of abalone

In the humoral immunity, the antimicrobial complexes, including phenoloxidase, SOD, ACP and ALP, have been revealed to participate in the degradation of pathogenic foreign proteins, carbohydrates, and lipids [34]. Detection of phenoloxidase is useful in investigating immune responses in several invertebrate species including oysters and arthropods, but it is of limited use in abalones when dealing with bacterial infection [35]. In order to examine the effects of MMP-1 on the humoral immunity of abalone, the cell-free hemolymph was collected and the activities of SOD, ALP and ACP were determined. Fig. 4A showed that *V. parahemolyticus* infection decreased the SOD activity, while MMP-1 silencing further reduced the SOD activity. However, the activities of ALP and ACP were significantly upregulated post bacterial infection. When MMP-1 was knocked down by MMP-1-specific siRNAs, the enzyme activities decreased significantly (Fig. 4B and C). These results indicated that MMP-1 was involved in the regulation of humoral immunity of abalone.

3.5. MMP-1 was engaged in the anti-Vibrio immune response by regulating AIF-1, MPEG-1 and Tis11FP expression

To further investigate the action mechanism of MMP-1 in the innate immunity of abalone, the effects of MMP-1 on the expression of some well-known immune factors obtained from transcriptome data [36,37] were determined by qPCR. As one of the key factors associated with inflammatory response and immune defense, AIF-1 has been identified as a pro-inflammatory factor related to immune response in various molluscs, including abalone [38,39]. MPEG-1 possesses antibacterial activity as a means to protect abalone from pathogens [40]. Tis11FP is crucial for many aspects of immune regulation by targeting mRNAs for degradation and modulation of signaling pathways [41]. TLRs are evolutionarily conserved molecules that play an essential role in microbial pathogens defenses [42], while NF-κB is an important transcription factor that can be activated upon pathogen infection [43].

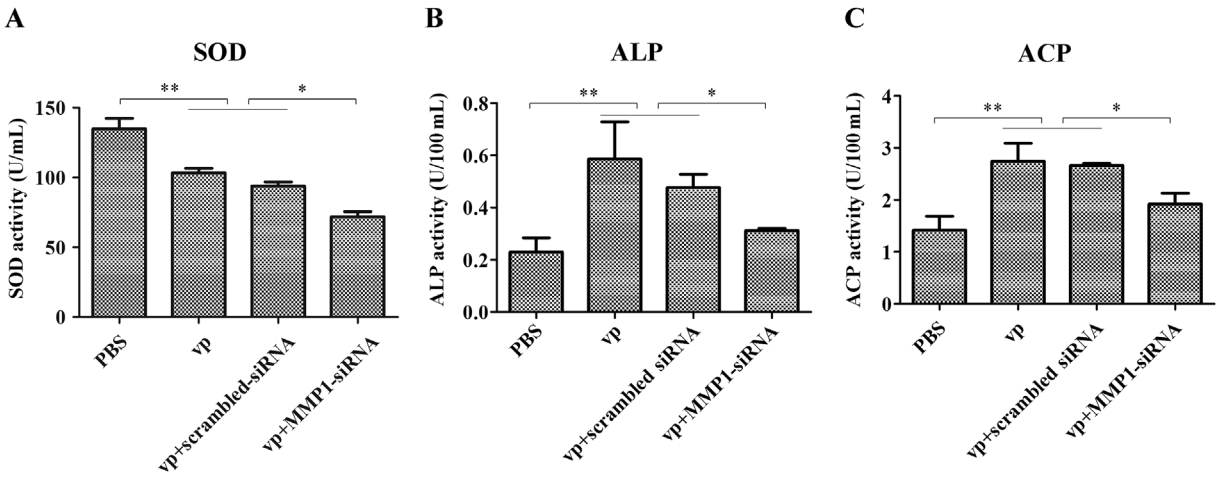

Fig. 4. The involvement of MMP-1 in the humoral immunity of abalone. *V. parahemolyticus* suspension and MMP-1-specific siRNAs were co-injected into abalone. As controls, PBS, *V. parahemolyticus* alone and scrambled siRNA were included. At 24 h post injection, hemolymph was extracted and centrifuged at 500 × *g* for 20 min. Afterwards, the cell-free hemolymph was collected and subjected to determination of SOD (A), ALP (B) and ACP (C) activity. Columns represented the mean of triplicate assays. vp represented *V. parahemolyticus*. In all panels, the significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

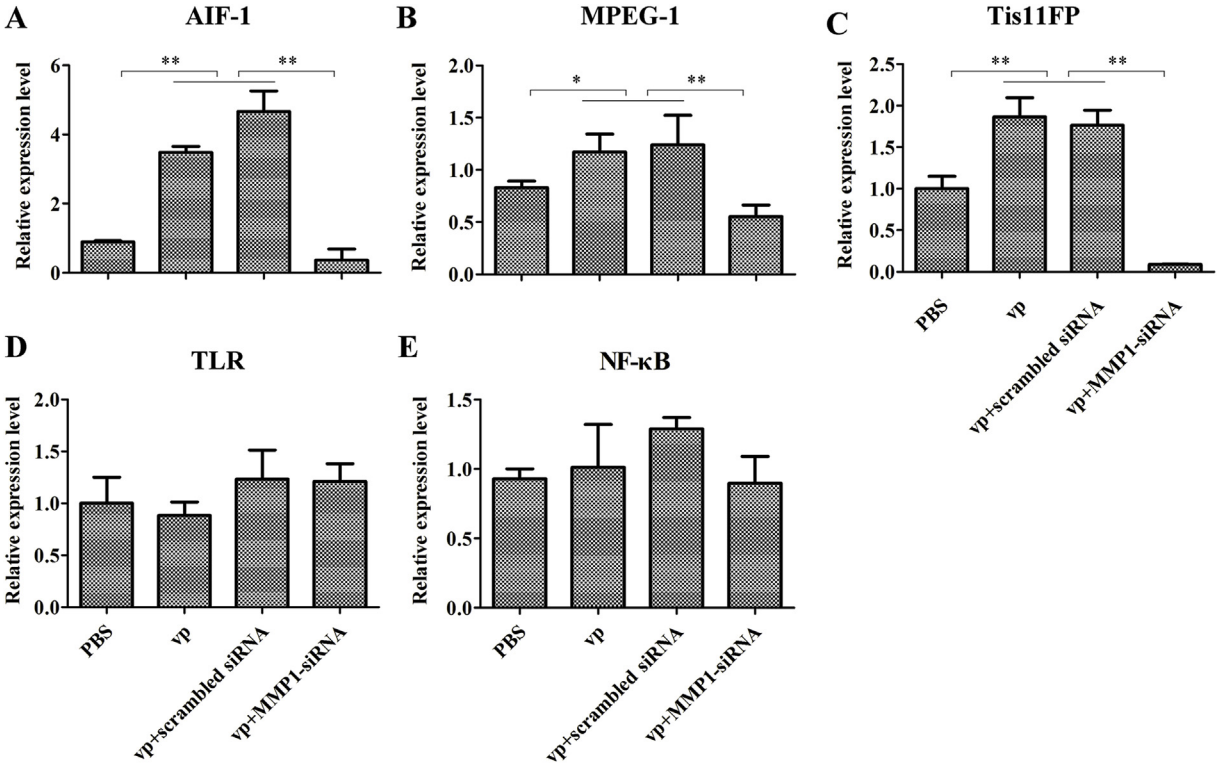

Fig. 5. Effects of MMP-1 on the expression of immune factors. *V. parahemolyticus* suspension and MMP-1-specific siRNAs were co-injected into abalone. As controls, PBS, *V. parahemolyticus* alone and scrambled siRNA were included. At 24 h post injection, hemocytes were collected and the total RNA was extracted. The transcript levels of AIF-1 (A), MPEG-1 (B), Tis11FP (C), TLR (D) and NF-κB (E) were determined by qPCR. β-actin was used as the reference gene for internal control. Columns represented the mean of triplicate assays. vp represented *V. parahemolyticus*. In all panels, plotted data points referred to the means ± standard deviations of triplicate assays and asterisks represented statistically significant differences (*$p < 0.05$, **$p < 0.01$).

Notably, bacterial infection induced the abundant expression of AIF-1, MPEG-1 and Tis11FP genes, while silencing of MMP-1 significantly decreased the expression of these genes (Fig. 5A, B and 5C). However, neither bacterial infection nor MMP-1 knock-down showed effects on the expression of TLR and NF-κB genes (Fig. 5D and E). These results revealed that MMP-1 was engaged in the innate immunity of abalone by regulating gene expression of specific immune factors.

3.6. MMP-1 was localized in the nuclei of abalone hemocytes

Based on the role of MMP-1 in the regulation of gene expression and innate immunity, immunofluorescence assay was conducted to detect the cellular localization of MMP-1. Fig. 6 showed that MMP-1 protein was barely expressed in uninfected hemocytes. As infection time prolonged, the expression levels of MMP-1 increased gradually (Fig. 6), which was consistent with the protein levels determined by Western blot analysis (Fig. 1C). Interestingly, the elevated MMP-1 protein level was accompanied with the continuous translocation of MMP-1 protein

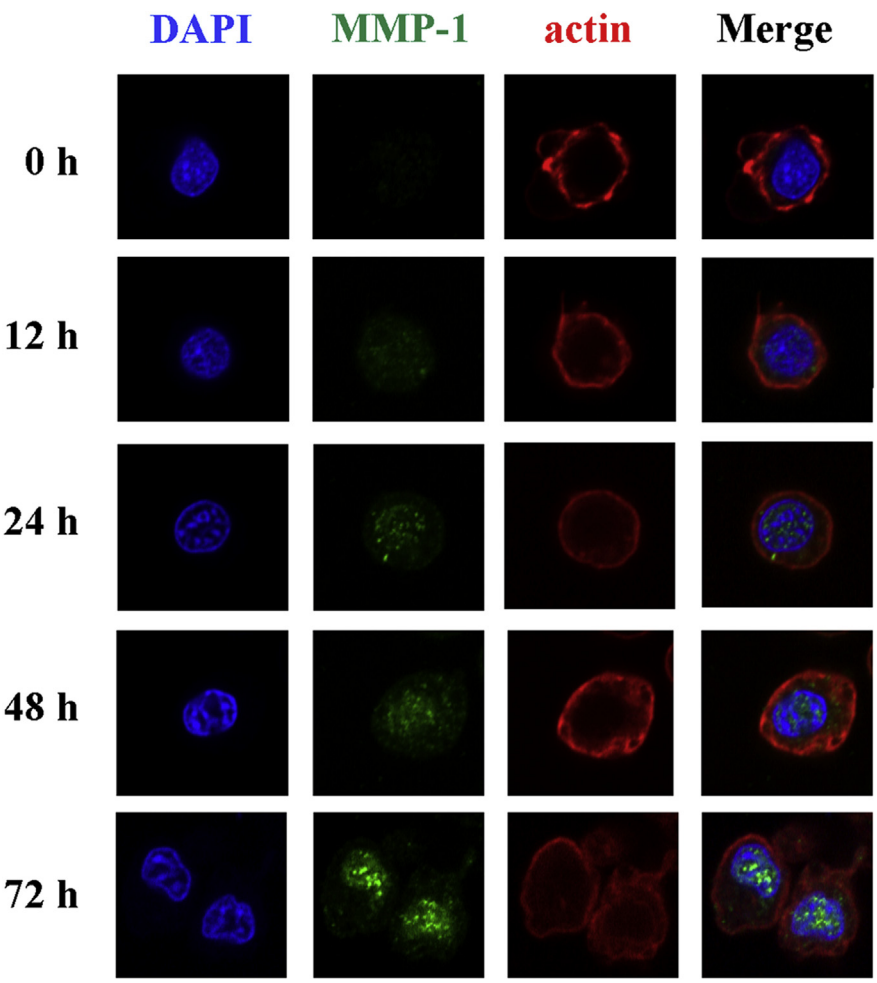

Fig. 6. Cellular localization of MMP-1 in abalone hemocytes. The abalone were injected with *V. parahemolyticus* suspension. At different time points post bacterial infection, the abalone hemocytes were collected, applied onto glass slide and stained with anti-MMP-1 IgG and tetramethylrhodamine-conjugated phalloidin. The distribution of MMP-1 and actin was evaluated under a confocal microscope. The numbers in hour indicated the time post infection.

to the nuclei (Fig. 6). From the above, MMP-1 might function as a transcriptional regulator by direct regulation of downstream gene expression or by hydrolyzing other proteins.

4. Discussion

MMPs are a family of zinc metalloendopeptidases that mainly function in the turnover of the extracellular matrix (ECM) components. They are secreted as inactive proenzymes, which can be activated by proteinases, chaotropic agents, thiol-modifying reagents and heat, resulting in the dissociation of the Cys-Zn interaction or the removal of the propeptide domain [44]. Recent studies have indicated that some MMPs are engaged in the immune response against pathogens infection in mammals, including MMP-12 [18], MT1-MMP [19], MMP-25 [20] and MMP-9 [21]. Moreover, MMPs are also widely present in invertebrates by participating in various tissue turnover processes. In Drosophila, MMPs are involved in tracheal growth, histolysis, axon guidance, tissue invasion, dendritic remodeling and tumor invasion. A Hydra MMP is necessary for the regeneration and maintenance of cell identity, and a sea urchin MMP could degrade matrix for hatching. An MMP is required for anchor cell invasion in nematode [22]. By construction of forward suppression subtractive hybridization (SSH) cDNA library, Wang et al. [36] reports that three MMPs, which are similar to MMP-1 and MMP-14, are significantly upregulated in hemocytes of bacteria-challenged variously colored abalone (*Haliotis diversicolor* Reeve, 1846). The innate immune response generated against *V. anguillarum* results in an upregulated expression pattern of MMP-1 in red abalone (*Haliotis rufescens*) [45]. In this study, we found that MMP-1 was constitutively expressed in all tissues of Pacific abalone, indicating that broad distribution of MMP-1 might be related to its involvement in diverse physiological processes of abalone. While hemocytes presented the highest transcript level of MMP-1, the highest level of MMP-1 protein was found in the gonad. In addition to the temporal and spatial variations of mRNAs in different tissues, the local availability of resources for protein biosynthesis also influences the relationship between protein levels and their coding transcripts [46]. As known, the gonad is a dynamic organ and the extracellular matrix suffers remodeling events during gametogenesis [47], which will further explain the high MMP-1 protein level in gonad. Our study also showed that the proenzyme and mature forms of MMP-1 were both present in different tissues of abalone, indicating that MMP-1 played an active role in intrinsic cell functions.

In molluscs, the hemolymph circulates systemically and could target pathogenic agents, activate the host's defense pathways and release defense proteins [48]. Gills are an ideal compartment for adhesion and entrance of pathogens, as they are covered with mucus, which could chemotactically attract or serve as a nutrient for pathogens [10]. As an important immune organ, the hepatopancreas of molluscs is engaged in the defense of pathogens by secreting various enzymes to hydrolyze microorganisms [26]. *V. parahemolyticus* infection of abalone induces the transcriptional upregulation of various genes, including defensin [49], cathepsin L [50], fasciclin 1-like protein [51], and cSP [26].

However, their protein levels are barely determined. In the present study, *Vibrio* copies increased gradually as the infection time prolonged, accompanied by upregulated transcript level of MMP-1 in disparate tissues, with the peak values at 24 h. Notably, the MMP-1 protein levels in hemocytes and hepatopancreas increased gradually post *V. parahemolyticus* stimulation, while it was upregulated significantly from 12 h to 48 h and reduced to the level of the control group at 72 h in the gill. It is reported that the protein contents in cells and tissues are influenced by translation rates, modulation of translation rate and proteins' half-life, and protein synthesis delay [52].

Cellular immunity is mediated primarily by the hemocytes distributed in the hemolymph and tissues, which are involved in cell migration, phagocytosis as well as the respiratory burst to the antimicrobial components [32]. Administration of beta-1,3–1,6-glucan induces an increment in the phagocytic activity of Taiwan abalone hemocytes [31]. Abalones subjected to low temperature stress suffers a decrease in the phagocytic capacity of hemocytes [53]. In this study, we found that *V. parahemolyticus* stimulation lead to a significant reduction of phagocytic rate of abalone hemocytes. Previous study has revealed that *Vibrio* infection inhibits phagocytosis ability by a mechanism that *Vibrio* is involved in the MAPK signal transduction pathway in abalone hemocytes [54]. Besides, the phagocytosis index of hemocytes is significantly reduced by exposure to *V. harveyi* [55], which is attributed to the decreased viability of hemocytes post pathogen infection. The activation of apoptosis in pathogen-infected cells is an essential host defense mechanism against invading pathogens [56]. As an active process of cell death, cell apoptosis has been reported as the cause of hemocytopenia [35]. The results of our study showed that hemocyte apoptosis was induced by bacterial infection, which was further enhanced by loss of MMP-1 gene expression. Evidence also indicates that intracellular MMP-1 in nuclei conferred resistance to apoptosis in human glia cells [57], indicating that nuclear MMP-1 is engaged in the regulation of cell apoptosis, which is similar with our findings in abalone hemocytes. Hence, the decreased viability of hemocytes caused by MMP-1 knock-down further resulted in reduced phagocytic activity, leading to increased *Vibrio* copies in abalone hemolymph.

Large amounts of reactive oxygen species (ROS) are generated to kill the internalized bacteria during phagocytosis, but damages will occur when the generation of ROS is excessive. As the first and most important line of defense against ROS, SOD can protect tissues from further oxidative damage [58]. In our study, SOD activity in hemolymph was significantly decreased when abalones were infected with *V. parahemolyticus*, which was consistent with the reduced phagocytic activity. Wang et al. [59] also reports a similar result whereby abalone injected with *V. parahaemolyticus* shows decreased SOD activity. Moreover, with decreased phagocytic ability caused by MMP-1 knockdown, the SOD activity was further inhibited to some extent, indicating that SOD activity was correlated with phagocytic ability. Our study verified that bacterial infection increased the activities of hydrolytic enzymes—ACP and ALP, but MMP-1 silencing impaired the upregulated hydrolytic activities. ACP, a typical lysosomal enzyme that can kill and digest foreign substances, has been used as a marker of macrophage activation in mammal [60]. The ALP is an important component of lysosomal enzymes that originate from hemocytes to destroy extracellular pathogens [61]. Chen et al. [62] report that both ACP and ALP are involved in immune defense mechanisms in abalone. As phagocytosis of pathogen was coupled with activation of lysosomal hydrolytic activity, MMP-1 silencing suppressed the phagocytic ability of hemocytes, in turn alleviating the augmented activities of ACP and ALP.

Previous work has revealed that the role of MMPs goes far beyond that of digesting extracellular matrix molecules alone, and these enzymes have unknown substrates, thus participating in host defense system. For example, MMP-12 is internalized by virus-infected cells, translocated to the nuclei and functions as a transcription factor, thus controlling host responses to viral infection [18]. Further research demonstrates that the activity of nuclear MMP-2 is increased as a result of ischemia-reperfusion (I/R) injury in rat heart tissue [63]. The present study indicated that MMP-1 was localized to the nuclei of abalone hemocytes, and the intracellular level of this enzyme increased gradually as the infection time prolonged. A probable cause is that the secreted MMP-1 could be taken up by the cells and localized to the nuclei [18]. It has been reported that nuclear MMP-1 expression could be detected in the stromal cells of breast cancer [64], and overexpression of nuclear MMPs is associated with aggressive cancer progression and poor survival rate [65]. Hence, the upregulated expression of MMP-1 in the nuclei of abalone hemocytes suggests that the abalone was not healthy. Furthermore, the findings of this study revealed that silencing of MMP-1 alleviated the augmented transcript levels of AIF-1, MPEG-1 and Tis11FP induced by bacterial infection. However, the mRNA levels of TLR and NF-κB were not affected by MMP-1, suggesting that these immune-related factors do not respond to MMP-1 at the transcriptional level. In this context, the nuclear localized MMP-1 might function as a transcriptional regulator or by selectively activating or inactivating other components through proteolysis, thereby regulating the expression of some downstream genes. This issue merits further investigation.

In conclusion, MMP-1 played an important role in the innate immunity of abalone by altering *Vibrio* infection and hemocytes activities. Nucleus-translocated MMP-1 might function as a transcriptional regulator or by selectively activating or inactivating other components through proteolysis, thus regulating downstream gene expression. Hence, our study established an important role of MMP-1 in abalone innate immunity against *V. parahemolyticus* infection and it represented the first report on the investigation of MMP in abalone.

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