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Tissue inhibitor of metalloproteinase gene from pearl oyster *Pinctada martensii* participates in nacre formation

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ABSTRACT

Tissue inhibitors of metalloproteinases (TIMPs) are nature inhibitors of matrix metalloproteinases and play a vital role in the regulation of extracellular matrix turnover, tissue remodeling and bone formation. In this study, the molecular characterization of TIMP and its potential function in nacre formation was described in pearl oyster *Pinctada martensii*. The cDNA of TIMP gene in *P. martensii* (Pm-TIMP) was 901 bp long, containing a 5′ untranslated region (UTR) of 51 bp, a 3′ UTR of 169 bp, and an open reading fragment (ORF) of 681 bp encoding 226 amino acids with an estimated molecular mass of 23.37 kDa and a theoretical isoelectric point of 5.42; The predicted amino acid sequence had a signal peptide, 13 cysteine residues, a N-terminal domain and a C-terminal domain, similar to that from other species. Amino acid multiple alignment showed Pm-TIMP had the highest (41%) identity to that from *Crassostrea gigas*. Tissue expression analysis indicated Pm-TIMP was highly expressed in nacre formation related-tissues, including mantle and pearl sac. After decreasing Pm-TIMP gene expression by RNA interference (RNAi) technology in the mantle pallium, the inner nacreous layer of the shells showed a disordered growth. These results indicated that the obtained Pm-TIMP in this study participated in nacre formation.

1. Introduction

Tissue inhibitors of metalloproteinases (TIMPs), a large family of endogenous specific inhibitors, can regulate matrix metalloproteinases (MMPs), zinc-dependent proteolytic enzymes [1,2]. A balance between TIMPs and MMPs is necessary for keeping the ECM (extracellular matrix) homeostasis, and the destruction of the balance may result in a number of pathological events, such as arthritis, tumor growth and metastasis [3]. Thus TIMPs are thought as important regulators of ECM turnover, tissue and bone remodeling [4,5]. In addition, TIMPs also have other biological activities that are independent of metalloproteinases, such as promoting cell growth and differentiation and osteoclast bone resorption [4,6,7].

In invertebrate, TIMPs have been found in *Drosophila melanogaster*, *nematodes*, *hydra*, *Crassostrea gigas* and *Tegillarca granosa*, and so on. Disruption of the TIMP gene in *Drosophila* induced inflated wings, bloated guts, tissue autolysis and early death [8]. Moreover, it was reported that TIMP in *Drosophila* was an endogenous inhibitor of MMPs and ADAMs in vivo [9]. These results revealed that TIMP in *Drosophila* involved in ECM turnover and cell–matrix adhesion or cell signaling pathways. To date, TIMP gene in mollusks has been found and characterized in *C. gigas* [10] and *T. granosa* [11], which was closely related to wound healing and defense mechanisms.

The nacre, also named "mother of pearl", is the inner nacreous layer of the shell and is a classical product of biomineralization as bone. Among the nacre components, matrix proteins are crucial in controlling crystal nucleation, crystal orientation and mineral polymorph selection. Inhibiting the expression of matrix proteins, such as dermatopontin [12], could disrupt the crystal polymorphisms and led to a disordered growth of the nacre. It has reported that TIMPs in the vertebrate play an important role in the regulation of ECM turnover, bone remodeling and resorption. So, we speculated TIMP in pearl oyster involved in nacre formation. As is well-known, pearl oyster *Pinctada martensii* is the main species cultured for marine pearl production. In our previous research of the transcriptome of pearl sac from *P. martensii* [13], we have got a partial sequence of TIMP gene. The aim of this study was to obtain the full length of TIMP and deliberated its exact functions in vivo by RNA interference (RNAi) technology.

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Table 1
Primer list used in this study.

Primer name	Primer sequence (From 5′ to 3′)	Application
TIMP-5′outer	GCGGCAGAATTTTTCTTGAGGGTGTGA	RACE
TIMP-5′inner	GCGCACGAGCTAGCAGACAGTTGTTT	RACE
TIMP-3′outer	TTACCAGACGGGACTTTGCTGACCACA	RACE
TIMP-3′inner	TTCTCATCAGAGGAGGGGGCTCAGGT	RACE
TIMP-F	ACTTGAGGAGGAGCTGACCTA	qRT-PCR
TIMP-R	GTAATGCAATGCCTGAAATGA	qRT-PCR
β-actin-F	GTGTAAGGCGGGGTTTGCT	qRT-PCR
β-actin-R	GGGTCCTTCAGCGTTAGTATCTT	qRT-PCR
dsRNA-TIMP-F	GCGTAATACGACTCACTATAGGGGG CTTCACTCCCTTGGTGTCAGA	RNAi
dsRNA-TIMP-R	GCGTAATACGACTCACTATAGGGTCG TTAGCCTCGTTGAATCGTCC	RNAi
dsRNA-RFP-F	GCGTAATACGACTCACTATAGGGGAG CTGGTTTAGTGAACCGTCAGA	RNAi
dsRNA-RFP-R	GCGTAATACGACTCACTATAGGGAA AACCTCTACAAATGTGGTATGGC	RNAi

2. Materials and methods

2.1. Animals, total RNA extraction and cDNA synthesis

Adult specimens of *P. martensii* (about 2 years of age, shell length ranging between 5 and 6 cm) were obtained from Liushagang, Zhanjiang, Guangdong Province, China. They were maintained at 25–27 °C in tanks with the recirculating seawater for one week before the experiment. Mantle pallium from pearl oysters were isolated and immediately stored in liquid nitrogen.

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The integrity of RNA was determined by electrophoresis on 1.2% agarose gel and staining with ethidium bromide. The quantity of RNA was determined by measuring OD260/OD280 with NanoDrop 2000 Spectrophotometer (Thermo). Altogether 1 μg total RNA was used as template for the RT-reaction with M-MLV reverse transcriptase (Promega, USA) and random primer.

2.2. Rapid amplification of cDNA ends (RACE)

Single strand cDNA for all RACE reactions were prepared from total RNA of mantle pallium. 5′ RACE and 3′ RACE were conducted using SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The gene specific primers were designed based on the TIMP cDNA fragment from the transcriptome of *P. martensii*. To increase the specificity and sensitivity of the amplification, nested-PCR was applied. The inner and outer PCR primers were listed in the Table 1.

2.3. DNA sequencing and sequence analysis

The PCR products after purification, including the 5′ and 3′ ends, were sub-cloned into pMD-18T vector (TAKARA) and sequenced. The full-length cDNA of TIMP gene was analyzed using the BLAST program available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The open reading fragment (ORF) was characterized using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The amino acid sequence of Pm-TIMP cDNA was calculated with DNAMAN. SignalP-4.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to analyze the signal peptide. The protein molecular weight and theoretical pI were analyzed using programe tool (http://web.expasy.org/cgi-bin/protparam/protparam). Multiple alignments were created using the Clustalx program.

2.4. Quantitative Real-Time PCR (qRT-PCR) analysis of Pm-TIMP gene expression

qRT-PCR assay was performed using Thermo Scientific DyNAmo Flash SYBR Green qPCR Kit (Thermo, USA) according to the manufacturer's protocol and was done with the Applied Biosystems 7500/7500 Fast real-time system (ABI, USA). In a 96-well plate, each sample was run in triplicate, along with the internal control gene. Sequences of the specific primers were shown in Table 1.

2.5. RNA interference (RNAi) experiment

RNAi was performed to test the Pm-TIMP effect on shell formation in vivo. Sequence specific primers (Table 1) were designed and used to amplify the specific sequences from the synthesized cDNA. The red fluorescent protein (RFP) template sequence, which is not existed in *P. martensii*, got from pDsRed2-N1 (Clontech) and dsRNA-RFP was generated as a control. Ten individuals were used in each treatment. The PCR products were purified using EasyPure Quick Gel Extraction Kit (TransGen). T7 RNA polymerase (Thermo) was used to synthesize the dsRNA. RNase free DNase I (Thermo) was used to digest the template DNA. The integrity and quantity of the dsRNA were verified as previously described. The dsRNA-Pm-TIMP was diluted to 80 μg 100 μL^{-1} with RNase-free water, 100 μL solutions were injected into the muscle of *P. martensii* for

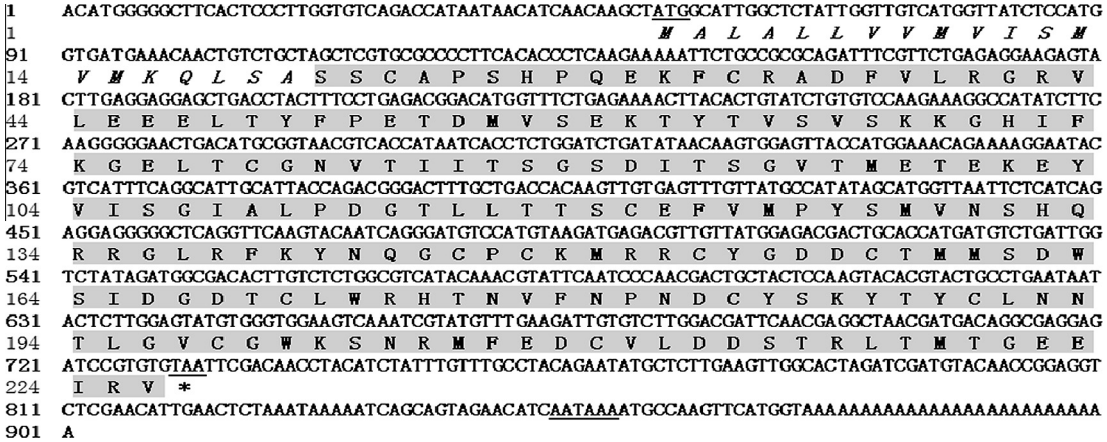

Fig. 1. Nucleotide and amino acid sequences of Pm-TIMP. The bold and normal numbers on the left indicate the positions of the Pm-TIMP cDNA sequence and the amino acid residues, respectively. The initiation codon (ATG), the stop codon (TAA), and the putative polyadenylation signal (AATAAA) are underlined, the putative signal peptide (1–20aa) is indicated in italic, the mature protein (21–226aa) is gray.

the first time, and the same doses (100 μL solutions) were injected five days after the first injection. For control groups, the same volume of RNase-free water and 80 μg of dsRNA-RFP in RNase-free water was separately injected each time.

Total RNA was extracted from mantle pallium eight days after the first injection and first-strand cDNA was synthesized. qRT-PCR was used to measure the expression levels of the Pm-TIMP gene and β-actin was used as an internal reference. The corresponding shells were collected, washed with Mili-Q water and air-dried, then were cut into pieces (1 cm × 1 cm), coated with gold and observed by an FEI Quanta 200 scanning electron microscope (SEM). The inner nacre structure of the shells was observed.

2.6. Statistical analysis

The data from the experiments were analyzed using one-way analysis of variance (ANOVA) in SPSS 19.0. A *P*-value less than 0.05 was considered as statistical significance.

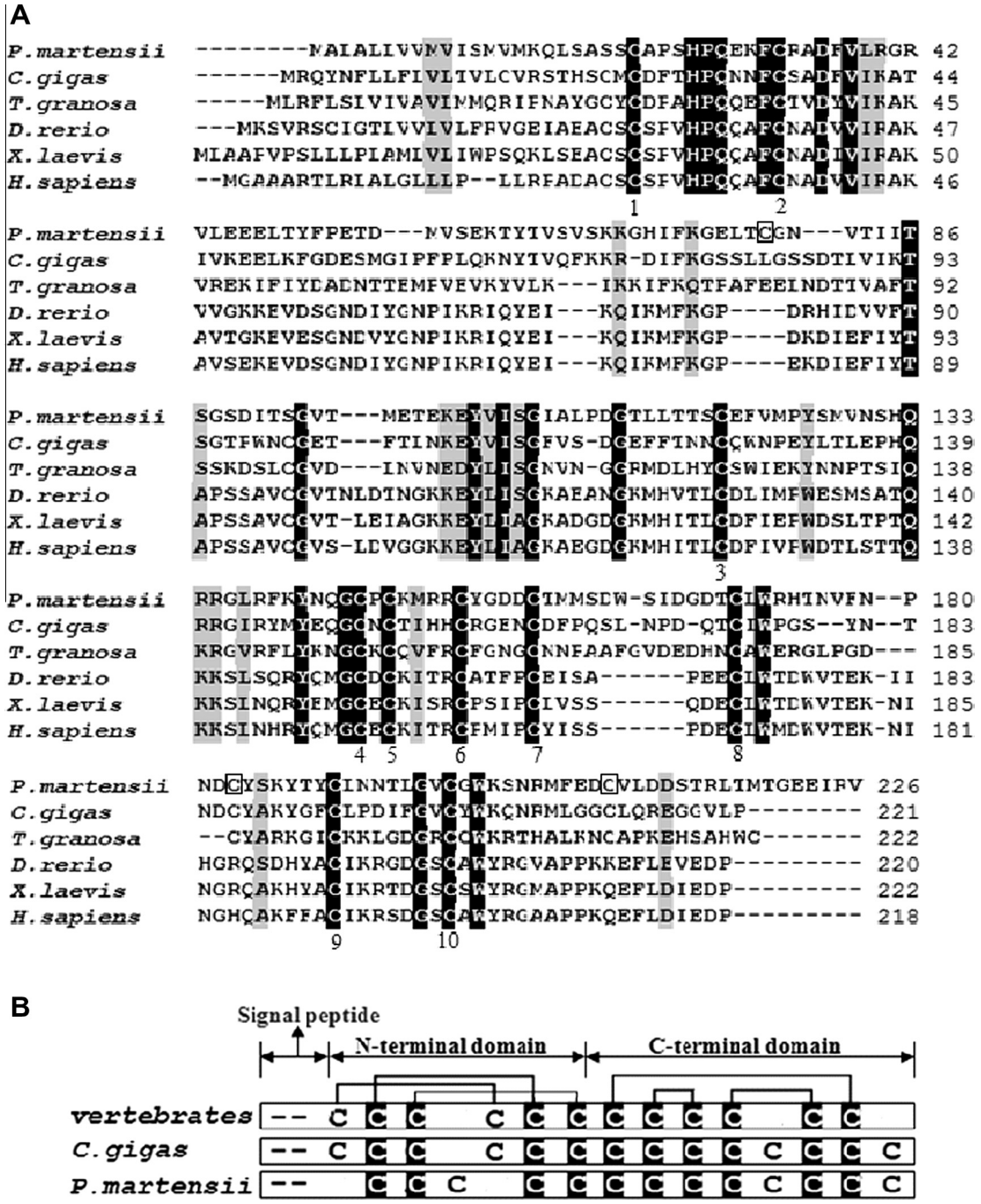

Fig. 2. Homology of the Pm-TIMP between vertebrates and invertebrates. (A) Multiple comparison of TIMP from different species (accession numbers: AGS32052.1, AAG42824.1, AFB81539.1, AAI15245.1, AAH81167.1, AAC50729.1). The conserved amino acids in all animal TIMPs are written in white on black background, and similar amino acids are shaded in gray; Numbers on the right refer to the total amino acid of each protein; The numbers at the lower part of alignment sequences indicate the shared cysteine residues between vertebrates and invertebrates; Additional cysteine residues in *P. martensii* are boxed. (B) A scheme depicting the structure of TIMP protein including a signal peptide, a N-terminal domain and a C-terminal domain. Vertebrate TIMP drawing derived from the analysis of the 21 amino acid sequences (accession numbers: ACN12788.1, CAP19941.1, NP_001015760.1, AAH81167.1, DAA18186.1, ACV04822.1, XP_003997297.1, AAF21942.1, XP_004432887.1, AAC50729.1, XP_001146829.2, AAA40446.1, EDM06753.1, XP_005069908.1, AAD28252.1, BAD99514.1, AFJ79969.1, ACK37365.1, AAI15245.1, AFM85816.1, BAE06264.1). Cysteine residues in black boxes are conserved in all TIMPs. Lines above the vertebrate TIMP drawing represented the disulfide bonding pattern.

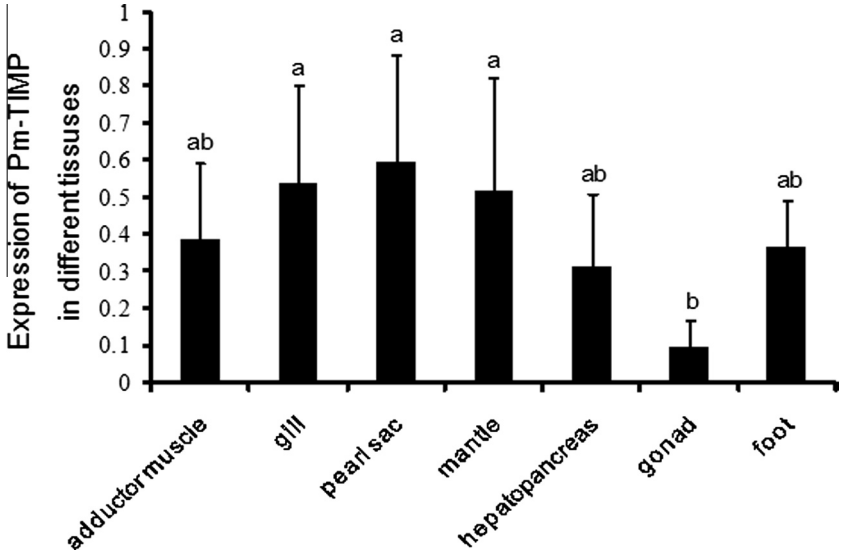

Fig. 3. Expression pattern of Pm-TIMP mRNA in different tissues by qRT-PCR. Each bar was a mean of five pearl oysters for different tissue (adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad and foot). The pearl oyster β-actin gene was used as the reference gene. Significant difference was indicated by different letters ($P < 0.05$).

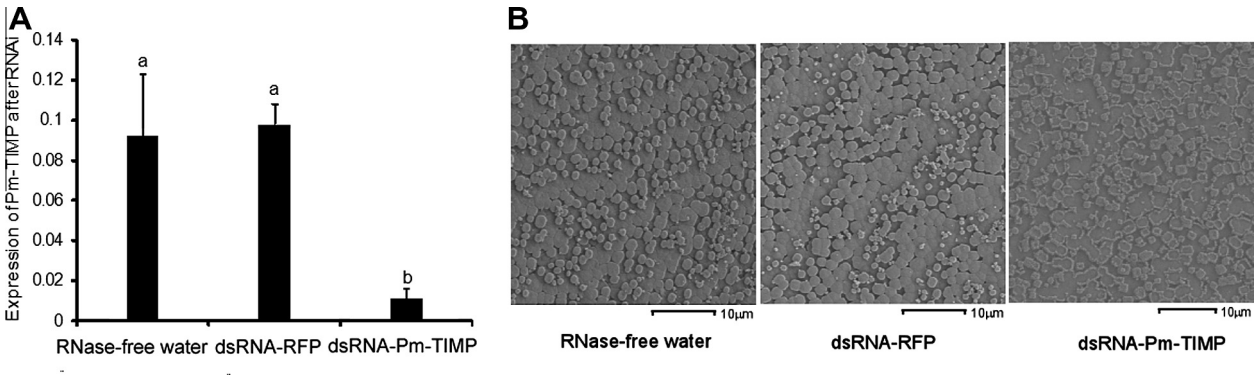

Fig. 4. Changes by dsRNA-Pm-TIMP interference. (A) Expression levels of Pm-TIMP mRNA by RNAi. qRT-PCR was done with RNA samples from controls (RNase-free water, dsRNA-RFP) and dsRNA-Pm-TIMP group eight days after injection, and five individuals were tested in each group. The pearl oyster β-actin gene was used as an internal control. Significant difference was indicated by different letters ($P < 0.05$). (B) SEM images of the surface of the nacreous layer of the oysters injected with RNase-free water, dsRNA-RFP and dsRNA-Pm-TIMP. Bars = 10 μm.

3. Results

3.1. Cloning and sequence analysis of the Pm-TIMP cDNA

Based on the TIMP cDNA fragment from *P. martensii* transcriptome database, gene-specific primers were designed to amplify the 5′ and 3′ nucleotide sequence using total RNA of mantle pallium as the template, then the PCR products were cloned and sequenced. The complete cDNA sequence of Pm-TIMP was 901 bp. It contained a 5′ untranslated region (UTR) of 51 bp, an open reading fragment (ORF) of 681 bp predicted to encode a 226 amino acid polypeptide, a 3′ UTR of 169 bp with 27 bp poly (A) tail and a typical polyadenylation signal (AATAAA). This cDNA sequence had been submitted to GenBank with the Accession No. AGS32052.1. The deduced molecular mass of the mature Pm-TIMP protein was 23.37 kDa and the theoretical isoelectric point was 5.42. The analysis of the deduced amino acid sequence by the SignalP 4.0 software revealed the presence of a 20-residue signal peptide with a predicted cleavage site located between residues 20 and 21, as shown in Fig. 1.

3.2. Homologous and structure analysis of Pm-TIMP

Search for homology was conducted with the deduced amino acid sequence using the BLASTx program. We found that Pm-TIMP shared the highest (41%) identity to the TIMP from *C. gigas*, 30% to *T. granosa*. In vertebrate, Pm-TIMP was closer to TIMP2, such as *Xenopus laevis* (33%), *Sparus aurata* (32%), *Homo sapiens* (31%), compared with the other three members in the TIMP family. Thus we used TIMP2 amino acid sequences of different species when conducted the homologous comparison and structure analysis.

Using Cluxtalx software to carry out the homologous comparison, 10 of 13 cysteine residues existing in the mature Pm-TIMP were found in the same position of vertebrate TIMPs, and the spacing between cysteine residues was well conserved among all TIMPs (Fig. 2A). According to the homology, we drew a structure scheme of Pm-TIMP, which included a signal peptide, a N-terminal domain and a C-terminal domain. As seen in Fig. 2B, in the Pm-TIMP, five disulfide bonds were conserved, whereas two additional cysteines in the C-terminal region may form an additional disulfide bond,

and another cystine in the N-terminal may not participate in the formation of intrachain disulfide bond.

3.3. Distribution and expression of Pm-TIMP gene in different tissues

To determine the tissue specific expression of Pm-TIMP mRNA, qRT-PCR analysis was employed using total RNA from adductor muscle, gill, pearl sac, mantle, hepatopancreas, gonad and foot with β-actin as an internal control. Pm-TIMP distributed widely in the detected seven tissues of *P. martensii* with highly expression in pearl sac, mantle and gill (Fig. 3). Pearl sac and mantle were nacre formation related-tissues.

3.4. Expression levels of Pm-TIMP and SEM observation after RNA interference (RNAi)

To further investigate the function of Pm-TIMP gene in nacre formation, we applied RNAi technology to inhibit the expression of the gene in vivo. Controls were RNase-free water and dsRNA-RFP injected-groups. Eight days after injection, we employed qRT-PCR to measure the mRNA levels of the Pm-TIMP in the mantle pallium. The expression of Pm-TIMP gene in the dsRNA-Pm-TIMP injected group was suppressed to approximately 12% compared with that from control groups (Fig. 4A).

We observed the microstructure of nacre from each group eight days after injection by SEM. The nacre in the RNase-free water and dsRNA-RFP injected-groups had the same normal orderly type of microstructure. Small hexagonal flat tablets of aragonite were packed together to produce a stair-like growth pattern. Whereas a disordered growth of the nacreous layer was observed in the dsRNA-Pm-TIMP injected-group. The change of tablet shape resulted in the disappearance of the stair (Fig. 4B).

4. Discussion

Both of nacre and bone formation are typical biomineralization processes. Research has indicated the complex machineries directing nacre or bone formation may be homologous [14]. Some proteins regulating bone formation have been verified to have functions in nacre formation, such as BMP-2 [15]. TIMPs are natural MMPs inhibitors. A balance between TIMP and MMPs was crucial in regulating bone formation. We inferred TIMP might involve in nacre formation, while need experimental elucidation. In this report, we cloned and identified Pm-TIMP from *P. martensii*.

In vertebrate, TIMP mature protein is produced by cleaving off the signal peptide and the cleavage site upstream of signal peptide is one conserved motif Cys-X-Cys (X represents amino acids). The mature peptides of TIMPs contain 12 highly conserved cysteine residues which can form six intrachain disulfide bonds that divide the protein into two unique domains, the N-terminal domain and the C-terminal domain. Each domain is stabilized by three disulfide bonds [16]. The N-terminal domain is capable of forming a stable native structure which has inhibitory activity against metalloproteinases [17], whereas the C-terminal domain confers the specific biochemical properties of the molecule, such as localizing the ECM [18]. Compared to vertebrate TIMPs, Pm-TIMP contained 13 cysteines among which 10 cysteines existed in the same location with that from vertebrate, formed intrachain disulfide bonds and folded the protein into two domains, C-terminal domain and N-terminal domain. The C-terminal domain of Pm-TIMP contained an additional pair of cysteine residues, which was the same as *C. gigas* [10]. Whereas, the N-terminal domain of Pm-TIMP missed a couple of disulfide bond and the cleavage site upstream of the signal peptide was Ser-X-Cys, both of which were different from the other species, as Keith Brew [4] reported TIMPs in invertebrates were more variable in sequence than those from vertebrates, and TIMP evolution did not appear to have been a linear process in invertebrate.

Tissue expression pattern of Pm-TIMP showed it distributed widely in all detected tissues of *P. martensii* and highly expressed in pearl sac and mantle, which were the main nacre formation related-tissues. It has been reported that TIMPs in the vertebrate played an important role in the regulation of bone modeling and remodeling in human bone [5], especially in osteoclastic bone resorption [6]. To further elucidate the role of the Pm-TIMP gene in nacre formation, RNAi technology was used to inhibit the expression of Pm-TIMP. The expression of Pm-TIMP gene was decreased by approximately 88%, compared with RNase-free water or dsRNA-RFP injected-group. Together with the SEM images of the nacre, we found a disordered growth of the nacre in dsRNA-Pm-TIMP injected groups. Therefore, these results suggested that the Pm-TIMP played a potential role in nacre formation.

TIMPs are natural MMPs inhibitors and inhibit the MMPs proteolytic activity by forming noncovalent stoechiometric complexes (1:1) that are resistant to proteolytic degradation [19]. Matrix metalloproteinases (MMPs) have the capacity to degrade most components of extracellular matrix. MMPs have found in the mollusks, such as *Crassostrea virginica* [20], *Haliotis diversicolor* [21], *Thais clavigera* [22] and so on. In nacre formation, matrix proteins play a key role in the nucleation, growth, shape and orientation of calcium carbonate crystal [23,24]. Consequently, we inferred the function of Pm-TIMP on nacre formation was by regulating the matrix proteins in nacre through inhibiting MMP activity.

As illustrated in the introduction, the function of TIMPs in bone resorption activity was independent of the inhibitory activity toward MMPs [6,25,26]. Some TIMP-2 binding proteins on the plasma membrane of osteoclasts were detected by a cross-linking experiment. Thus we speculated Pm-TIMP might also act directly on organic matrix proteins secreted from the mantle pallium, which mediated molluscan nacre formation.

In summary, we have got the full length of Pm-TIMP cDNA, analyzed the characteristic of its ORF and peptide sequence, and detected the expression pattern of different tissues in *P. martensii*. We further verified the function of Pm-TIMP by RNAi technology. The results indicated that the obtained Pm-TIMP in this study involved in nacre formation. To better understand how Pm-TIMP regulates these processes, further studies on the signaling pathways are required.

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