Functional characteristic and differential expression of myostatin in Chlamys nobilis

Kecheng Zhu, Huayang Guo, Nan Zhang, Yundong Li, Shigui Jiang and Dianchang Zhang

Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, People’s Republic of China.

ABSTRACT
Myostatin (MSTN) was a conserved negative regulatory protein of muscle growth and development in numerous species. In this study, the MSTN gene was cloned and characterized from a noble scallop, Chlamys nobilis, and it was named CN-MSTN. To investigate the molecular characterization of MSTN and its gene expression profile of spatio-temporal, we isolated the MSTN cDNA sequence in C. nobilis and analysed expression patterns using quantitative real-time PCR. CN-MSTN cDNA contained a 1,374 bp open reading frame that encoded a 458 amino acids. Furthermore, the result of 3D model indicated that CN-MSTN mature peptide was similar to that of Chlamys farreri. Additionally, based on both the nucleotide and amino acid datasets, phylogenetic trees indicated that C. nobilis has the highest similarity with homologues of C. farreri. The expression patterns of different stages showed that MSTN expression was markedly higher in the gastrulae period. Additionally, remarkable expression of MSTN was observed in adductor muscle than with other tissues. Our data would provide expression and phylogenetic information of this economical important sea food, noble scallop.

1. Introduction
Myostatin (MSTN), which negatively regulated muscle development, was a member of the transforming growth factor beta (TGF-β) superfamily in a variety of species (McPherron et al. 1997; Thomas et al. 2000). Moreover, increasing skeletal muscle quality in mice, cattle and humans was associated with the lack of its functions, especially to the ‘double muscle’ feature observed in breeding cattle.

Due to its important function, so far, MSTN gene has been well characterized in a wide variety of vertebrates including birds, mammals and aquatic species (Rodgers and Garikipati 2008). Among aquatic species, Sparus aurata (Maccatrozzo et al. 2001), Salmo salar (Østbye et al., 2001), Oncorhynchus mykiss (Rescan et al. 2001), Argospecten iradians (Guo et al. 2012), Argospecten purpuratus (Morales-Collio et al. 2014), Chlamys farreri (Hu et al. 2010) have also been cloned and identified. Furthermore, low mRNA levels of MSTN were found in the gland (Ji et al. 1998). Nevertheless, there was differential expression of MSTN in non-mammalian species. Two MSTN hypotypes are expressed in skeletal muscles of Danio rerio (Biga et al. 2005; Kerr et al. 2005) and O. mykiss (Garikipati et al. 2007) with identical amino acid sequences. In particular, MSTN was also detected in the intestine and liver of Lctalurus punctatus (Kocabas et al., 2002), yet not in the same tissues of Umbrina cirrosa and chicken (Maccatrozzo et al. 2001; Bhattacharya et al. 2016).

The scallop Chlamys nobilis (Pectinidae, Pterioida) is a vital and commercial marine mollusk mostly cultured in the southern sea of China (Zheng et al. 2010). In the present study, the cDNA sequences of MSTN gene were cloned and characterized. Moreover, C. nobilis MSTN of spatio-temporal expression and 3D model was structured. Additionally, the phylogenetic analysis based on nucleotide and amino acid datasets of 16 species was established. This research could provide useful MSTN evolution and function.

2. Materials and methods
2.1. Samples collection
Healthy adult C. nobilis (body weight 26.11 ± 2.52 g; shell length 5.30 ± 0.13 cm; shell width 5.01 ± 0.19 cm) used in this study were collected from Lingshi in Hainan Province, China. Those scallops were retained (50 shell/tank) in 300 L tanks with circulating seawater (temperature 21 ± 0.5°C) for two weeks before experiment.

To research the distribution of CN-MSTN mRNA expression, eight tissues (including the mantle, pleopod, digestive gland, heart, gill, adductor muscle, female gonad and male gonad) were collected from five scallops. Furthermore, embryos and larvae (at least 20 each stage) at different developmental stages were collected for analysis of temporal expression. All samples were stored at 80°C after...
addition of 1 ml Trizol reagent (Invitrogen) for subsequent RNA extraction.

2.2. Total RNA isolation and reverse transcription

We used a Trizol kit (Promega, Madison, WI, USA) to extract total RNA from various tissues. Moreover, RNA quantity and quality (concentration) were determined with NanoDrop 2000 spectrophotometer (ThermoScientific, Waltham, USA), and to confirm integrity, it was visualized in 1.2% sepharose gel with 1× TBE buffer (Tris-Sodium acetate-EDTA). To synthesize cDNA, A PrimeScript™ RT reagent Kit was accompanied by gDNA Eraser (TaKaRa, Japan). Total RNA (2 µg) and 0.5 µg of Oligod (T)16 was reacted for 5 min at 70°C. After incubation for 2 min on ice, the mixture was reversely transcribed depending on the manufacturer’s instructions and stored at −20°C until further use.

2.3. Molecular cloning and sequencing

Based on the conserved sequences of *Nodipecten subnodosus* and *C. farreri* MSTN (KF471120.1, EU563852.2), we designed gene-specific primers (MSTN-R1: AAGAGGAGTATGGGCAAC; MSTN-L1: CTCGCACAACCACATC; MSTN-R2: CTAACAC- CACGGCTAAAC; MSTN-L2: CGCAGACACTTCCACCTTC) and used existing primers from Hu et al. (2010) to amplify the open reading frame (ORF) of CN-MSTN. The PCR products were tested by a 1.5% agarose gel and notarized by sequencing using Sequencing Analysis 5.2.

2.4. Bioinformatic analysis

BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) is used to calculate amino acid and nucleotide sequence similarity searches. The coding sequence of MSTN was forecasted by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Moreover, to obtain theoretical isoelectric point, molecular weight and features of the predicted proteins and signal peptide, ExPASy analysis (http://us.expasy.org/tools) was utilized and SignalP 4.1 Server was used for prediction (http://www.cbs. dtu.dk/services/). A homology model of MSTN from *C. nobilis* was constructed by the SWISS-MODEL Protein Modelling Server (Kopp and Schwede 2004; Arnold et al. 2006). Multiple sequence alignments and phylogenetic trees of the deduced nucleotide and amino acid sequences were performed using MEGA 6.0 program with Maximum parsimony (MP), Neighbor-Joining (NJ) and Bayesian inference (BI) methods (Lartillot et al. 2009; Tamura et al. 2013).

2.5. Gene expression analysis

Quantitative real-time PCR (qRT-PCR) was performed to analyse the mRNA levels of MSTN (Bustin et al. 2009). Specific primer pairs for MSTN (F: GACAGCACAAGCGATTACC; R: TTTCCGATGT- CATTCCA-GC) and reference gene *b-actin*-F/R were obtained (Lu et al. 2016). The system of qRT-PCR was referred to Zhu et al. (2016). Furthermore, the relative expression was determined using the 2^ΔΔCT method (Livak and Schmittgen 2001).

2.6. Statistical analysis

MSTN transcripts were expressed as mean ± SE. The results were performed by one-way Analysis of Variance (ANOVA), and Duncan’s test was used to establish the difference between treatments. Differences were considered at a significant level of *p* < .05 and *p* < .01.

3. Results

3.1. Cloning and sequence analysis of CN-MSTN

Fragments of MSTN were amplified by PCR. After identification of the fragments, it revealed that CN-MSTN [GenBank accession no. KY197473] consisted of a 1423 bp fragment which contained a 1374 bp ORF encoding a polypeptide of 458 amino acids. The expected CN-MSTN protein had a molecular mass and an isoelectric point of 53 kDa and 5.35, respectively. Moreover, the grand average of hydropathicity of predicted CN-MSTN protein was −0.592. The result of SignalP indicated that a putative signal peptide of 19 amino acids which was estimated to be an extracellular targeting sequence was contained in the deduced amino acid sequence.

Like additional TGF-β superfamily members, a potential proteolytic processing site RXXR (amino acids 327–330) was in the CN-MSTN (Figure 1). Moreover, a propeptide and a mature peptide were produced by this site. Nine conserved cysteine residues were detected in the carboxy-terminus portion. Both the full amino acid sequences and carboxy-terminal showed higher homologies with those species in Table 1 and Figure 1.

3.2. 3D structure of CN-MSTN

A 3D model of CN-MSTN mature peptide was constructed by the SWISS-MODEL Protein Modelling Server. To compare three closely species, the model of *C. farreri* and *M. musculus* were also established. The result indicated that a 3D model of CN-MSTN mature peptide was resemblance among *C. farreri* and *M. musculus* MSTN which revealed the classics TGF-β family hand-shaped frame (Figure 2) (Lin et al. 2006; Guo et al. 2012). Moreover, it suggested that MSTN of both scallops had a longer α-helix region than that of *M. musculus* (Lin et al. 2006).

3.3. Phylogeny

To know the phylogenetic relationships among molluscs, a dataset of nucleotide and amino acid sequences of 16 marine mollusk MSTN was generated (Figure 3). Based on NJ, MP and BI analyses of the nucleic acid and amino acid sequences, respectively, the results showed consistency in the topologies’ structure, with a close relationship between *C. nobilis* and *C. farreri*.

3.4. Spatio-temporal expression analysis of MSTN

Spatio-temporal expression patterns indicated that MSTN expression in the gastrulae period was markedly higher than at other development stages (*p* < .01). After this period, the expression gradually decreased (Figure 4(A)). Additionally, the
highest MSTN expression was shown in adductor muscle compared with the other tissues, with the second-best expression noted in mantle (Figure 4(B)).

4. Discussion

In this study, we cloned and characterized the MSTN gene (which contained 1374 bp ORF encoding 458 amino acids) from C. nobilis. Moreover, the structure of CN-MSTN was similar to other scallop MSTN, which contained 3 exons and 2 introns (Kim et al. 2004; Guo et al. 2012). Therein, the first exon contained N-terminal signal for secretion, whereas exon 3 consisted of the conserved C-terminal mature peptide, common to the A. irradians MSTN proteins (Guo et al. 2012). Furthermore, nine conserved cysteine residues and a RSKR proteolytic processing site were in the C-terminal (Guo et al. 2012).

Nucleotide and protein alignments with other molluscs prominently showed that the C. nobilis MSTN shared the highest homology with C. farreri, consistent with the fact that both C. nobilis and C. farreri were members of the Pectinidae superfamily. Therefore, five adjacent species' MSTN were chosen for alignment; the result indicated that C-terminal region was highly conserved in MSTN (Hu et al. 2010; Morales-Collio et al. 2014).

Table 1. Comparison of deduced amino acid region of C. nobilis MSTN with other MSTN.

| Species                        | Full deduced amino acid identity (%) | GenBank No.  |
|--------------------------------|--------------------------------------|--------------|
| Chlamys nobilis                | 100                                  | KY197473     |
| Chlamys farreri                | 93                                   | EU563852.2   |
| Nodilpecten subnodosus         | 87                                   | KF471120.1   |
| Argopeuten iradians MSTN-like | 90                                   | AY533362.1   |
| Argopeuten iradians MSTN      | 87                                   | JN684903.1   |
| Argopecten purpuratus          | 83                                   | KC771285.1   |
| Mytilus chilensis              | 83                                   | KF040495.1   |
| Pinctada fascata               | 47                                   | KJ772159.1   |
| Tegillarca granosa             | 47                                   | KP250872.1   |
| Pinctada martensi              | 45                                   | KJ579132.1   |
| Sinonovacula constricta        | 37                                   | AHH32929.1   |
| Cairina moschata               | 34                                   | ACL79915.1   |
| Hama sapiens                   | 33                                   | AB848419.1   |
| Cyprinus carpio                | 31                                   | G0214769.1   |
| Oryzias latipes                | 31                                   | NM_001201499.1 |
| Mus musculus                   | 33                                   | NM_010834.3   |

Figure 1. Comparison of deduced amino acid sequences of C. nobilis of MSTN with published MSTN in other species. The black-boxed area is labelled to indicate the proteolytic processing site (RXXR). Moreover, nine conserved cysteine residues are shown with imaginary boxes. The accession numbers of the sequences used are listed in Table 1.
Comparison of the 3D structure of MSTN among the C. nobilis, C. farreri and M. musculus suggested that both scallops’ MSTN has a longer α-helix region than that of M. musculus (Guo et al. 2012). Therefore, CN-MSTN may contain more components for effecting on other DNA sequences or amino acids, which may not be an important function in M. musculus (Cash et al. 2009).

Tissue-specific expression profile indicated that MSTN mRNA levels were found to be highest in adductor muscle, which was in keeping with the result of C. farreri, A. purpuratus, A. irradians, N. subnodosus and mammalian MSTN (Hu et al. 2010; Guo et al. 2012; Morales-Collio et al. 2014; Morelos et al. 2015). It was suggested that a highly conservation of role in muscle development. Unlike C. farreri MSTN first
detected in 2–4 cells period (Hu et al. 2010), in *C. nobilis*, the **MSTN** expression was detected at the fertilized eggs period.

### 5. Conclusion

In conclusion, a close relationship between *C. nobilis* and *C. farreri* was supported by phylogenetic analysis based on both the nucleotide and amino acid sequences. Furthermore, the 3D model of CN-MSTN mature peptide was similar to that of *C. farreri*.

Additionally, spatio-temporal expression patterns indicated that **MSTN** expression was found to be remarkable in the gastrulae period and adductor muscle than in other stages and tissues, respectively.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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