

## Characterization of a Myostatin-like Gene from the Scallop *Patinopecten yessoensis*

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Myostatin (GDF8) is a growth factor that limits muscle tissue growth and development in vertebrates. We isolated a myostatin-like gene (Py-MSTN) from the marine invertebrate, the scallop *Patinopecten yessoensis*. Py-MSTN was highly expressed in the adductor muscle and in the gill unexpectedly. Amino acid analysis showed that Py-MSTN has 49% amino acid sequence identity and 64% similarity to human myostatin (Hs-MSTN), and 42% identity and 61% similarity to myoglianin, the only invertebrate homolog. These results indicated that Py-MSTN may be functionally similar to the vertebrate MSTN than the invertebrate homolog. Phylogenetic analysis suggested that Py-MSTN is an ancestral form of vertebrate MSTN and GDF11 and does not belong to other TGF- $\beta$  family members. Molecular modeling showed that Py-MSTN exhibits a similar tertiary structure to mammalian BMP7, a member of TGF- $\beta$  family. In addition, the amino acid residues which contact extracellular domain of the receptor were relatively conserved. Given these results, we propose that Py-MSTN is a functionally active member of the TGF- $\beta$  family and is involved in muscle growth and regulation.

Key words: *Patinopecten yessoensis*, Scallop, Myostatin, TGF- $\beta$ , Muscle

### Introduction

Myostatin is a member of the TGF- $\beta$  superfamily that are responsible for growth and development of tissue. Originally identified as growth and differentiation factor-8 (GDF-8), its mutation resulted in a double-sized muscle in mouse (McPherron et al. 1997) and in cattle (McPherron and Lee, 1997), which indicated its inhibitory roles in muscle growth and development. Given its potential commercial and medicinal applications, many myostatin genes have been isolated and characterized from a variety of vertebrates including human (Jespersen et al., 2006), pork (Stratil and Kopečný 1999), chicken (Yang et al., 2001b), and fish (Kocabas et al., 2002; Amali et al., 2004; Ko et al., 2006). Interestingly, most fishes appear to have two copies of the myostatin genes as a result of the gene duplication (Biga et al., 2005).

The inhibitory roles of myostatin in muscle development appear to be conserved throughout all vertebrates in that muscle mass increases by eliminating its function (Yang et al., 2001a; Nishi et al., 2002). It has been recently demonstrated that knocking-

down of myostatin-1 gene in *Danio rerio* enhances the expression of muscle-specific transcription factors such as MyoD and myogenin as well as IGF-II (Amali et al., 2004). More recently, a dsRNA injection in early development stage in zebrafish produced hyperplasia or hypertrophy caused by drastic decrease of myostatin mRNA (Acosta et al., 2005). Myostatin has been well studied in the vertebrates, however, few studies have examined invertebrate myostatin homologs. One of the most closely related gene identified thus far is myoglianin in *Drosophila melanogaster* (Lo and Frasch, 1999). However with 598 amino acid residues myoglianin is much larger than its vertebrate homologs.

The scallop *Patinopecten yessoensis* is a cold-tolerant species inhabiting the northern Pacific Ocean. The northeastern regions of the peninsula, mostly belonging to Kangwon province, are a key place for the scallop production in Korea. This species contributes significant value to the Korean seafood market, and given that the scallop requires 2-3 years before it is ready to harvest, the regulation of growth and development of skeletal muscle is of commercial as well as scientific importance. For example, by

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understanding the molecular mechanisms governing muscle development, it may be possible to manipulate and control muscle growth for commercial applications. In addition, this study would contribute to the understanding of the regulation of muscle development in invertebrates in general.

We isolated and characterized the invertebrate myostatin-like gene (Py-MSTN) from scallop *Patinopecten yessoensis*. Py-MSTN was highly expressed in skeletal muscle as in the other MSTNs. In addition, the sequence alignment and structural model analysis suggested that Py-MSTN is the ancestral form of vertebrate MSTNs and that it uses a similar signaling pathway by binding the same type of receptor family. Therefore, we suggest that Py-MSTN may be used to regulate muscle growth in scallop aquaculture.

## Materials and Methods

### Materials

Scallops were purchased from a local seafood market and were stored in a autoclaved and filtered seawater tank at 15°C until dissection. The *E. coli* strain XL-1 Blue was used for cloning and sequencing.

### Cloning Py-MSTN gene

Total RNA was extracted from the adductor muscle tissue of the scallops using an RNeasy Protect Mini Kit (Qiagen). mRNA was isolated from the total RNA using the Oligotex mRNA Mini Kit (Qiagen) according to the manufacturer's instructions. Purified mRNA was quantified and stored at -80°C until cDNA synthesis. mRNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen) and an anchored oligo-dT primer.

Myostatin cDNAs were initially obtained by nested RT-PCR using degenerate primers (Table 1) targeted to conserved sequences in the mature peptide regions of a wide variety of myostatins from the GenBank database (<http://www.ncbi.nlm.nih.gov>). The First round of PCR was performed with *mstnF1* and

*mstnR1* (94°C, 30 s; 50°C, 20 s; 72°C, 30 s; 35 cycles) and the second nested PCR was carried out under the same PCR conditions using 1 µL of the first round PCR product as a template. Amplified PCR products were identified by 2% agarose gel electrophoresis and excised, purified, and cloned in TOPO/pCR 2.1 (Invitrogen). Positive colonies were selected and cultured in LB broth medium. Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen) and their DNA sequences were determined using an automated DNA sequencer (Applied Biosystems). Rapid amplification of cDNA ends (RACE) was used to obtain the 3' region of mRNA using the RACE system (Invitrogen Inc.) which has been used previously (Kim et al. 2005b; Lee et al. 2007). The second round nested PCR was carried out using two forward primers for 3' RACE (Table1)

### Expression Analysis of Py-MSTN gene

We used RT-PCR to examine the tissue type expression profiles of Py-MSTN. cDNAs were synthesized and used for cloning the Py-MSTN gene. cDNA samples were treated with DNase I (Promega Inc.) to prevent genomic DNA contamination of genomic DNA and then quantified. A 500 ng aliquot of cDNA from each tissue type was used as a template and specific forward and reverse primers were designed (Table 1). Each sample was amplified by PCR while considering the melting temperatures of the primers and the PCR product sizes (94°C, 30 s; 62°C, 20 s; 72°C, 30 s; 35 cycles). PCR products were confirmed by 2% agarose gel electrophoresis.  $\beta$ -actin was amplified as a positive control.

Real-time PCR was carried out using the DNA Engine Opticon 2 Real-Time PCR Detection System (Bio-Rad) to measure the Py-MSTN expression levels in various tissues. cDNA synthesized from each tissue was quantified and 100 ng was used for each reaction. SYBR premix Ex Taq™ (TaKaRa Bio Inc.) was used to detect fluorescence during PCR cycles. Real-time PCRs were carried out under the same conditions as in the standard PCR described

Table 1. Oligonucleotide primers used for the study of scallop myostatin

| Name       | Sequence                         | Description                 |
|------------|----------------------------------|-----------------------------|
| MSTN F1    | 5'-RTNGARGCNYTNGAYGAR-3'         | Degenerate forward primer-1 |
| MSTN F2    | 5'-TGYTGYMGNTAYCCNYT-3'          | Degenerate forward primer-2 |
| MSTN R1    | 5'-SWRCANCCRCANCKATC-3'          | Degenerate reverse primer-1 |
| MSTN R2    | 5'-RCANCCRCANCKATCNAC-3'         | Degenerate reverse primer-2 |
| Py-MSTN F1 | 5'-TTTGGGTGGGACTTTGTGATAGCTC-3'  | First primer for 3' RACE    |
| Py-MSTN F2 | 5'-TGATTCAGCAAGCACCGTCCCCAACA-3' | Nested primer for 3' RACE   |
| Py-MSTN F3 | 5'-AAAGTTCCAACCAAACGAGAAATCGG-3' | Specific primer for RT-PCR  |
| Py-MSTN R1 | 5'-CTATCACAAAGTCCCACCCAAACGC-3'  | Specific primer for RT-PCR  |

above. All PCR products were reconfirmed by agarose gel electrophoresis after evaluating expected melting temperatures to assure proper amplification. Standard curves were constructed to quantify copy numbers as described previously (Kim et al., 2005a).  $\beta$ -actin gene expression was used to normalize expression levels.

### Amino acid sequence analysis

The deduced Py-MSTN amino acid sequence was obtained from the web-based open reading frame (ORF) finder program (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The amino acid sequence was then aligned with previously identified other TGF- $\beta$  family members. From the amino acid sequence similarity, seven MSTNs (gi6016121, gi48314966, gi6754752, gi113912357, gi47825371, gi14646885, gi14646883), four GDF11s (gi6649914, gi47085753, gi109480148, gi6649923), three bone morphogenetic proteins (BMP3) (gi8392993, gi27734166, gi4557371) and myoglianin (gi4580679), the only invertebrate homolog, were selected. Given that fish contain at least two copies of MSTN genes, we included two MSTN amino acid sequences (om-MSTN1 & 2) from rainbow trout as a representative sample. Expected mature peptides of each protein were selected and analyzed by submitting them to internet-based clustalW program (<http://www.ebi.ac.uk/clustalw/index.html>). Analyzed data were then edited and annotated using GeneDoc program (<http://www.psc.edu/biomed/genedoc>). A phylogenetic tree was constructed using clustalW and was annotated graphically using PhyloDraw (<http://pearl.cs.pusan.ac.kr/phylo draw>).

### Molecular modeling

In order to construct the structural model of Py-MSTN, we used MODELLER which is a comparative protein structure modeling program by satisfaction of spatial restraints (<http://salilab.org/modeller>). We used the program's default values and scripts unless specified. The modeling is based on a single template and consists of five comparative modeling steps: identification of related structures, selection of template(s), alignment of structures, building a model based on template structure(s), and model evaluation. Briefly, sequences of known structures potentially related to Py-MSTN were searched in the non-redundant PDB sequences. The template was selected based on two criteria: sequence identity and e-values, which are representative indicators of similarity. Typically, a sequence identity greater than approximately 25% is considered a

potential template. We were able to obtain ten possible templates. These templates were further analyzed to select the most appropriate template, and the bone morphogenic protein-7 (1 bmp) sequence was selected. The Py-MSTN sequence was then aligned against 1 bmp. The 3D model of the Py-MSTN was constructed automatically based on the structure of 1 bmp using the automodel module in MODELLER. Out of five models generated, the model with lowest value of the MODELLER objective function was selected and further evaluated for DOPE potential using MODELLER and the program PROCHECK.

## Results and Discussion

### Cloning of the Py-MSTN gene

We isolated an invertebrate myostatin, gene sequence of Py-MSTN from a scallop. Several PCR amplicons were produced using a combination of degenerative primers designed from amino acid alignments (Table 1). Two products were identified as MSTN homologs and their sequences contained an overlapping region. By combining the two sequences, 678 bp of partial fragment was generated. We used 3'RACE with two consecutive sequence specific primers and confirmed that "TAA" was the stop codon of the sequence. 5' RACE was also performed to obtain 5' upstream sequences, but was not successful. Although we were unable to obtain the full ORF, we identified sufficient mature sequence of the functional form of MSTN (Fig. 1). Analysis of Py-MSTN revealed a putative proteolytic processing site (RSKR). Vertebrate MSTN is processed by the serine protease, furin, which belongs to a family of proprotein convertases (PC) (Lee and McPherron 2001). Furin recognizes a conserved RXXR sequence and cleaves it into an N-terminal latency associated peptide (LAP) and a C-terminal mature MSTN peptide (Thomas et al. 2000). This result suggested that Py-MSTN should be the substrate for a furin-like protein in the scallop. Humans contain at least nine different PC but there has been no previous report about the scallop homolog.

We found that Py-MSTN contains nine conserved cysteine residues (Fig.1). MSTN, like other TGF- $\beta$ s, contains six highly conserved cysteine residues that are involved in the formation of three disulfide linkers. Cysteines 2 and 3 form disulfide bonds with cysteines 5 and 6, respectively and the third bond is formed between cysteines 1 and 4 (Fig. 2). The conserved cysteine residues of Py-MSTN indicate that Py-MSTN has a similar three-dimensional structure

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GCA GAG GCT TTG GAT GAG ACT GGC CGC AAC GTG GTA GTG CTT CCC CCA ACT TTT GGG GTA
A E A L D E T G R N V V V L P P T F G V

AAT GAC GGA TAC GAA CCA ATG TTG GAC ATG CGT ACA TCT CTA AGG AAG AGT ACA CGC TCC
N D G Y E P M L D M R T S L R K S T R S

AAG AGA TCA ACA GAG CTC TAT TGT GAC CAG CGA GAA GAG ACC GCC TGC TGC AGA TAT CCG
K R S T E L Y C D Q R E E T A C C R Y P

TTG GAA GTT GAT TTT GTT GCG TTT GGG TGG GAC TTT GTG ATA GCT CCT TTA ACT TAC GCT
L E V D F V A F G W D F V I A P L T Y A

GCC TAT CAC TGC GCC GGT GAA TGC AAG GGT GAA CAA TTG GAC GAT ACT CTT CAT GCT CAC
A Y H C A G E C K G E Q L D D T L H A H

GTG ATT CAG CAA GCA CCG TCC CCA ACA CTA AGC CCG CCA CAG TCA GCG CTG AGC AAT GTC
V I Q Q A P S P T L S P P Q S A L S N V

GGT CCG TGC TGC ACC CCG ACT AAA ATG TCC GAC CTA GCC ATG CTT TTC TTC GAT CAT AAC
G P C C T P T K M S D L A M L F F D H N

TCC AAC ATA GCA TTA ACG CGA TTG CCC AGG ATG AAA GTA GAC AGA TGT GGT TGT GCG TAA
S N I A L T R L P R M K V D R C G C A *
    
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Fig. 1. Nucleotide and deduced amino acid sequences of the C-terminus of Py-MSTN. The proteolytic processing site (RXXR) is underlined. All nine conserved cysteine residues are boxed. The asterisk indicates the stop codon.

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          1          2          3
Mm-MSTN : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVF---LQKYPETHLVHQA : 50
Hs-MSTN : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVF---LQKYPETHLVHQA : 50
Ss-MSTN : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVF---LQKYPETHLVHQA : 50
Gg-MSTN : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVF---LQKYPETHLVHQA : 50
Bt-MSTN : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVF---LQKYPETHLVHQA : 50
Om-MSTN2 : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVMH---LQKYPETHLVNKA : 50
Om-MSTN1 : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVMH---LQKYPETHLVNKA : 50
Hs-GDF11 : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVMF---LQKYPETHLVQQA : 50
Rn-GDF11 : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVMF---LQKYPETHLVQQA : 50
Mm-GDF11 : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVMF---LQKYPETHLVQQA : 50
Dr-GDF11 : CCRYPLTVDEEAGW-DWIIAPRYKANYCSGCECEVMF---LQKYPETHLVQHA : 50
Py-MSTN : CCRYPLEVDEEAGW-DVVIAPLTYAAMHQAEGCKGEQ---LDDTLHAHVIQQA : 50
Dm-MGLN : CCRYELKVNETSAGW-HEFVVAPTSFDAYECSDCKVGY---LQKYPETHLVHQA : 50
Rn-BMP3 : CARRYLKVDEADIGWSEWIIISPSEFDAYCSGACQSPMPKSKPSNHEATIQSIV : 54
Mm-BMP3 : CARRYLKVDEADIGWSEWIIISPSEFDAYCSGACQSPMPKSKPSNHEATIQSIV : 54
Hs-BMP3 : CARRYLKVDEADIGWSEWIIISPSEFDAYCSGACQSPMPKSKPSNHEATIQSIV : 54

          4          5 6
Mm-MSTN : N-----PR---CSAGPCCTPKMSPINMLYFNGKEQLIYGKIPAMVVDRCCCS : 95
Hs-MSTN : N-----PR---CSAGPCCTPKMSPINMLYFNGKEQLIYGKIPAMVVDRCCCS : 95
Ss-MSTN : N-----PR---CSAGPCCTPKMSPINMLYFNGKEQLIYGKIPAMVVDRCCCS : 95
Gg-MSTN : N-----PR---CSAGPCCTPKMSPINMLYFNGKEQLIYGKIPAMVVDRCCCS : 95
Bt-MSTN : N-----PR---CSAGPCCTPKMSPINMLYFNGEGQLIYGKIPAMVVDRCCCS : 95
Om-MSTN2 : N-----PR---CTAGPCCTPKMSPINMLYFNKKEQLIYGKIPAMVVDRCCCS : 95
Om-MSTN1 : N-----PR---CTAGPCCTPKMSPINMLYFNKKEQLIYGKIPAMVVDRCCCS : 95
Hs-GDF11 : N-----PR---CSAGPCCTPKMSPINMLYFNKQQLIYGKIPAMVVDRCCCS : 95
Rn-GDF11 : N-----PR---CSAGPCCTPKMSPINMLYFNKQQLIYGKIPAMVVDRCCCS : 95
Mm-GDF11 : N-----PR---CSAGPCCTPKMSPINMLYFNKQQLIYGKIPAMVVDRCCCS : 95
Dr-GDF11 : N-----PR---CSAGPCCTPKMSPINMLYFNKQQLIYGKIPAMVVDRCCCS : 95
Py-MSTN : PSPILSPPQSALSNVGPCTPKMSDLAMLFFDHNSNIALTRLPKVVDRCCCA : 104
Dm-MGLN : T-----SATPCCSPKMSLSILLYFDDNHNVLVLSVLPNMSVGGCCS : 92
Rn-BMP3 : R-----AVGVVSIPPECCVPEKMSLSILFFDENKNVVLKVYPNMTVDSACR : 103
Mm-BMP3 : R-----AVGVVSIPPECCVPEKMSLSILFFDENKNVVLKVYPNMTVDSACR : 103
Hs-BMP3 : R-----AVGVVPIPECCVPEKMSLSILFFDENKNVVLKVYPNMTVESACR : 103
    
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Fig. 2. Comparison of the deduced amino acid sequences of the TGF- $\beta$  family. The sequence of Py-MSTN was aligned with other vertebrate myostatins (MSTNs), growth and development factors (GDF11s), and bone morphogenic factors (BMP-3s) using ClustalW program. Arabic numerals indicate the conserved cysteine residues for intra-molecular disulfide bonds.

to other MSTNs and members of the TGF- $\beta$  family. Compared with MSTNs from vertebrates, Py-MSTN has nine additional amino acid residues between cysteine 3 and 4, which contain the  $\alpha$  helix region 'heel', which is important for binding to the specific receptor.

### Amino acid sequence comparison

To examine the possible role of Py-MSTN in muscle growth, deduced amino acid sequence of Py-MSTN was aligned with other previously reported MSTN, GDF11, and BMP proteins from a variety of species (Fig. 2). The mature peptides of Py-MSTN consist of 104 amino acid residues which are nine residues longer than all other MSTN and GDF11 proteins discovered thus far. There are no gaps in the amino acid sequence between MSTN and GDF11, and more than 90% of amino acid sequences are similar among all vertebrates. This suggests that in vertebrates, MSTN and GDF11 may be functionally closer to each other than to Py-MSTN. Py-MSTN exhibited 67% similarity to human MSTN and 65% similarity to human GDF11 respectively that is little different. GDF11 in mammals is expressed in skeletal muscle and in several other tissues such as brain, limb bud, and dental pulp (Nakashima et al. 1999). Although its detailed function in each tissue and developmental stage remains unclear, GDF11 may be involved in axial skeleton patterning in developing vertebrates (McPherron et al. 1999). Our results suggest that Py-MSTN, vertebrate GDF11, and MSTN may share the same ancestral gene and that GDF-11 and MSTN have recently diverged. Our phylogenetic analysis supported this hypothesis (Fig. 5). Py-MSTN appears to be more closely related to vertebrate MSTNs and GDF11s than to BMP3 and other TGF- $\beta$  family member. This suggests that Py-MSTN shares the same ancestral gene with MSTN and GDF11 but not with other TGF- $\beta$ s. In addition, it appears that after GDF11 and MSTN diverged into two groups, the fish MSTNs experienced a whole genome duplication event, resulting in two copies of MSTN gene, which explains why fish possess an additional MSTN gene.

### Expression of Py-MSTN

The expression of Py-MSTN was detected in the adductor muscle and the gill tissue, which is mostly composed of skeletal muscle by RT-PCR (Fig. 3). Low expression levels were also detected in the ovary, testis, and mantle. This strongly suggests that like its vertebrate homolog, Py-MSTN is involved in the development of skeletal muscle in invertebrates.

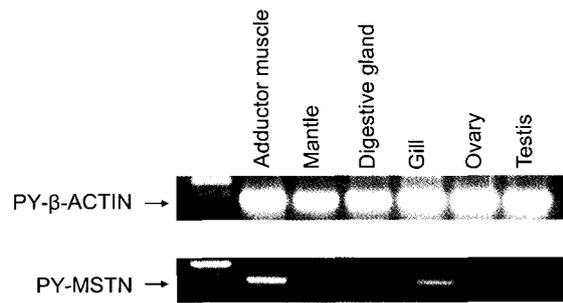


Fig. 3. Expression of Py-MSTN genes in various tissues. Regular RT-PCR was carried out and amplicons were run on 2% agarose gel for 20 min. The  $\beta$ -actin gene was used as a positive control (a).

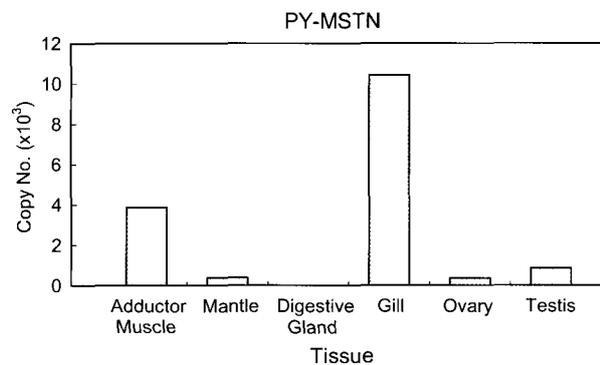


Fig. 4. Real-time RT-PCR analysis of Py-MSTN mRNA expression in different tissues. Each copy number out of 100 ng of total RNA was measured by constructing the standard curves referring threshold (Ct) values of Py-MSTN. Tm values were used to reconfirm each value.

However, further analyses including detailed reactions in development of muscle are necessary to confirm this hypothesis. The inhibitory roles of MSTN in the development of mammalian muscle tissue are clear, however fish contain at least two copies of the MSTN gene and their expression patterns differ considerably (Rescan et al. 2001). Our real-time PCR data indicated a 100-fold increase in Py-MSTN expression in the adductor muscle and gill compared to the digestive gland (Fig. 4). In addition, a small amount of Py-MSTN mRNA was detected in other tissues including the mantle, testis, and ovary. The expression of Py-MSTN in reproductive organs may be important for the successful development of embryos as in fish (Vianello et al. 2003).

### Molecular modeling of Py-MSTN

To model Py-MSTN, we began by aligning the Py-MSTN target sequence with templates of known related three-dimensional structures. We then search

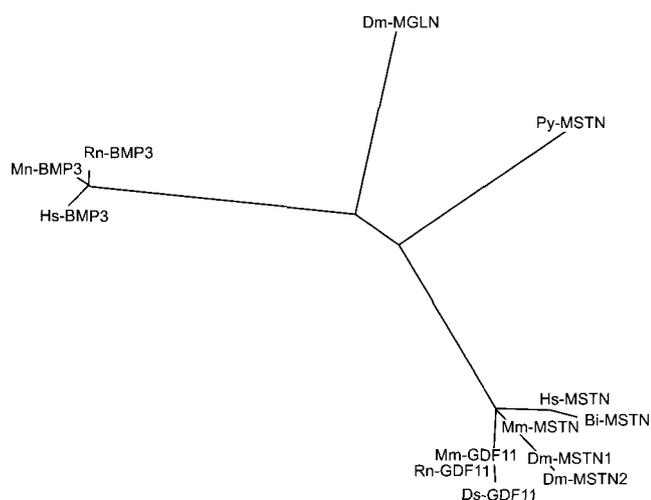


Fig.5. Phylogenetic tree of the TGF- $\beta$  family members based on the amino acid sequence similarity. The phylogenetic tree for TGF- $\beta$  was generated using the neighbor-joining method with the GeneDoc program. The distance along each branch represents the unbiased unrooted evolutionary distance. Genebank accession numbers: gi6016121, gi48314966, gi6754752, gi113912357, gi47825371, gi14646885, gi14646883, gi6649914, gi47085753, gi109480148, gi6649923, gi8392993, gi27734166, gi4557371, gi4580679.

ched for structures related to the Py-MSTN sequence, and of the 35 sequences retrieved from the database, ten structures (PDB ID codes 1bmp, 3bmpA, 1es7a, 1es7C, 1lx5A, 1lx1A, 1reuA, 1rewA, 1rewB, and 1m4uL) exhibited 40% sequence identity to Py-MSTN-with e-values of zero. These structures were further compared and clustered. Finally, 1 bmp was selected as a single template for its relatively long alignment lengths, high sequence identity, and slightly better crystallographic resolution. The 40% primary sequence identity between Py-MSTN and bone morphogenic protein-7 (1 bmp), is well above the minimal potential value (25%). This indicates that the crystal structure of BMP-7 would act as a good template for Py-MSTN. This alignment, which is very crucial to obtain a high quality model structure, was carefully examined and used as the input to calculate the three-dimensional model for the target sequence.

The quality of the models was assessed by the model evaluation module in the MODELLER, and the program PROCHECK. The energy level obtained using the former module was comparable to that of the template 1 bmp (data not shown). A Ramachandran plot for the Py-MSTN structure calculated using the latter program, was produced to compare the overall stereochemical quality of Py-MSTN

model against the crystal structure of BMP-7. Analysis of the Ramachandran plot of the modeled PyMSTN structure indicated that 88.6% of the residues were in the most favorable regions, and the additional 10.2% were in the allowed regions. These evaluations confirmed that overall the model was reasonable. Therefore, given its similar three-dimensional structure, it appears that Py-MSTN may be a functional member of the TGF- $\beta$  family.

We determined that Py-MSTN is a functional member of the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily of proteins. Many X-ray crystal structures of the TGF- $\beta$  superfamily are available (Griffith et al. 1996; Allendorph et al. 2006). The C-terminal regions of this superfamily share sequence similarity, and our Py-MSTN model has the same fold as its relatives (Fig. 6). Our model has three structural elements; finger 1 and 2 and a heel. The four strands of the antiparallel  $\beta$ -sheet form two finger-like projections. An  $\alpha$ -helix position perpendicular to the axis of the two fingers forms the heel of the hand. The N-terminus of finger 1 corresponds to the thumb of the hand.

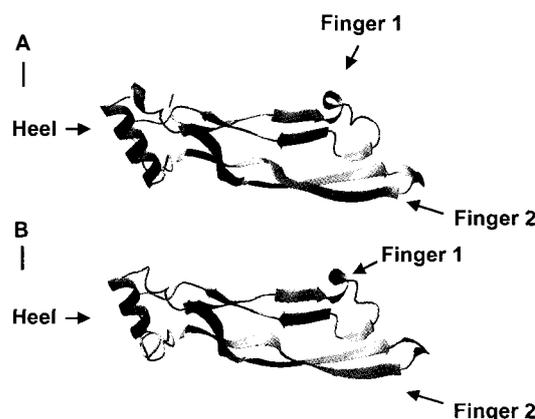


Fig. 6. Ribbon representation of 3D structures of (A) BMP-7 (PDB ID code 1bmp, template) and (B) Py-MSTN (target). The  $\beta$ -sheets are displayed as arrows and the  $\alpha$ -helix is represented as a tube.

The member of the TGF- $\beta$  superfamily perform their biological functions by interacting with specific type I and type II serine/threonine kinase receptors. The residues of BMP-7 involved in receptor binding are highlighted in cyan in Figure 7A and corresponding residues of Py-MSTN are indicated. A comparison of the two ligand-binding interfaces clearly shows that Py-MSTN is a member of the TGF- $\beta$  family and has similar receptors to BMP-7. The residues highlighted indicate that the specificity of binding between the Py-MSTN and receptor should

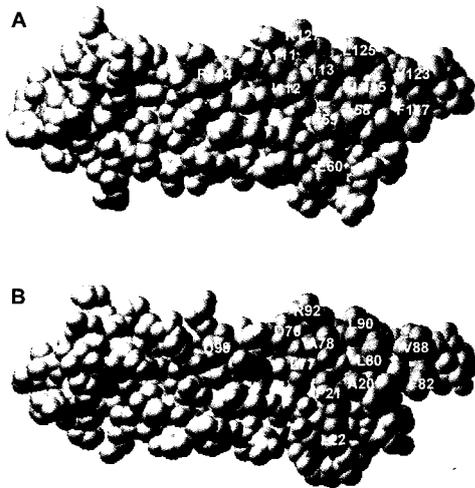


Fig. 7. Schematic drawing of the ligand-binding interface of BMP-7 to ActRII-ECD (Activin receptor type II extracellular domain) (A) and corresponding residues of Py-MSTN to BMP-7 (B).

be different from that of BMP-7.

In conclusion, we isolated a myostatin homolog (Py-MSTN) from *Patinopecten yessoensis* and demonstrated that it shares many features with vertebrate myostatins. Our data strongly suggest that Py-MSTN is involved in growth and development of muscle in invertebrate. Further studies should examine possible commercial applications of this protein.

### Acknowledgements

This research was supported by a Research Fund provided by the Pukyong National University, Support for Faculty Research Abroad. H. J. Kim was funded by KOPRI grant No. PE06230.

### References

- Acosta, J., Y. Carpio, I. Borroto, O. Gonzalez and M.P. Estrada. 2005. Myostatin gene silenced by RNAi show a zebrafish giant phenotype. *J. Biotechnol.*, 119, 324-331.
- Allendorph, G.P., W.W. Vale and S. Choe. 2006. Structure of the ternary signaling complex of a TGF-beta superfamily member. *Proc. Natl. Acad. Sci. USA*, 103, 7643-7648.
- Amali, A.A., C.J. Lin, Y.H. Chen, W.L. Wang, H.Y. Gong, C.Y. Lee, Y.L. Ko, J.K. Lu, G.M. Her, T.T. Chen and J.L. Wu. 2004. Up-regulation of muscle-specific transcription factors during embryonic somitogenesis of zebrafish (*Danio rerio*) by knock-down of myostatin - I. *Dev. Dyn.*, 229, 847-856.

- Biga, P.R., S.B. Roberts, D.B. Iliev, L.A. McCauley, J.S. Moon, P. Collodi and F.W. Goetz. 2005. The isolation, characterization, and expression of a novel GDF11 gene and a second myostatin form in zebrafish, *Danio rerio*. *Comp. Biochem. Physiol. B.*, 141, 218-230.
- Griffith, D.L., P.C. Keck, T.K. Sampath, D.C. Rueger and W.D. Carlson. 1996. Three-dimensional structure of recombinant human osteogenic protein. 1: structural paradigm for the transforming growth factor beta superfamily. *Proc. Natl. Acad. Sci. USA*, 93, 878-883.
- Jespersen, J., M. Kjaer and P. Schjerling. 2006. The possible role of myostatin in skeletal muscle atrophy and cachexia. *Scand. J. Med. Sci. Sports*, 16, 74-82.
- Kim, H.W., E.S. Chang and D.L. Mykles. 2005a. Three calpains and ecdysone receptor in the land crab *Gecarcinus lateralis*: sequences, expression and effects of elevated ecdysteroid induced by eyestalk ablation. *J. Exp. Biol.*, 208(Pt 16), 3177-3197.
- Kim, H.W., S.G. Lee and D.L. Mykles. 2005b. Ecdysteroid-responsive genes, RXR and E75, in the tropical land crab, *Gecarcinus lateralis*: differential tissue expression of multiple RXR isoforms generated at three alternative splicing sites in the hinge and ligand-binding domains. *Mol. Cell. Endocrinol.*, 242, 80-95.
- Ko, C.F., T.T. Chiou, T.T. Chen, J.L. Wu, J.C. Chen and J.K. Lu. 2007. Molecular cloning of myostatin gene and characterization of tissue-specific and developmental stage-specific expression of the gene in orange spotted grouper, *Epinephelus coioides*. *Mar. Biotechnol.*, 9, 21-32.
- Kocabas, A.M., H. Kucuktas, R.A. Dunham and Z. Liu. 2002. Molecular characterization and differential expression of the myostatin gene in channel catfish (*Ictalurus punctatus*). *Biochim. Biophys. Acta*, 1575, 99-107.
- Lee, K.J., H.W. Kim, A.M. Gomez, E.S. Chang, J.A. Covi and D.L. Mykles. 2007. Molt-inhibiting hormone from the tropical land crab, *Gecarcinus lateralis*: Cloning, tissue expression, and expression of biologically active recombinant peptide in yeast. *Gen. Comp. Endocrinol.*, 150, 505-513.
- Lee, S.J. and A.C. McPherron. 2001. Regulation of myostatin activity and muscle growth. *Proc. Natl. Acad. Sci. USA*, 98, 9306-9311.
- Lo, P.C. and M. Frasch. 1999. Sequence and expression of myoglianin, a novel *Drosophila* gene of the TGF-beta superfamily. *Mech Dev.*, 86, 171-175.
- McPherron, A.C., A.M. Lawler and S.J. Lee. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature.*, 387, 83-90.
- McPherron, A.C., A.M. Lawler and J. Lee. 1999. Regulation of anterior/posterior patterning of the axial

- skeleton by growth/differentiation factor 11. *Nat. Genet.*, 22, 260-264.
- McPherron, A.C. and S.J. Lee. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Proc. Natl. Acad. Sci. USA*, 94, 12457-12461.
- Nakashima, M., T. Toyono, A. Akamine and A. Joyner. 1999. Expression of growth/differentiation factor 11, a new member of the BMP/TGFbeta superfamily during mouse embryogenesis. *Mech. Dev.*, 80, 185-189.
- Nishi, M., A. Yasue, S. Nishimatu, T. Nohno, T. Yamaoka, M. Itakura, K. Moriyama, H. Ohuchi and S. Noji. 2002. A missense mutant myostatin causes hyperplasia without hypertrophy in the mouse muscle. *Biochem. Biophys. Res. Comm.*, 293, 247-251.
- Rescan, P.Y., I. Jutel and C. Ralliere. 2001. Two myostatin genes are differentially expressed in myotomal muscles of the trout (*Oncorhynchus mykiss*). *J. Exp. Biol.*, 204, 3523-3529.
- Stratil, A. and M. Kopecny. 1999. Genomic organization, sequence and polymorphism of the porcine myostatin (GDF8; MSTN) gene. *Anim. Genet.*, 30, 468-470.
- Thomas, M., B. Langley, C. Berry, M. Sharma, S. Kirk, J. Bass and R. Kambadur. 2000. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *J. Biol. Chem.*, 275, 40235-40243.
- Vianello, S., L. Brazzoduro, L. Dalla Valle, P. Belvedere and L. Colombo. 2003. Myostatin expression during development and chronic stress in zebrafish (*Danio rerio*). *J. Endocrinol.*, 176, 47-59.
- Yang, J., T. Ratovitski, J.P. Brady, M.B. Solomon, K.D. Wells and R.J. Wall. 2001a. Expression of myostatin pro domain results in muscular transgenic mice. *Mol. Reprod. Dev.*, 60, 351-361.
- Yang, W., H.X. Wang, Y. Chen, Y. Zhang and D.H. Zhu. 2001b. Cloning, expression and purification of the chicken growth and differentiation factor - 8. *Sheng Wu Gong Cheng Xue Bao*, 17, 460-462.

(Received January 2007, Accepted March 2007)