Characterization and Expression Pattern of the Partial Myostatin cDNA in Shrimp, *Fenneropenaeus chinensis*

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Abstract Muscle tissue expresses many muscle-specific genes, including myostatin (also known as GDF8) that is a member of the transforming growth factor-beta superfamily. Myostatin (MSTN) negatively regulates mammalian skeletal muscle growth and development by inhibiting myoblast proliferation. Mice and cattle possessing mutant MSTN alleles display a 'double muscling' phenotype characterized by extreme skeletal muscle hypertrophy and/or hyperplasia. In this study, we first have characterized partial cDNA of a MSTN gene from the muscle tissue in the *F. chinensis* and examined its expression pattern in various tissues. The partial MSTN gene (GenBank accession number EU 131093) in the *F. chinensis* was 1134 bp, encoding for 377 amino acids that showed 63-93% amino acid similarity to other vertebrate MSTNs, containing a conserved proteolytic cleavage site (RXRR) and conserved cysteine residues in the C-terminus. Based on a RT-PCR, the MSTN gene was expressed in the all tissues of *F. chinensis* used in this study.

Key words : Myostatin; GDF-8; TGF-β; *Fenneropenaeus chinensis*

Introduction

The transforming growth factor β (TGF-β) superfamily includes a number of factors that are responsible for growth and development of tissues. One member of this superfamily, originally identified as growth and differentiation factor-8 (GDF-8), was first characterized in the mice when it was discovered that disrupting the GDF-8 gene resulted in a dramatic increase in muscle mass without notable effects on other tissues [12]. This protein was renamed myostatin (MSTN) based on the phenotype of GDF-8 null mice and the predominant expression of the GDF-8 in muscle [12]. Naturally occurring MSTN mutations were attributed to the 'double muscle' phenotype observed in some breeds of cattle [5,6,12].

MSTN gene has been cloned and identified from a wide variety of vertebrates including human [3], mice, cattle [12], chicken [9,12], and several fish species such as zebrafish [1,2,20], Atlantic salmon [13], rainbow trout [15], brook trout [16], striped bass, tilapia [17], gilthead sea bream [10,11], channel catfish [7], and European sea bass [19]. While MSTN gene is expressed primarily in myogenic linage cells in mammals, MSTN gene in fish is expressed in a variety of tissues including muscle, gill, eyes, tongue, spleen, heart, stomach, intestine, kidney, liver, ovaries, brain, and testes [4,8, 10,11,13-15,19]. This diverse pattern of expression in fish suggests that the biological actions of MSTN may not be restricted to the skeletal muscle but may influence other tisssues as well.

Stduies in mammalian and avian species have shown

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that manipulation of myostatin activity is a potential means to improve skeletal muscle growth of these species (Lee and McPherron, 2001; Bogdanovich et al., 2002; Kim et al., 2006 and 2007; Yang et al., 2001; Whittemore et al., 2003). In fish, two recent studies examined the effect of modulating myostatin on muscle growth in zebrafish by over-expressing MSTN prodomain [20] or disrupting MSTN production with anti-sense morpholinos [1]. Although the results of these two studies in zebrafish could not unequivocally demonstrate the effect of MSTN inhibition on fish muscle growth, it was suggested that MSTN has a potential as a muscle growth inhibitor also in fish. The successful manipulation of MSTN expression and/or its bioactivity in commercially important fishes, thus, appears to have a profound impact on the efficiency of aquaculture production. *Fenneropenaeus chinensis* has become one of the important aquaculture species in Koean, and herein we report the cloning and characterization of MSTN gene from the *F. chinensis,* as well as its expression in various tissues.

Materials and Methods

Sample Collection

Three-months-old *F. chinensis* were obtained from the West Sea Mariculture Research Center (NFRDI, Korea), and tissues samples (muscle, gill, hepatopancreas, gut, heart and stomach) were dissected, rapidly frozen in liquid nitrogen and stored at -80℃ until analysis.

Cloning of the partial F. chinensis MSTN cDNA.

Total RNA was extracted from six tissues using Trizol Reagent (Invitrogen, USA), respectively, and following the manufacturer's instructions. The RNA quality and quantity were checked by spectrophotometry (Ultrospec 3100pro, Biosciences). One microgram of total RNA extracted from muscle tissue was reverse transcribed using a reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA, USA) and oligo dT primers to obtain first-strand cDNA. The cDNA was used as template for the subsequent PCR reactions. All PCR reactions were performed using a iCycler (Bio-Rad, Hercules, CA, USA), under the following conditions: 3 min at 94℃ (initial denaturation), 35 cycles with 40 sec at 94℃, 40 sec at 58℃, 1 min at 72℃, and 10 min at 72℃ (final extension). Two primer sets, NF13, NR24 and NF14, NR23 (Table 1), were designed on the basic of MSTN sequences in other species. A 5ul aliquot of the PCR product was loaded to electrophoresis on a 1.0 % agarose gel, stained with ethidium bromide and visualised under UV light. Two partial fragments (330 bp) and (804 bp) were amplified with the primer sets. The PCR product was cloned into pGEM-T vector (Promega, SanLuis Obispo CA, USA) and sequenced.

Sequence analysis and alignment

Similarity searches of the sequenced DNA fragment and the deduced amino acid sequence were carried out by BLAST program (http://www.ncbi.nlm.nih.gov/blast/). Species representatives for mammalian (mouse AY204900), avian (chicken AY448007) and fishes (*M. saxatilis* AF290910 and zebrafish AF540956) were selected for the sequence alignments, and Poisson-corrected distances were estimated for all possible pairs. A phylogenetic tree was constructed using the neighbor-joining method based on the obtained distance matrix, and the node robustness was assessed using the bootstrap method (1000 replications). All phylogenetic analyses were carried out using the PHYLIP program and the phylogenetic tree of MSTN was constructed with the programs CLUSTAL W 1.83, BioEdit, PHYLIP, and TreeView software.

Expression analysis of the MSTN gene

Expression analysis by reverse transcriptase polymerase chain reaction (RT-PCR) was carried out on total RNA that was extracted using the same method described above, from the muscle, gill, hepatopancreas, gut, heart, and stomach of the *F. chinensis*. The same amount of RNA (1 ug) for each sample was reverse transcribed using oligo $dT_{(16)}$ as a primer. The cDNA was amplified mature fMSTN gene (NF14 and NR23) by PCR reaction under the following conditions; denaturation at 94℃ for 40 sec, annealing 40℃ for 40 sec,

Table 1. Sequence of primers used in this study

Primer	Direction	Sequence (5' - 3')
Primer for RT-PCR		
NF13	Forward	ATG CAT CTG TCT CAC ATT GTG CT
NF14	Forward	GAG ACG CGG GCC TGG ACT GTG
NR23	Reverse	TCA AGA GCA CCC GCA ACG GTC
NR24	Reverse	TCC TGA CTC GCT TTG GGC CGT

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M  H  L  S  H  I  V  L  Y  L  S  L  L  V  A  L  G  P  V  V  L  S  D  Q  E     25
ATGCATCTGTCTCACATTGTGCTCTATCTCAGTTTGCTGGTTGCTTTGGGTCCAGTAGTTCTGAGCGACCAAGAG   75

T  H  H  Q  Q  P  S  A  S  S  P  E  D  A  E  Q  C  A  T  C  D  V  R  Q  Q     50
ACGCACCATCAGCAGCCCTCGGCCAGCAGCCCGGAGGACGCGGAGCAGTGCGCCACCTGCGACGTCCGGCAGCAG  150

I  K  T  M  R  L  N  A  I  K  S  Q  I  L  S  K  L  R  M  K  E  A  P  N  I     75
ATAAAAACCATGCGACTAAACGCGATCAAATCTCAGATTCTGAGCAAACTGCGAATGAAGGAAGCTCCGAACATC  225

S  R  D  I  V  K  Q  L  L  P  K  A  P  P  L  Q  Q  L  L  D  Q  Y  D  V  L    100
AGCCGAGACATTGTGAAGCAGCTCCTGCCCAAAGCACCGCCGCTGCAGCAGCTTCTCGACCAGTACGACGTGCTG  300

G  D  D  N  R  D  V  V  M  E  D  D  D  E  H  A  T  T  E  T  I  M  M  M  A    125
GGAGACGACAACAGGGATGTGGTCATGGAGGATGATGATGAGCACGCCACCACAGAGACAATCATGATGATGGCC  375

T  E  P  E  P  I  V  Q  V  D  A  E  P  K  C  C  F  F  S  F  T  Q  K  F  Q    150
ACCGAACCCGAGCCCATCGTCCAGGTGGATGCGGAGCCCAAGTGCTGCTTTTTCTCTTTTACCCAAAAGTTTCAA  450

A  S  R  I  V  R  A  Q  L  W  V  Y  L  R  P  A  D  E  A  T  T  V  F  L  Q    175
GCCAGTCGCATCGTGCGGGCGCAGCTCTGGGTCTACTTGCGGCCGGCGGACGAGGCGACCACCGTGTTCCTGCAG  525

I  S  R  L  M  P  V  T  D  G  S  R  H  I  R  I  R  S  L  K  I  D  V  N  A    200
ATCTCCCGCCTGATGCCGGTCACGGACGGGAGCAGGCACATACGGATCCGTTCCCTGAAGATTGACGTGAACGCC  600

G  L  S  S  W  Q  S  I  D  V  K  Q  V  L  T  V  W  L  R  Q  P  E  T  N  W    225
GGGCTCAGCTCCTGGCAAAGTATAGACGTCAAACAAGTGTTGACTGTGTGGCTGCGGCAGCCGGAGACCAACTGG  675

G  I  E  I  N  A  F  D  S  R  G  N  D  L  A  V  T  S  T  E  P  G  E  E  G    250
GGCATCGAGATTAACGCCTTCGATTCGAGGGGAAACGACTTGGCCGTGACCTCCACAGAGCCCGGAGAGGAAGGA  750

L  Q  P  F  M  E  V  K  I  T  D  G  P  K  R  V  R  R  D  A  G  L  D  C  D    275
CTGCAACCATTCATGGAGGTGAAGATCACCGACGGCCCAAAGCGAGTCAGGAGAGACGCGGGCCTGGACTGTGAC  825

E  N  S  P  E  T  R  C  C  R  Y  P  L  T  V  D  F  E  D  F  G  W  D  W  I    300
GAAAACTCCCCAGAGACCCGGTGCTGCCGCTATCCGCTCACAGTTGACTTTGAGGACTTTGGCTGGGACTGGATT  900

I  A  P  K  R  Y  K  A  N  Y  C  S  G  E  C  E  Y  M  H  L  Q  K  Y  P  H    325
ATTGCCCCAAAGCGCTACAAGGCCAACTATTGCTCTGGGGAGTGTGAGTACATGCACTTGCAGAAGTATCCCCAC  975

T  H  L  V  N  K  A  N  P  R  G  T  A  G  P  C  C  T  P  T  K  M  S  P  I    350
ACCCACCTGGTGAACAAAGCCAACCCCAGAGGGACCGCAGGCCCCTGCTGTACCCCCACCAAGATGTCGCCCATC  1050

N  M  L  Y  F  N  R  K  E  Q  I  I  Y  G  K  I  P  S  M  V  V  D  R  C  G    375
AACATGCTCTACTTTAATCGCAAAGAGCAGATCATCTACGGCAAGATCCCCTCCATGGTGGTGGACCGTTGCGGG  1125

C  S  *     377
TGCTCTTGA   1134
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Fig. 1. The nucleotide and deduced amino acid sequences of *F. chinensis* myostatin (fMSTN). The proteolytic processing site (RXRR) is underlined.

extension at 72℃ 1 min, for a total of 35 cycles. Five microliters of PCR product were subjected to electrophoresis on a 1.0 % agarose gel. The *F. chinensis* β -actin gene (GenBank accession number DQ205426) was used as a positive control.

Results and Discussion

The partial cloning of *F. chinensis* MSTN (fMSTN) has resulted in 1134 bp (GenBank accession number EU131093), coding for a 377-amino-acid protein (Fig. 1). The fMSTN sequence has an MSTN RXRR cleavage site and nine conserved cysteine residues (Fig. 2).

Alignment and phylogenetic analysis of the MSTN amino acid sequence from the *F. chinensis* and other vertebrates are shown in Fig. 2 and Fig. 3. The deduced amino acid sequence of unprocessed, prodomain, and mature fMSTN region had homology with unprocessed MSTN region: *Mus musculus*(63%), *Gallu gallus* (64%), *Danio rerio* (80%), and *Moron saxatilis* (93%); prodomain MSTN region : *M. musculus* (52%), *G. gallus* (53%), *D. rerio* (74%), and *M. saxatilis* (91%); mature MSTN region *: M. musculus* (89%), *G. gallus* (89%), *D. rerio* (94%), and *M. saxatilis* (100%) (Table 2). The multiple sequence alignment showed that mature region, which was situated between the RXXR motif (residues 265-268, RARR, matching the RXXR consensus site) and the carboxy terminal, was highly conserved from fish to mouse.

Expression of the mature fMSTN gene in various *F. chinensis* tissues such as muscle, gill, hepatopancreas,

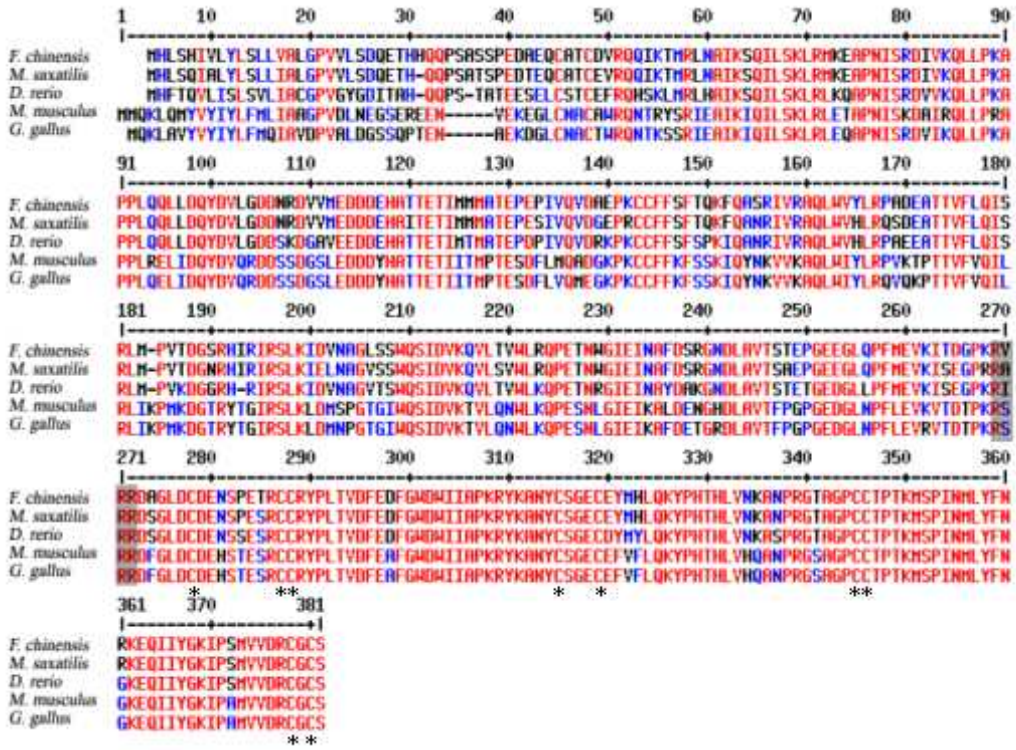

Fig. 2. Amino acid alignment of mouse (*M. musculus*; accession number AY204900), chicken (*G. gallus*; accession number AY448007), zebrafish (*D. rerio*; accession number AF540956), strip sea-bass; accession number AF290910), and shrimp (*F. chinensis*; accession number EU131093) MSTNs. Grey boxes represent the putative RXRR proteolytic processing site. Conserved cysteine residues are denoted with asterisks.

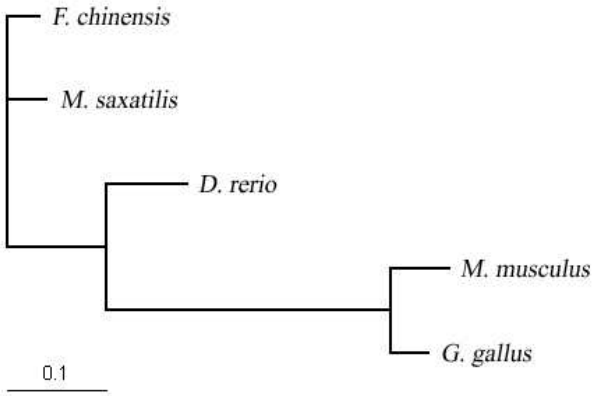

Fig. 3. Phylogenetic tree was constructed by the neighbor-joining method, base on the alignments of the coding regions of MSTNs. The tree was generated via CLUSTAL W 1.83 and depicted visually via Tree-View 1.6.6. Positions containing gaps were excluded from the analysis. Numbers at tree nodes refer to bootstrap values after 1000 replicates. The scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site.

gut, heart, and stomach was analyzed by RT-PCR. fMSTN region was expressed in all tissues (Fig. 4).

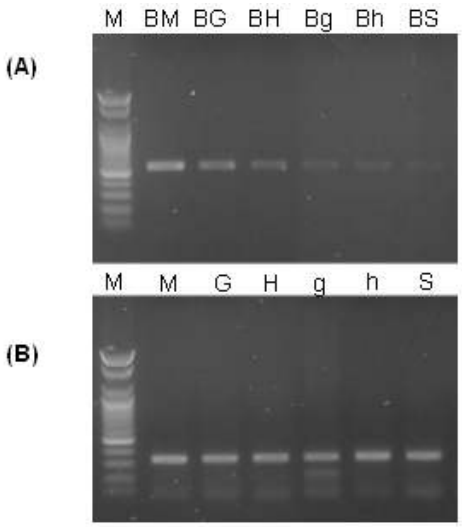

Fig. 4. Expression pattern of MSTN mRNA in various tissues of *F. chinensis*. RNA samples were isolated from various tissues. One microliter of first strand cDNA was used as the template for PCR amplification of MSTN (panel B) and a β-actin cDNAs fragments (panel A). The PCR products were subjected on 1.0 % agarose gels in TAE buffer. M, 100 bp ladder marker; B, β-actin; M, muscle; G, gill; H, hepatopancreas; g, gut; h, heart; S, stomach.

Table 2. Amino acid sequence identities of myostatin from various organisms

	F. chinensis	*M. musculus*	*G. gallus*	*D. rerio*	*M. saxatilis*
Unprocessed amino acid					
F. chinensis	100				
M. musculus	63	100			
G. gallus	64	90	100		
D. rerio	80	66	66	100	
M. saxatilis	93	62	63	81	100
Prodomain					
F. chinensis	100				
M. musculus	52	100			
G. gallus	53	87	100		
D. rerio	74	58	57	100	
M. saxatilis	91	52	52	75	100
Mature					
F. chinensis	100				
M. musculus	89	100			
G. gallus	89	100	100		
D. rerio	94	88	88	100	
M. saxatilis	100	88	88	95	100

Identities are given in relation to the unprocessed amino acids to the regions upstream and downstream of the proteolytic processing site. Sources include: mouse MSTN (*M. musculus*; accession number AY204900), chicken MSTN (*G. gallus*; accession number AY448007), zebrafish MSTN (*D. rerio*; accession number AF540956), strip sea-bass MSTN (*M. saxatilis*; accession number AF290910), and shrimp MSTN (*F. chinensis*; accession number EU131093).

In this study, we first isolated and characterized partial cDNA of a MSTN gene in the *F. chinensis*, which shared similar structures with other vertebrate MSTN genes. The putative amino acid sequence showed a highly conserved carboxy-terminal portion, corresponding to the mature processed protein. A potential proteolytic processing site (RARR, matching the RXXR consensus site) and nine cysteine residues were present, as in all vertebrate MSTN genes [4,5,12].

Although the fMSTN gene shares high structural similarity with mammalian counterparts, the expression pattern of MSTN in fish is quite different from that in mammals. In mice, MSTN is strongly expressed in skeletal muscles and weakly expressed in cardiomyocytes, mammary glands, and adipose tissue [12, 18]. However, the MSTN expression of tilapia was detected in several tissues including muscle, eyes, gill filaments, brain, gut, and gonads, but not in the liver, kidney, stomach, or heart [17]. In our study, the fMSTN was expressed in all tissues as other fishes [13,15-17]. These data suggest that biological actions of MSTN in lower vertebrates may not be restricted to negative muscle growth regulation, but may also help to regulate the growth and activity of supplemental tissues as well. Therefore, future studies need to elucidate the physiological and molecular mechanisms of MSTN *in F. Chinensis*, and such an understanding will be useful in manipulating the fMSTN to improve the production efficiency of *F. chinensis* in aquaculture industry.

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