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A Myostain-like Gene Expressed Highly in the Muscle Tissue of Chinese mitten crab, *Eriocheir sinensis*

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A complete cDNA, which encodes for a myostatin-like protein (Es-MSTN), was isolated from the Chinese mitten crab, *Eriocheir sinensis*. Es-MSTN was composed of 2,397 nucleotides and the open reading frame (ORF) specified a protein containing 468 amino acids. Es-MSTN exhibited 32% amino acid sequence identity and 52% similarity to human myostatin. Multiple sequence alignment analysis indicated that Es-MSTN possessed the conserved proteolytic cleavage site (RXXR) for maturation of the protein and nine cysteine residues for disulfide bridges. Besides the conserved structural features, Es-MSTN also exhibits its unique characters; a longer N-terminal domain which is involved in protein folding and latent form of myostatin and absence of the cleavage site for BMP-1/tolloid family of metalloproteinase to activate mature myostatin. Phylogenetic analysis suggests that Es-MSTN showed the closely related to both vertebrate myostatin and GDF11. Es-MSTN is expressed highly in the claw muscle, leg muscle, thoracic muscle and heart, and moderately in the hindgut suggesting that Es-MSTN may play important roles in the muscle tissues. As homolog of mammalian myostatin and GDF11, Es-MSTN may be involved in development of muscular tissue and further study will help to produce high-quality seafood.

Key words: Myostatin, Muscle, Chinese mitten crab, *Eriocheir sinensis*, Crustacean, Molting

Introduction

Myostatin, a negative regulator of muscle growth, was discovered by geneticists A. McPherron and Lee (McPherron et al., 1997a). They also produced myostatin-null mice which have about twice as much muscle as normal mice and a significant reduction in body fat (McPherron et al., 1997b; Lin et al., 2002; McPherron et al., 2002). The myostatin genes have been isolated and characterized in many different mammals and fish species (Rescan et al., 2001; Vianello et al., 2003; Garikipati et al., 2006; Ostbye et al., 2007). Those vertebrate primary coding sequence is highly conserved among all vertebrates (Rodgers et al., 2001). Myostatin gene in fish has been duplicated as most fish species possess two distinct myostatin genes while salmonids have four (Garikipati et al., 2006; Rodgers et al., 2007). Although its gene structure is very similar among the vertebrates, the physiological and developmental functions of fish myostatins are still unclear from the different expression patterns and knock-out experiments. Signaling pathway of myostatin is also well established in mammals. As a member of the TGF-β superfamily, myostatin-induced transcription requires the participation of regulatory Smads (Smad2/3) and Co-Smads (Smad4). Conversely, inhibitory Smad7, but not Smad6, dramatically reduces the myostatin-induced trans-cription (Zhu et al., 2004; Forbes et al., 2006). This Smad7 inhibition is enhanced by co-expression of Smurf1(Wicks et al., 2006).

Compared with vertebrate little has been known about invertebrate myostatin. There are only a couple of reports about myostatin homologs in invertebrates (Lo et al., 1999; Kim et al., 2004). Those reports strongly support the idea that developmental process of invertebrate muscular tissue is controlled by myostatin-like genes as in vertebrates. The major purpose of this study is to understand roles of crustacean myostatin homolog in muscle plasticity during the molting cycles. This study includes cloning and characterization of crustacean myostatin homolog and determination of expression profile.

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Investigation of roles of crustacean myostatin in the muscle fiber plasticity is necessary to understand difference in growth and development of muscle fiber between other vertebrates and crustaceans. Furthermore, knowledge of molecular mechanism of muscle growth may be applied for production of high-quality crustacean products.

Materials and Methods

Experimental animals

Live adult crabs were purchased from a crab farm which is located in Cheongyang, South Korea and held in a tank with circulating aerated fresh water for 2-3 days until use.

Cloning Es-MSTN cDNA

Total RNA from the claw muscle tissue was extracted using the Trizol reagent according to the manufacturer's instructions (Invitogen, USA) and quantified with a ND-1000 NanoDrop UV spectrophotometer (Nanodrop Technologies, Inc. USA). cDNA was synthesized in a reaction containing reverse transcriptase and oligo-dT primer. A 12 μL mixture containing 3 μg total RNA, 1 μL 20 μM oligo dT primer and 4 μL dNTPs was heated to 70°C for 5 min and chilled on ice for 2 min. First-strand buffer (5 X, 4 μL), 2 μL 0.1 M DTT and 1 μL RNaseout were added to the mixture, which was incubated at 37°C for 2 min. MMLV reverse transcriptase (1 μL) was added and then the mixture was incubated at 37°C for 50 min.

Myostatin cDNAs were initially obtained by nested RT-PCR using degenerate primers (Table 1) targeted to conserved sequences in the mature peptide regions of various myostatins including from the GenBank database (http://www.ncbi.nlm.nih.gov). The first round of PCR was carried out with MSTNF1 and MSTNR1 primer pair. The reactions contained 3 μL first-strand cDNA (1,500 ng), 1 μL 100 μM MSTNF1, 1 μL 100 μM MSTNR1, 3 μL 10X buffer, 2 μL dNTPs, 0.2 μL *Takara Ex Taq* polymerase (Takara Bio Inc., Japan), and 19.8 μL distilled water. The second nested PCR was carried out using 1 μL of the first round PCR product as a template. First and second PCR conditions were 1 min at 94°C, followed by 40 cycles at 94°C for 30 sec, 52°C for 40 sec, and 72°C for 1 min.

PCR products were separated with 1.5% agarose gel electrophoresis and stained with ethidium bromide. The PCR products with expected size were purified using Gel Extraction Kit (Geneall Inc., Korea), ligated into TA plasmid vector with the pGEM-T Easy Cloning Kit (Promega, USA), and transformed into One Shot Top 10 *E. coli* strain (Invitrogen, USA). Nucleotide sequences of the cloned cDNAs were determined using the automated DNA sequencer (ABI Biosystem, USA). The nucleotide sequence similarities were examined by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

Rapid amplification of cDNA ends (RACE) was used to obtain full-length cDNA. The DNA Walking SpeedUp™ Kit (Seegene, Korea) was used according to the manu-facturer's instructions. Briefly, first, 50 ng cDNA, 2 μL of 5 μM DW2-ACP (one of DW2-ACP from 1 to 12), 5 μM specific primer 1, 10 μL 2X SeeAmp™ ACP™ master Mix II and 6 μL distilled

Table 1. Oligonucleotide primers used for the study of Chinese mitten crab myostatin

Name	Sequence	Description
MSTNF1	5'-ATHAAYCCNCCNAAYAT-3'	Degenerate forward primer-1
MSTNF2	5'-AARGGNAAYTGGGTNAA-3'	Degenerate forward primer-2
MSTNF3	5'-AAYCCNCCNAAYATGACNGG-3'	Degenerate forward primer-3
MSTNF4	5'-CCNCCNAAYATGACNGGNGT-3'	Degenerate forward primer-4
MSTNR1	5'-TCNACNGTNARRTGRTANC-3'	Degenerate reverse primer-1
MSTNR2	5'-NGTNARRTGRTACCGNCKRCARC-3'	Degenerate reverse primer-2
ESMSTNR1	5'-AGCCACTTGATTCCCCTGCGTGTC-3'	Specific reverse primer-1
ESMSTNR2	5'-ACCACGCCTAGGTTATCCTCCGG-3'	Specific reverse primer-2
ESMSTNRTF1	5'-AATCTATAACCCTGACGAGCCGGA-3'	Specific forward primer for RT-PCR
ESMSTNRTR1	5'-TCCTCCGTGTGGATCTCAAGAA-3'	Specific reverse primer for RT-PCR
ESMSTNF1	5'-TCACTGCTGCAAGAGTGGCTGACGA-3'	First primer for 3' RACE
ESMSTNF2	5'-CCGGAGGATAACCTAGGCGTGGT-3'	Second primer for 3' RACE
ESMSTNF3	5'-GACACGCAGGGGAATCAAGTGGCT-3'	Third primer for 3' RACE
ESMSTNR3	5'-TTGACATCCGGCTCGTCAGGGTTATAG-3'	First primer for 5' RACE
ESMSTNR4	5' -TAGATTGGCTCGTTGCTGGGCATGG-3'	Second primer for 5' RACE
ESMSTNR5	5'-GATAATGCCCTGCACGTCAGGGTTG-3'	Third primer for 5' RACE
28SrRNAF870	5'-CCCGTCTTGAAACACGGACCA-3'	Forward primer for positive control
28SrRNAR1200	5'-TTCGATTAGTCTTTCGCCCCTAT-3'	Reverse primer for positive control

water were mixed. The mixture was placed in a preheated (94°C) thermal cycler and the PCR was carried out for one cycle (94°C for 5 min, 42°C for 1 min and 72°C for 2 min), for 30 cycles (94°C for 30 sec, 60°C for 30 sec and 72°C for 100 sec) and for one cycle (72°C for 7 min). The PCR products were purified using a PCR purification Kit (Geneall Inc., Korea) to remove the DW-ACP and specific primer1 present in the first PCR reaction. Second, 20 μL of a mixture containing 3 μL purified first PCR products, 2 μL 5 μM DW2-ACPN, 1 μL 5 μM specific primer2, 10 μL 2X SeeAmp™ ACP™ master Mix II and 4 μL distilled water was preheated (94°C) thermal cycler. The second PCR was carried out for one cycle (94°C for 3 min), for 35 cycles (94°C for 30 sec, 60°C for 30 sec and 72°C for 100 sec) and for one cycle (72°C for 7 min). Third, a mixture containing 2 μL second PCR products, 1 μL 5 μM UniP2, 1 μL 5 μM specific primer, 10 μL 2X SeeAmp™ ACP™ master Mix II and 6 μL distilled water was placed in a preheated (94°C) thermal cycler. The third PCR was carried out for one cycle (94°C for 3 min), for 30 cycles (94°C for 30 sec, 65°C for 30 sec and 72°C for 100 sec) and for one cycle (72°C for 7 min). PCR products were purified, cloned, and sequenced as described above. Finally 5' sequence was amplified from genomic DNA referring mammalian myostatin gene structure. Open reading frame was estimated by ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). After each cDNA fragment was put together, complete full-length ORF was reconfirmed by RT-PCR. All primers used this experiment were designed using IDT-SciTools program (http://www.idtdna.com/SciTools/SciTools.aspx) and were synthesized by Bioneer Company, Korea (Table 1).

Expression of Es-MSTN

The tissue expression profile of Es-MSTN was examined using end-point RT-PCR. Total RNA was isolated and quantified as used for cloning Es-MSTN. The total RNAs of heart, gill, hindgut, hepatopancreas, epidermis, thoracic muscle, leg muscle and claw muscle tissues were treated with DNase I (Promega, USA) to eliminate genomic DNA conamination. cDNA was synthesized using random hexamers for the reverse transcription, instead of the oligo dT primer. 20 μL PCR reaction mixture conains 2 μL cDNA (100 ng), 2 μL 2 μM ESMSTNRTF1, 2 μL 2 μM ESMSTNRTR1 primer, 0.2 μL *Takara Ex Taq* polymerase, dNTP and buffer (Takara Bio Inc., Japan). PCR conditions were 1 min at 94°C, followed by 35 cycles at 94°C for 1 min, 60°C for 30 sec, and 72°C for 40 sec. PCR products were analyzed by 1.5% agarose gel electrophoresis. PCR using primers to the 28S rRNA served as a positive control (Table 1).

Data analysis and Statistics

Multiple amino acid sequence alignment was performed using ClustalW program (http://www.ebi.c.uk/Tools/ clustalw2/index.html) and represented by GeneDoc program (http://www.nrbsc.org/gfx/enedoc/index.html). Signal peptide and proposed cleavage site were estimated by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Phylogenetic diagram of the MSTN family members was generated by the neighbor-joining method with ClustalW program and bootstrapping test was performed by Molecular Evolutionary Genetics Analysis (MEGA4) and replications were 500 (Tamura et al., 2007).

Results and Discussion

Cloning of the Es-MSTN gene

We isolated a crustacean myostatin gene sequence, Es-MSTN, from the Chinese mitten crab. Several PCR products were initially obtained using a combination of degenerative primers targeted to conserved sequences in the mature peptide regions of a wide variety of myostatins from the GenBank database (Table1). The PCR product of 270 bp exhibited the highest amino acid sequence similarity to piscine myostatin from *Sebastes schlegelii* (GenBank accession number: ABD96100). We then carried out PCR reaction using a combination of degenerative forward primers and specific reverse primers designed from that partial nucleotide sequence. Its PCR product was also identified as a part of MSTN sequence. We used 3' and 5' RACE with three consecutive sequence-specific primers and obtained full-length of Es-MSTN cDNA. We reconfirmed the full-length open reading frame (ORF) of Es-MSTN as a single transcript by RT-PCR. A cDNA encoding Es-MSTN was composed of 2,397 nucleotides and ORF specified a protein containing 468 amino acids (Fig. 1). Mammalian myostatin protein is a 375-amino acid propeptide, which is proteolytically processed at the RSRR (263) site to give rise to a 26-kDa active processed peptide as dimeric form (Sharma et al., 1999). The longer peptide length of Es-MSTN was mainly due to the additional N-terminal amino acid residues. Es-MSTN exhibited 32% amino acid sequence identity and 52% nucleotide similarity with human myostatin.

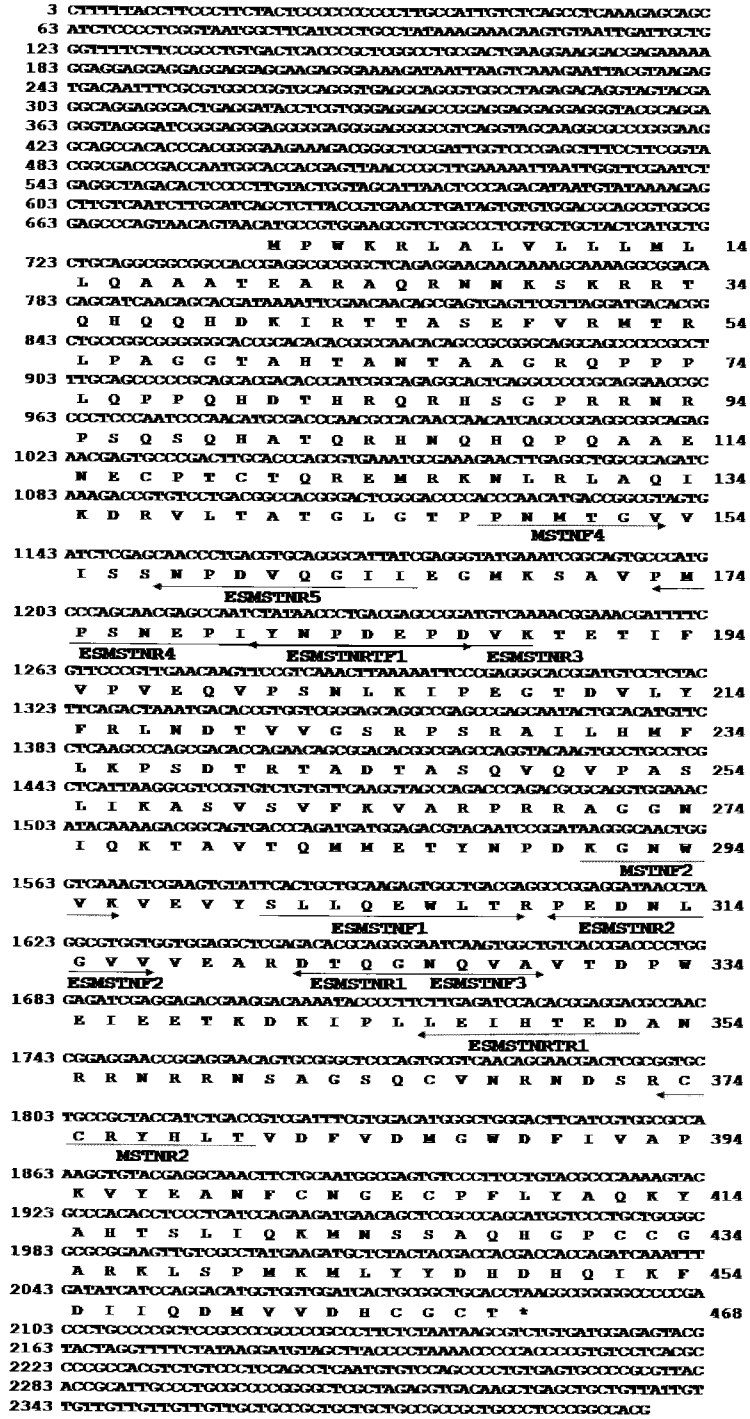

Fig. 1. The nucleotide and deduced amino acid sequences of Es-MSTN (GenBank accession number: EU650662). Locations of PCR primers used to obtain Es-MSTN fragment are indicated with arrows. An asterisk (*) indicates the stop codon. Numbers of nucleotide (left) and amino acid (right) are included.

Amino acid sequence comparison

Multiple amino acid alignment was carried out to understand structural character of Es-MSTN (Fig. 2). Es-MSTN appears to contain the most of conserved structural features. First, its hydrophobic core amino acids signal peptides, which functions as a secretory signal, is well conserved followed by relatively longer N-terminal region. Proposed signal peptide cleavage site was between 24 Ala and 25 Gln from the N-terminus. Putative proteolytic processing site

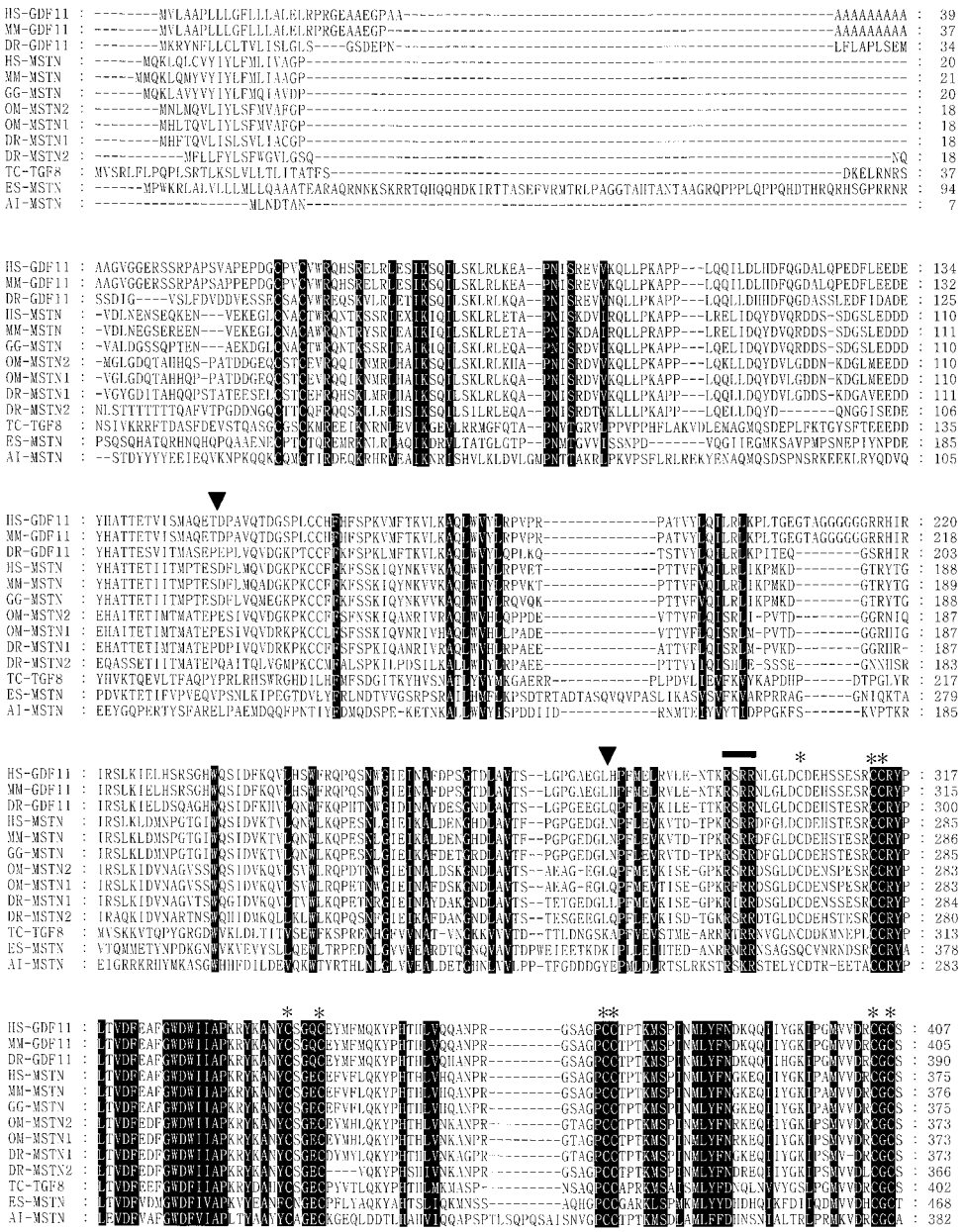

Fig. 2. Comparison of the deduced amino acid sequences of the TGF-β family members. Conserved amino acid residues among different TGF-β family members shaded in black colors. Conserved proteolytic cleavage site (RXXR) is overlined. Two inverted arrow heads indicate the conserved exon-intron boundaries. Asterisks are nine conserved cysteine residues. HS-GDF11 (*Homo sapiens*, growth differentiation factor 11, gi|5031613|ref|NP_005802.1|); MM-GDF11 (*Mus musculus*, growth differentiation factor 11, gi|112807180|ref|NP 034402.1|); DR-GDF11 (*Danio rerio*, growth differentiation factor 11, gi|47085753|ref|NP_998140.1|); HS-MSTN (*Homo sapiens*, myostatin, gi|4885259|ref|NP_005250.1|); MM-MSTN (*Mus musculus*, myostatin, gi|6754752|ref|NP_034964.1|); GG-MSTN (*Gallus gallus*, myostatin, gi|47825371|ref|NP_001001461.1|); OM-MSTN2 (*Oncorhynchus mykiss*, myostatin2, gi|185134856|ref|NP_001117755.1|); OM-MSTN1 (*Oncorhynchus mykiss*, myostatin1, gi|185134736|ref|NP_001117754.1|); DR-MSTN1 (*Danio rerio*, myostatin1, gi|18858751|ref|NP_571094.1|); DR-MSTN2 (*Danio rerio*, myostatin2, gi|66472830|ref|NP_001018627.1|); TC-TGF8 (*Trachysalambria curvirostris,* transforming growth factor8 gi|91085397|ref|XP_966819.1|); Es-MSTN (*Eriocheir sinensis*, myostatin, this study); AI-MSTN (*Argopecten irradians*, myostatin, gi|47606686|gb|AAT36326.1|)

(RXXR) in the C-terminal was also found. Mammalian myostatins undergo the proteolytic processing by a calcium-dependent serine protease called furin (Lee et al., 2001). Furin belongs to a family of mammalian processing enzymes called proprotein convertases (PCs), of which seven members have so far been identified (Thomas, 2002). The proteolytic processing of myostatin results in both a N-terminal Latency Associated peptide (LAP, also referred to as pro-peptide) and a C-terminal mature myostatin peptide (Thomas et al., 2000). LAP region is believed to be involved in proper folding and inhibitory role of myostatin as in other TGF-β family members (Saharinen et al., 1999; Lee et al., 2001). The latent complex with LAP and mature peptide then undergo proteolytic process on the LAP region to release the dimeric mature peptide and consequently binding to specific receptor (Lee et al., 2005). BMP-1/tolloid family of metalloproteinase appears to recognize Asn76 residue of LAP domain and activate mature myostatin (Wolfman et al., 2003). Interestingly, none of invertebrate genes contain the sequence at the corresponding amino acid residue suggesting that invertebrate myostatin-like proteins may not be the substrate for the protease in vertebrate at least (Fig. 2). Although Es-MSTN shares the recognition site for furin (RXXR), the overall process of the crustacean protein may be different from vertebrate model from the difference of LAP region. Unlike LAP region, C-terminal mature peptide of Es-MSTN shows much higher amino acid similarity to mammalian myostatins. Es-MSTN shares 71% amino acid sequence similarity to puffer myostatin (Genbank accession number; AAR88255) and 70% to human myostatin (Genbank accession number; ABI48386). The numbers of amino acid residues were 109, which is the same number to mammalian myostatins. In addition, nine cysteine residues are also well conserved (Fig. 1). These results suggest that Es-MSTN can form similar ternary structure to mammalian myostatins. Unfortunately, no crystal structure of mammalian myostatin has been reported; however, we can have a clue from the general structure of the ternary signaling complex of a TGF-β superfamily member (Allendorph et al., 2006). The overall structure of the C-terminal mature domain is conserved as a covalently disulfide-linked dimer throughout the super-family. Each ligand subunit contains three intradisulfide bonds, forming the characteristic "cystine knot" motif. We analyzed structural motif based on the conserved structure of TGF-β family member (Fig. 3). Seven cysteines are well conserved and there is no gap within the cystine knot space, which suggests overall framework of tertiary structure of Es-MSTN, myostatin, and GDF11 is similar each other and amino acid substitution of each protein may allow specificity for each receptor.

Phylogenetic tree was constructed to understand evolutional relationship of Es-MSTN (Fig. 4). Inhibin B, which is one of the most similar proteins to myostatin (Lee, 2004), was branched out from other myostatin-like proteins including myostatins, GDF11s, and invertebrate myostatin-like genes. Invertebrate myostatin-like genes then formed a cluster from vertebrate myostatins and GDF11s. These results indicated that invertebrate myostatin-like proteins is similarly closely related to both mammalian myostatin and GDF11. Generally two copies of piscine myostatins exist and those genes were group together different from GDF11s suggesting that genomic duplication event of myostatin genes was after GDF11 had been evolved as different proteins. Although amino acid sequences are similar each other, functions are fun-damentally different between myostatin and GDF11. Whereas myostatin functions mostly in muscle growth and development, GDF11 has been shown to regulate several patterning events, including anterior–posterior regionali-zation, kidney development and closure of the palate (Gad et al., 1999; Esquela et al., 2003). Es-MSTN was diverged from scallop myostatin-like protein and grouped together with Tc-MSTN, which is from the insect, *Tribolium castaneum*. Tc-MSTN is the putative cDNA sequence from a genome project and its functional study has not been carried out. Collectively, phylogenetic analysis suggests that the functions of the myostatin-like genes from invertebrate may not be limited in muscle growth and development as an ancestral gene of both myostatin and GDF11.

Expression of Es-MSTN

End-point RT-PCR result showed that Es-MSTN is expressed highly in the claw muscle, leg muscle, thoracic muscle and heart (Fig. 5). This result suggests that Es-MSTN may play an important role in the muscle tissues as in mammalian myostatins. Moderate expression in the hindgut was also identified suggesting additional roles in other than muscle tissues. In mammals, myostatin actions may not be limited to skeletal muscle but may additionally influence other tissues including cardiac muscle, adipo-cytes, and the brain (Rodgers et al., 2008). In mice, myostatin mRNA is strongly expressed in developing somite and skeletal muscles, and weakly

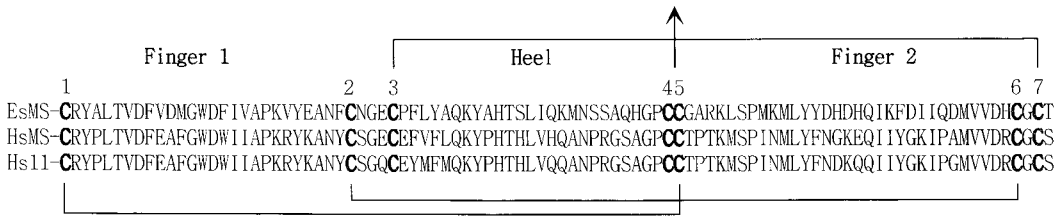

Fig. 3 The proposed seven-cysteine spacing motifs in the myostatin cysteine-knot domain. Seven cysteine residues are bolded and numbered. Three disulfide bonds are connected according to conserved model. Cysteine 4 (arrow) is utilized for inter-chain disulfide bond formation.

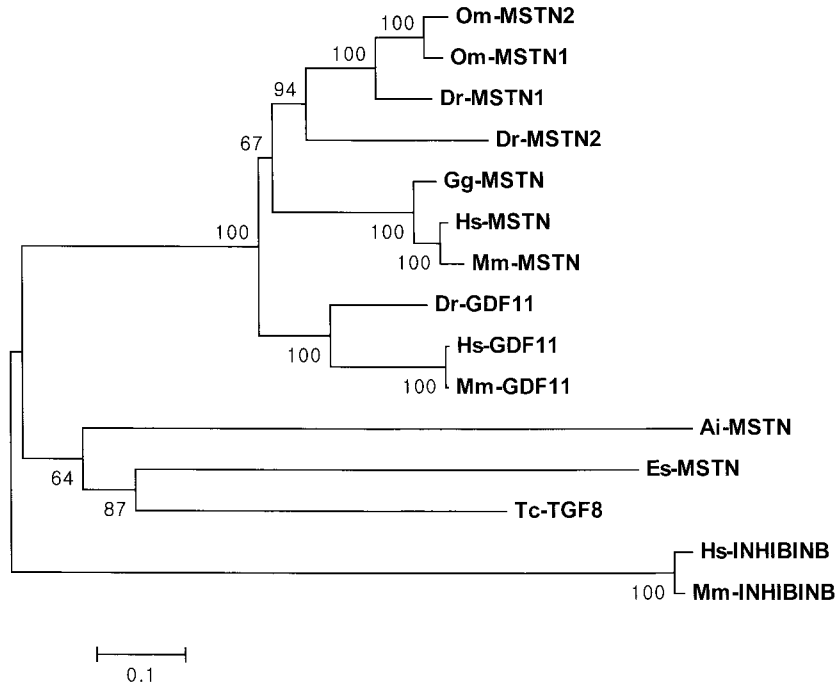

Fig. 4. Phylogenetic diagram of the TGF-β family members based on the amino acid sequence similarity. Phylogenetic tree was generated by the neighbor-joining method with ClustalW program and represented by MEGA4 program (See the legend to Fig. 3 for species). The bootstrapping replication was 500.

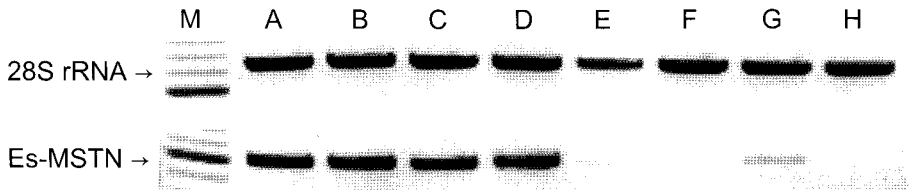

Fig. 5. Expression of Es-MSTN genes in various tissues.
Regular end-point RT-PCR was carried out and amplicons were run on a 1.5% agarose gel. The 28S rRNA gene was used as a positive control (top). M, marker; A, claw muscle; B, leg muscle; C, thoracic muscle; D, heart; E, epidermis; F, gill; G, hindgut; H, hepatopancreas.

expressed in cardiomyocytes, mammary glands and adipose tissue (McPherron et al., 1997; Ji et al., 1998; Sharma et al., 1999). Detailed roles in those tissues are still not clear and need to be elucidated.

Proposed role of Es-MSTN during molting cycle

Crustacean growth and development is associated with cyclical molting (ecdysis). Unlike mammals, most deca- pods exhibit very dramatic muscle fiber

change according to molt stages which is believed to be mediated by ecdysteroids. Muscle protein synthesis rates are elevated with peak during the premolt and postmolt, which suggested that elevated ecdysteroid level positively affect its expression (El Haj et al., 1987). 20-HE injection induced the protein synthesis in the claw, leg and abdominal muscle of the lobster after three days (Haj et al., 1996). These elevated muscle proteins are not always involved in increasing muscle mass. The large claw muscle undergoes atrophy by 30-60% prior to ecdysis in both lobster and land crabs (Skinner, 1966; Mykles et al., 1982). This process appears to be necessary for pulling throughout the relatively small aperture of the basi-ischial joint. This molt-induced claw muscle atrophy is the protein turn-over by several calcium-dependent proteinases (Mykles et al., 1986). These findings indicate that muscle dynamics in crustacean is a complex process coordinated by transcript-tional or translational regulations in different tissue types. In present study, we found additional regulator in muscle dynamics, Es-MSTN. We also found that its structural and expressional character is much similar to mammalian myostatins. Further study would be necessary to know the relationship between 20-HE, myostatin and mass of muscle. This result will help to understand dynamics of muscular fibers in commercially important crustacean and help to facilitate to build up muscle fiber to produce the high-quality seafood in the future.

Acknowledgements

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