



Molecular characterization of the myostatin gene and the effect of fasting on its expression in Chinese perch (*Siniperca chuatsi*)

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ABSTRACT. Myostatin (MSTN) is an important member of the transforming growth factor- β (TGF- β) superfamily and is a muscle growth inhibitor. In the present study, we cloned the Chinese perch MSTN cDNA sequence and analyzed its expression patterns under various conditions. The MSTN full cDNA sequence was 3347 bp long, including an open-reading frame of 1131 bp, which encoded 376 amino acids. Sequence analysis demonstrated that the MSTN shared a highly conserved signal peptide, a TGF- β functional peptide, a hydrolytic site (RARR), and nine conservative cysteine residues with other members of the TGF- β superfamily. Sequence alignment and phylogenetic tree analyses indicated that the MSTN had a close relationship with teleostean fish, but they are far separated from mammals. Real-time polymerase chain reaction analysis revealed that the MSTN was strongly expressed in the skeletal muscle and heart tissues. Temporal expression analysis demonstrated that the MSTN gene was expressed

in very low levels, from 20 to 90 dph (post-hatching development), and was at its highest level at 150 dph ($P < 0.05$). The fasting-re-feeding experiment showed that the expression of the MSTN gene was initially decreased in response to a single meal, after seven days of fasting, and subsequently increased significantly, and finally decreased back to its original level. Together, our results provided valuable knowledge regarding the regulation of MSTN gene expression in Chinese perch.

Key words: Chinese perch; Myostatin; cDNA cloning; Gene expression; Fasting-re-feeding

INTRODUCTION

Myostatin (MSTN), a member of the transforming growth factor- β (TGF- β) superfamily, acts as a negative regulator to inhibit skeletal muscle growth during myoblast proliferation and differentiation in teleost fish (Lee and McPherron, 2001). Knocking out the MSTN gene resulted in both the hypertrophy and hyperplasia of skeletal muscle, which was first discovered in cattle by McPherron and Lee (1997). Oppositely, its overexpression or systemic administration caused muscle atrophy (Rodriguez et al., 2014). MSTN plays a role in skeletal muscle growth by binding its receptors (activin type II receptors) on the cell surface (Bassi et al., 2015) and downregulating myogenic gene expression. *In vitro* studies demonstrated that MSTN cDNA sequences and expression patterns were characterized in a variety of teleost fish species including *Oreochromis mossambicus*, *Morone chrysops* (Rodgers et al., 2001), *Zebrafish* (Xu et al., 2003), and *Lates calcarifer* (De Santis et al., 2008).

After the discovery of the MSTN gene and its function in teleost species, its expression profiles were continually being confirmed in various tissues and developmental stages (Otto et al., 2010). The MSTN gene was primarily expressed in the skeletal muscle, and was present at lower levels in the adipose tissue (McPherron and Lee, 1997) and cardiac muscles (Gonzalez-Cadavid et al., 1998). Some studies have reported that the fish MSTN gene was extensively expressed in various tissues such as the muscles, eyes, gill filaments, spleen, ovaries, gut, brain, and testes (Rodgers et al., 2001; Østbye et al., 2001). The MSTN gene plays a negative regulatory role in embryonic skeletal muscle growth and the development of channel catfish, and is expressed strongly in the larvae, juveniles, and adults, but weakly in the embryos and older fish (Kocabas et al., 2002). In two-year old rainbow trout, the expression of the MSTN gene appeared to be tissue specific, and was high in the brains, testes, eyes, spleen, and skeletal muscles, but was weaker during the gastrulation, and then increased before hatching and yolk sac absorption (Garikipati et al., 2007).

Fish have the ability to adapt to survive for long periods without food and through metabolic depression (Cook et al., 2000). It has been reported that the MSTN gene acts as an important factor in tissue-regulation and energy conservation. Its abundant levels in the skeletal muscle of *Lates calcarifer* confirmed this genes involvement in the compensatory growth induced by refeeding. The MSTN mRNA expression increases with fasting and subsequently decreases after re-feeding (Terova et al., 2006). Nutritional status significantly influenced the MSTN gene expression levels in the skeletal muscle, resulting in an up-regulation during fasting and a down-regulation during the recovery after nutritional deprivation (De Santis and Jerry, 2011). However, the MSTN mRNA levels of *Coilia nasu* decreased in the muscle and increased in the brain when fasting (Du et al., 2014).

The Chinese perch (*Siniperca chuatsi*) is a freshwater fish and one of the most commercially important carnivorous fish species that is found in China as well as in eastern Asia, as (Chu et al., 2010). Its high nutritional value, high protein content, and appealing taste are responsible for its large-scale culture. In this study, we focused on the cloning, characterization, and expressional profile of the MSTN gene in various tissues and at various development stages and nutritional statuses.

MATERIAL AND METHODS

Experimental fish

The Chinese perch individuals used in this study were provided by the Fisheries Research Institute of Hunan Province, Changsha, China. The brain, muscle, intestine, heart, spleen, liver, and kidney tissues were collected from five fish [average body weight 500 g, 150 dph (post-hatching development)] and immediately preserved in liquid nitrogen. The muscle samples were used for the full-length cDNA Library Constructing Sequencing and for investigating the developmental ontogeny of the MSTN gene. Additionally, all tissues were surveyed for the mRNA expression of the MSTN gene by using a quantitative, real-time polymerase chain reaction in Chinese perch. Moreover, the muscles were dissected from the dorsal myotome of individuals in various developmental stages including 20 dph (equal to the weight of 5 g), 30 dph (10 g), 50 dph (50 g), 70 dph (100 g), 90 dph (150 g), and 150 dph (500 g) to analyze the temporal expression of the MSTN gene. All samples were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

Fasting-re-feeding experiment

In order to investigate the MSTN gene signature in response to nutrient restriction and re-feeding in the skeletal muscle of Chinese perch, 50 Chinese perch juveniles (average body weight 150 g, 90 dph) were reared in a net cage (5 m x 5 m x 2 m) for the fasting experiment. All juveniles were fed under standard conditions for two weeks. Juveniles were then fasted for one week, and fed a single meal, distributed to all individuals, to the point of visual satiation. Samples (fast muscles) were also collected at 0 h (before the recovery meal), and at 1, 3, 6, 12, 24, 48, 96 h (hours after the single meal), from seven random fish each time. All collected muscle samples were snap-frozen in liquid nitrogen and stored at -80°C to await further processing.

All of the procedures in our experiment were approved by the Hunan Province Animal Welfare Committee.

First strand cDNA synthesis

All of the tissue samples were kept in liquid nitrogen. Total RNA was extracted using the TRIzol® reagent (Invitrogen, USA) according to the manufacturer protocols. The quality of the RNA integrity was assessed using a 1.0% agarose gel electrophoresis. Approximately 1 µg total RNA was used for the first strand cDNA synthesis with a Prime Script 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) according to the specifications instructions. Negative controls contained neither a cDNA template nor transcriptase.

Rapid amplification of cDNA ends (RACE)

Two pairs of Specific primers (Table 1) were designed to amplify the ORF of the MSTN mRNA according to the *Siniperca scherzeri* mRNA sequence cloned by our laboratory (GenBank accession No. JN399996) by using the software package of primer 5 and being synthesized at the Yingjun Biotech Company (Shanghai, China). The standard PCR protocol was carried out as previously described (Zhu et al., 2016).

Table 1. Primers used for cloning and quantifying the MSTN genes.

Items	Name	Primer sequence (5'→3')	PCR objective
ORF	MSTN-F1	ATGCATCTCTCTCAGATTGTGCTGTA	ORF for the MSTN
	MSTN -R1	CCCAGCCAAAGTCTTCAAAGTCCAC	ORF for the MSTN
	MSTN -F2	ACTCCGGGCTCAGCTCTGGGTGAT	ORF for the MSTN
	MSTN -R2	TCAAGAGCATCCACAACGGTCCAC	ORF for the MSTN
3'-UTR	3'RACE outer	TACCGTCGTCCACTAGTGATTI	Universal primer for 3'RACE
	3'RACE inner	CGCGGATCCTCCACTAGTGATTTCACTATAGG	Universal primer for 3'RACE
	M-3'RACE GSP1	GAAGACTTTGGCTGGGACTGG	3'RACE for the MSTN
	M-3'RACE GSP2	TCGCCATCAACATGCTCTAC	3'RACE for the MSTN
5'-UTR	5'RACE outer	CATGGCTACATGCTGACAGCCTA	Universal primer for 5'RACE
	5'RACE inner	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	Universal primer for 5'RACE
	M-5'RACE GSP1	ACATCCTTGTGTCATCTCCAGCA	5'RACE for the MSTN
	M-5'RACE GSP2	GATATTAGGAGCTTCTTTCATTTCGC	5'RACE for the MSTN
RT-PCR	MSTN-RT-F	GCACATACGCATCCGCTCCCT	Real-time PCR for MSTN
	MSTN-RT-R	GTCACGGCCAAGTCATTTCCTA	Real-time PCR for MSTN
	RPL13-F	CACAAGAAGGAGAAGGCTCGGGT	Internal control (housekeeping gene)
	RPL13-R	TTTGGCTCTCTGGCACGGAT	Internal control (housekeeping gene)

F = Forward primer; R = Reverse primer.

To obtain the full-length cDNA sequences, the gene specific primers in Table 1 were designed according to the ORF cDNA sequence we obtained. Then, a semi-nested PCR was used to obtain the 5' and 3' unknown regions using a 3' and 5'-Full Race Kit (Takara). The obtained products were isolated using an agarose Gel-Purified Kit (OMEGA, USA). The DNA fragments were ligated into the pMD19-T vector (TaKaRa) and subsequently transformed into *Escherichia coli* DH5 α competent cells. The recombinants were eventually identified by blue and white spot selection. Putative clones were further screened by PCR amplification, and selected positive clones were sequenced using the BioSune Biotech Company (Shanghai, China). All sequenced fragments were assembled using the software DNASTAR.

Bioinformatic analysis

After sequencing, the result of the cDNA sequences of the MSTN gene was checked for homology among other entries by comparing it with the BLAST database from NCBI (NCBI, <http://www.ncbi.nlm.nih.gov>). The open-reading frame (ORF) sequences were predicted using an ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/orFigure.cgi>) and translated into the amino acid sequence via a translation tool (<http://web.expasy.org/translate/>). Analyzing the computation of various physical and chemical parameters was done using a ProtParam tool (<http://web.expasy.org/protparam/>). The database analysis of protein domains was conducted using the website <https://www.predictprotein.org/>, <http://prosite.expasy.org/>. Phylogenetic analyses were carried out using the software Mega 4.0 and Clustal X to determine the evolutionary relationship between the different related species by utilizing the neighbor-joining (N-J) and bootstrapping (for 1000 replicates) methods (Tamura et al., 2013).

Expression by RT-PCR

Quantitative, real-time PCR (RT-qPCR) was performed to analyze the expression patterns of the MSTN gene in Chinese perch under different conditions including the gene expression in various tissues and at various developmental stages as well as the effects of fasting-re-feeding. The total RNA was extracted from the samples by utilizing the first strand cDNA synthesis according to the manufacturer protocols as previously described. The SYBR Premix Ex Taq™ RT-PCR kit (TaKaRa) was used for Quantitative RT-PCR (RT-qPCR) which was performed in 25- μ L reaction mixtures containing 12.5 μ L SYBR Green mix, 2 μ L template cDNA, 1 μ L each primer (10 mM), and 8.5 μ L sterile water. This cycle was performed using fluorescent, quantitative instruments (Bio-Rad CFX96 system, USA): pre-incubation at 95°C for 60 s, amplification and quantification for 39 cycles of 95°C for 5 s, 60°C for 30 s, melting curve analysis from 65 to 95°C with a heating rate of 0.1°C/s and a fluorescence measurement to ensure consistency and the specificity of the amplified product. All of the RT-qPCR primers were designed using Primer Premier 5 software and synthesized by the Shanghai Yingjun Biology CompanyI (Table 1). RPL13 was believed to be a stable and suitable housekeeping gene and was to be used as an inner control. A no-template reaction was always used as a negative control to ensure the accuracy of the results, and each tissue sample was analyzed in triplicate. The relative expression ratio (R) of MSTN gene mRNA was calculated by using the comparative Ct method (known as the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where Ct is the cycle threshold. The basic equation was as follows:

$$\Delta\Delta Ct = (Ct_{\text{targetgene}} - Ct_{\text{housekeeping gene}})_{\text{experiment}} - (Ct_{\text{targetgene}} - Ct_{\text{housekeeping gene}})_{\text{control}}$$

(Equation 1)

Statistical analysis

All data are reported as means \pm SE. Statistical significance in the control and experimental groups was assessed by one-way ANOVA procedures using the Duncan test and regression analysis of SPSS17.0 Data Editor. The differences were considered statistically significant when $P < 0.05$ and represented with a, b, c, d superscripts or with an asterisk. All experiments were repeated at least three times.

RESULTS

Molecular characterization of the Chinese perch MSTN gene

Initially, we obtained a cDNA fragment of 1131 bp using the two primer pairs of MSTN-F1 and MSTN-R1, and MSTN-F2 and MSTN-R2. We then cloned the unknown ends of cDNA by rapid amplification of the cDNA ends (RACE). The full-length sequence of the Chinese perch MSTN cDNA (GenBank accession No. JF896453) was obtained by splicing and merging overlapping PCR fragments (Figure 1). The MSTN gene had 863 bp of the upstream sequence (5'-UTR), the open reading frame (ORF) of 1131 bp encoding a 376-amino acid protein, 1353 bp of the downstream sequence (3'-UTR) containing the termination signal (TGA), and a typical polyadenylation signal site (AATAAA).

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1      CGATGGCGCTGTCTTCTCATGTGCTGCAACCAGGTTTCAGCAACAAGGATTTGGACTAA
60     GCATTTTCTTTATCTTTGCTGACTTTTTTTTTTTTTTTTATTCCCTGTACGGGAGACGATGT
120    ATTACCGTTTGTGGTTTTACATGAGCCGATAATTCAGCTTCTCAGAAGGGAACCTGGTCTA
180    AACAGACGACACCTAGAGAAGGACTTGACTTGCACTGGAGAAAACCTGTAACTCTTATTTT
240    TCTTCCTCAAGGATGAAATGTATTTGCACAACCAGGCAGACA CAGCTG CATCCATGTCCC
300    TGTCATTAAAGA CAATTAATGTTTGACGGACTTATCGTGCCCGCAGTTAAAAAGTTTCCT
360    TTGTTTTCTTTGGAAACTAAATGTATTCACAGCACAACTGCACACGAAGTCGCACATCT
420    TGCTGTCTTTCATGATATGCAGCAGGCTCTGTAGCCTAAGCTGTTTGTGTTTAGTTTTG
480    ATCTGGGCTGATTGAGGTGCATCAGGGCGTTCCATATAACACAACAGTCCGGTCTGCGCC
540    CCTTCCCCTCACTGCGCAGTCACTGCAAGAAAAGTGAATTTATC CATCTG TGGACACGTT
600    CCGCACACGCTCACAGTCTCCGTCCTTTATGGTTTGACAACGAAAAAAGTTTTTCATGT
660    CAGTCGGTTAAAATTCATTGTTGCCTGTCCAGC CAATCATAGTTTTTGACGACACAAAAG
720    AGGCTAAAGTTGGAGTATAAAAAGGTGTGCGCTAATAAAGTATGATGCCTATCAGTGTGG
780    GACATTAATCCAAACCCAGTCCAGTCGCCTATCAGGTCCAGCACACACCAAGGGATCTTT
840    TTGTAAC TTCACACCTTAGAGACA ATGCATCTCTCTCAGATTGTGCTGTATCTTAGCTTG
1      M H L S Q I V L Y L S L
900    CTGCTTGCTTTGGGTCCAGTAGTTTTGAGTGACCAAGAGACGCACCAGCAGCCCTCCGCC
13     L L A L G P V V L S D Q E T H Q Q P S A
960    ACCAGCCAGAAGACACGGAGCAGTGCGCCACCTGCGAGGTCCGGCAGCAGATTAAAACT
33     T S P E D T E Q C A T C E V R Q Q I K T
1020   ATGCGATTAAACGCGATAAAATCTCAGATTTTGTAGCAAACCTGCGAATGAAAGAAGCTCCT
53     M R L N A I K S Q I L S K L R M K E A P
1080   AATATCAGCCGAGATATAGTGAAGCAGCTCCTGCCCAAAGCGCCGCGCTGCAGCAGCTT
73     N I S R D I V K Q L L P K A P P L Q Q L

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Figure 1. Nucleotide and deduced amino acid sequences of the Chinese perch MSTN gene. Gray-shaded area indicates the conservative cysteine residues and the polyadenylation signal of myostatin (TATAAA).

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


Figure 1. Continued.

1140 CTCGACCAGTACGACGTGCTGGGAGATGACAACAAGGATGTGGCTATGGAGGAGGACGAT
 93 L D Q Y D V L G D D N K D V A M E E D D
 1201 GAGCATGCCATCACGGAGACAATAATGATGATGGCCACTGAACCCGAGTCCATCGTCCAG
 113 E H A I T E T I M M M A T E P E S I V Q
 1260 GTGGATGGGGAACCAAAGTGTGCTTTTTCTCTTTTAATCAAAGTTTCAAGCCAATCGC
 133 V D G E P K C C F F S F N Q K F Q A N R
 1320 ATACTCCGGGCTCAGCTCTGGGTGTATCTGCGCCCGGCGCCGAGGCGACCACCGTGTTC
 153 I L R A Q L W V Y L R P A A E A T T V F
 1380 CTGCAAATCTCCCGCTGATGCCGGTCACAGACGGGAACAGGCACATACGCATCCGCTCC
 173 L Q I S R L M P V T D G N R H I R I R S
 1440 CTGAAGATCGACGTGAATGCTGGGGCCGGCTCTTGGCAAAGTATAGACGTCAAACAAGTG
 193 L K I D V N A G A G S W Q S I D V K Q V
 1500 TTGACTGTGTGGCTGCGGCAGCCGAGACCAACTGGGGCATCGAGATTAACGCCTTCGAT
 213 L T V W L R Q P E T N W G I E I N A F D
 1560 TCGAGTGGAAATGACTTGGCCGTGACCTCCGCAGAGCCTGGAGAGGAAGGACTGCAACCA
 233 S S G N D L A V T S A E P G E E G L Q P
 1620 TTCATGGAGGTGAAGATCTCAGAGGGCCCCAAGCGCGCCAGAAGAGACTCAGGCCTGGAC
 253 F M E V K I S E G P K ~~E A E R~~ D S G L D
 1680 TGTGACGAGAACTCTCCAGAGTCCCGGTGCTGCCGCTATCTGCTCACAGTGGACTTTGAA
 273 C D E N S P E S R C C R Y L L T V D F E
 1740 GACTTTGGCTGGGACTGGATTATTGCCCCAAAGCGCTACAAGGCCAACTATTGCTCCGGG
 293 D F G W D W I I A P K R Y K A N Y C S G
 1800 GAGTGTGAGTACATGCACTTACAGAAGTACCCGCACACCCACCTGGTGAACAAGGCCAAC
 313 E C E Y M H L Q K Y P H T H L V N K A N
 1860 CCCAGAGGGACCGCAGGCCCTGCTGTACCCCAAGATGTGCCCCATCAACATGCTC
 333 P R G T A G P C C T P T K M S P I N M L
 1920 TACTTTAACCGAAAAGAGCAGATCATCTATGGCAAGATCCCTTCCATGGTGGTGGACCGT

Continued on next page

Figure 1. Continued.

353 Y F N R K E Q I I Y G K I P S M V V D R
 1980 TGTGGATGCTCT**TGA**GTTGGGACGGAGAGCCTGGCGAGAGGGAGGGGAGGACGGAGGGGC
 373 **C G C S** *
 2040 TGTGGCTCAGTCCGGCCTCCAACCTTCAGACTTTTTGACACAACCAGTCCACCAGTTCAG
 2100 TGCTTTCCTGCAGAACACGGTGAATAGAACCAGAGTAGAGGCCACAAACAGCCCGACCT
 2160 TCCTGCAGGGCAGCGCTTTCACAACCGGCATAGCTCTTACTTTTCTTCTCCAGTGAAA
 2220 TCTTAGCCATATAGAGGCTTGAAGTCAGATGGATGCAGGAACACACATACACACATGC
 2280 TGGACCTTGGAGTGAATGTAGACAGACATTATCAAAGTTATCCAAAACTTTTCTTCCCC
 2340 TTTGTCTCTGTGTGTGTGTCTGTCCAATTATACAAACAACACTGACAAATTATACTCGTA
 2400 CGCCATCTACTCCACTCCACTCGTAGCCAACATAACAAGCTACTACATTTTTTGTAAAGCT
 2460 ATATGTTTGTGATAATCATTTTTCAAGTTATGTTTTTCAGAGTGAAACCAGGAATCCTCAT
 2520 GGACCTTTTGAAGGGCTTGGAAAAACAAAATGGAGACTCTTTAGAGTGATATTTTACAA
 2580 CTGGTAGAACATGTTTTAGTACACAGGTTTCGTAAGAATTGATGAAAACCTGGCAAAACCTT
 2640 AGTCACCCCACTTTATTACTGCCATT**TATAAAA**CCTAGCCACAAATTGCAACATAACAC
 2700 AGGTCATCATCTCTCCTGTACTGGATCAAACCTTTAAGTTCTAATTTAGTTTTTTGATTAA
 2760 CCATACTGAGAACTTGTGAATAAAAATCCTAAGTGTAGGTGGTGAGATGCTGGTTGATCAC
 2820 TCCTGAGATCCTATGGACACAAAATGTTCCACCAGGGTGAAGTGCCACTTTAGGTCTTG
 2880 ACAGGTCTTCATGTACTCCATACATACACGCAGCAACATACCTGACCGAGGAGCGTTATT
 2940 GCTCAGTCATAATCTTCACTATCCATCAGAACAGCCTGACCTGCCCGATACACTTGAATT
 3000 ATTCTGATTGTAAATTCACATTACTGGACAGAAGGACTTGAACGAAGGCACAATGAATG
 3060 CAGCCTACAAATGAAAAATGTGTTTAAGACAGAGAAAATGGATTGTTGAAATGTATGAAT
 3120 GTTGAGATCATGCTTAAACTCTGTCTTACAACAACACAGTTTGCACATATGGCACACCAA
 3180 TAGAAAGAATTGGTTGCTACAAAAGTTGAAAAAAGTGAATTTGATATGTTTGCTAATTTG
 3240 TATTGTATACATATGCTATTGTTTCCATTAGGAGTTGCCTTTCTAAACCACTGTTGGTAA
 3300 ATGTATAAAACCACAACCTAGCAAGATAAAAAGATGTAATACAGCAACT

-  E-box domain
-  I-box domain
-  CAAT-box domain
-  Start codon
- * Termination codon
-  Conservatively hydrolytic
- TGF-β functional peptide area
- - - - - Signal peptide

The results of the analysis of the promoter prediction indicated that the promoter domain contained three E-boxes, one I-box, and two CAAT-boxes (Figure 1). The protein sequence of the Chinese perch MSTN gene was examined using the ExPasy online software. The results of the analysis indicated that the size of the protein mass was 42.56 kDa, the theoretical pI was 5.56 at PH 7.00, and that the molecular formula of its translated protein sequences was $C_{1865}H_{2960}N_{518}O_{568}S_{26}$. Among the 20 different amino acids, there was a relatively high proportion of leucine (Leu) (8.8%) and glutamic acid (Glu) (6.9%), as well as a low proportion of tryptophan (Try) (1.6%) (Table 2, Figure 2A). Additionally, it contained 49 delicious amino acids consisting of 23 aspartic acid (Asp) and 26 glutamic acid (Glu). For its property, it was predicted that the amino acids contained 49 strongly acidic (-, D, E) amino acids, 48 strongly basic (+, K, R, H) amino acids, 114 hydrophilic amino acids (S, Q, T, G, N, C), and 165 hydrophobic amino acids (A, I, L, P, F, M, W, V). This indicated that the hydrophobic amino acids were significantly greater in number than the hydrophilic amino acids, where they were evenly distributed in the N-terminal of the peptide chain (Figure 2B). For this function, the first 22 amino acids (M¹ to S²²) of the protein constituted the putative signal peptide, the middle 113 amino acids of the protein (R²⁶⁴-S³⁷⁶) consisted of a single conserved bioactive domain (TGF- β functional peptide area). Among the 376 amino acids, it also had a hydrolytic site (RARR) for releasing the mature peptide and nine conservative cysteine residues in the C-lobe bioactive domain (Figure 3). By using phosphorylation and glycosylation site analysis, we found that they contained 13 serine sites, three threonine sites, and two tyrosine sites, and their positions among the amino acids were Ser^{22,31,34,129,192,232,234,242,259,269,277,280,347}, Thr^{38,241,336}, and Tyr^{316,362}, respectively. Additionally, there was an N-link glycosylation propeptide motif site (NISR⁷³⁻⁷⁶) and an O-link glycosylation propeptide motif site (TSPE³³⁻³⁶). The results of the analysis of the secondary structure protein indicated that the composition of the MSTN gene precursor protein consisted of 18.35% H (helix), 59.57% E (strand), and 22.07% L (loop) whereas the Solvent Accessibility contained 54.52% exposed proteins and 45.48% buried proteins. In the maturation protein, there were 6.42% H (helix), 25.69% E (strand), and 67.89% L (loop), the Solvent Accessibility contained 63.30% exposed proteins and 36.70% buried proteins. The 3D structure of the protein aligned well with the protein templates and shared 88.07% identities with the N-terminal mature peptide of the modeled structure (3sekB) in the protein templates.

Table 2. Amino acid composition of the MSTN protein of Chinese perch.

Name of amino acid	Triplet abbreviation	One-letter abbreviation	Number	Mol/mol (%)	Property
Glutamic acid	Glu	E	26	6.90	Acidic
Aspartic acid	Asp	D	23	6.10	Acidic
Arginine	Arg	R	21	5.60	Basic
Lysine	Lys	K	20	5.30	Basic
Histidine	His	H	7	1.90	Basic
Serine	Ser	S	25	6.60	Hydrophilic
Glutamine	Gln	Q	23	6.10	Hydrophilic
Threonine	Thr	T	19	5.10	Hydrophilic
Glycine	Gly	G	18	4.80	Hydrophilic
Asparagine	Asn	N	16	4.30	Hydrophilic
Cysteine	Cys	C	13	3.50	Hydrophilic
Leucine	Leu	L	33	8.80	Hydrophobic
Alanine	Ala	A	24	6.40	Hydrophobic
Isoleucine	Ile	I	24	6.40	Hydrophobic
Proline	Pro	P	23	6.10	Hydrophobic
Valine	Val	V	22	5.90	Hydrophobic
Methionine	Met	M	13	3.50	Hydrophobic
Tyrosine	Tyr	Y	10	2.70	Hydrophobic
Phenylalanine	Phe	F	10	2.70	Hydrophobic
Tryptophan	Trp	W	6	1.60	Hydrophobic

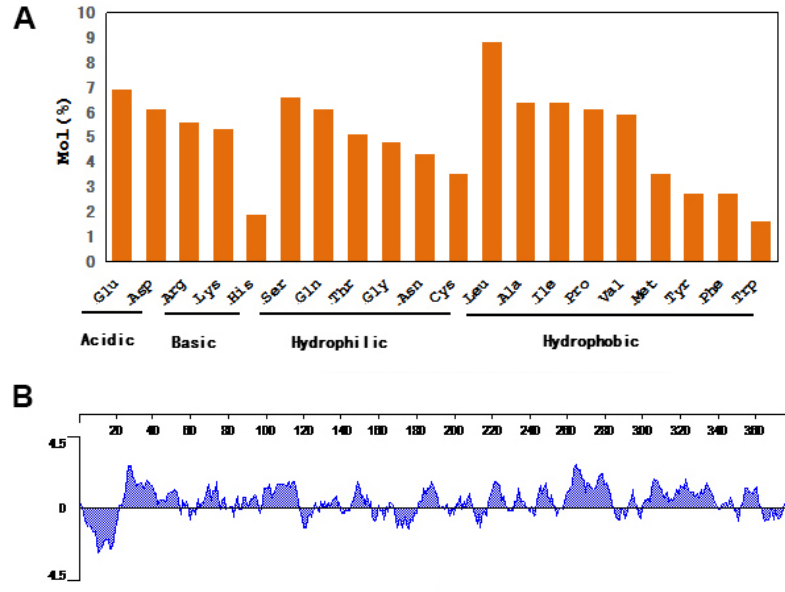


Figure 2. Analysis of the compositions and features of deduced amino acids of the Chinese perch MSTN gene. **A.** Amino acid composition in the protein encoded by the *Siniperca chuatsi* MSTN gene, the composition of 20 amino acids are different, the highest percentage is leucine (Leu) and the lowest is tryptophan (Trp). **B.** Prediction of the hydrophobic and hydrophilic areas of the protein sequence of the *Siniperca chuatsi* MSTN gene. The hydrophobic amino acids are significantly greater in number than polar amino acids.

Micropterus	MHLSQIVLYLSLLIALGPPVLSQETHQQQPSATSPIETECCATCEVRQIKTRMLNAIK	60
Lates	MHPSQIVLYLSLLIALGPPVLSQETHQ-QPSATSPEDECCVTCEVRQIKTRMLNAIK	59
Siniperca	MHLSQIVLYLSLLIALGPPVLSQETHQ-QPSATSPEDECCATCEVRQIKTRMLNAIK	59
Umbrina	MHLSQIVLYLSLLIALGPPVLSQETHQ-QPSATSPEDECCATCEVRQIKTRMLNAIK	59
Larimichthys	MHLSQIVLYLSLLIALGPPVLSQETHQ-QPSATSPEDECCATCEVRQIKTRMLNAIK	59
Micropterus	SQLSKLRMEAPNISRDIVKQLPKAPPLQQLDQYDVLGDNKDVVMEEDDEHAITET	120
Lates	SQLSKLRMEAPNISRDIVKQLPKAPPLQQLDQYDVLGDNKDVVMEEDDEHAITET	119
Siniperca	SQLSKLRMEAPNISRDIVKQLPKAPPLQQLDQYDVLGDNKDVVMEEDDEHAITET	119
Umbrina	SQLSKLRMEAPNISRDIVKQLPKAPPLQQLDQYDVLGDNKDVVMEEDDEHAITET	119
Larimichthys	SQLSKLRMEAPNISRDIVKQLPKAPPLQQLDQYDVLGDNKDVVMEEDDEHAITET	119
Micropterus	IMMMATEFESIVQVDEPKCCFFSFTQKFOANRIVRAQLMVLRLQETDEATTVFLQISRIM	180
Lates	IMMMATELESIVQVDEPKCCFFSFTQKFOANRIVRAQLMVLRLQETDEATTVFLQISRIM	179
Siniperca	IMMMATEFESIVQVDEPKCCFFSFTQKFOANRIVRAQLMVLRLQETDEATTVFLQISRIM	179
Umbrina	IMMMATEFESIVQVDEPKCCFFSFTQKFOANRIVRAQLMVLRLQETDEATTVFLQISRIM	179
Larimichthys	IMMMATEFESIVQVDEPKCCFFSFTQKFOANRIVRAQLMVLRLQETDEATTVFLQISRIM	179
Micropterus	PVTDGSRHIRIRSLKIDVNAVGSWSQSIDVRQVLSVWLRQFETNMGIEINAFDSRGNDA	240
Lates	PVTDGSRHIRIRSLKIDVNAVGSWSQSIDVRQVLSVWLRQFETNMGIEINAFDSRGNDA	239
Siniperca	PVTDGSRHIRIRSLKIDVNAVGSWSQSIDVRQVLSVWLRQFETNMGIEINAFDSRGNDA	239
Umbrina	PVTDGSRHIRIRSLKIDVNAVGSWSQSIDVRQVLSVWLRQFETNMGIEINAFDSRGNDA	239
Larimichthys	PVTDGSRHIRIRSLKIDVNAVGSWSQSIDVRQVLSVWLRQFETNMGIEINAFDSRGNDA	239
Micropterus	VTSAEFGEEGLQPFMEVKISEGPHARRDSGLDCDENSPESSCCRYPLTVDFEDFGWDI	300
Lates	VTSVEFGEEGLQPFMEVKISEGPHARRDTGLDCDENSPESSCCRYPLTVDFEDFGWDI	299
Siniperca	VTSAEFGEEGLQPFMEVKISEGPHARRDSGLDCDENSPESSCCRYPLTVDFEDFGWDI	299
Umbrina	VTSAEFGEEGLQPFMEVKISEGPHARRDSGLDCDENSPESSCCRYPLTVDFEDFGWDI	299
Larimichthys	VTSAEFGEEGLQPFMEVKISEGPHARRDSGLDCDENSPESSCCRYPLTVDFEDFGWDI	299
Micropterus	IAPKRYKANYCSGECEYMLQRYFHTHLVNKANFRGTAGPCCTFTKMSFINMLYFNREKQ	360
Lates	IAPKRYKANYCSGECEYMLQRYFHTHLVNKANFRGTAGPCCTFTKMSFINMLYFNREKQ	359
Siniperca	IAPKRYKANYCSGECEYMLQRYFHTHLVNKANFRGTAGPCCTFTKMSFINMLYFNREKQ	359
Umbrina	IAPKRYKANYCSGECEYMLQRYFHTHLVNKANFRGTAGPCCTFTKMSFINMLYFNREKQ	359
Larimichthys	IAPKRYKANYCSGECEYMLQRYFHTHLVNKANFRGTAGPCCTFTKMSFINMLYFNREKQ	359
Micropterus	IYYGKIPSMVVDRCGCS	377
Lates	IYYGKIPSMVVDRCGCS	376
Siniperca	IYYGKIPSMVVDRCGCS	376
Umbrina	IYYGKIPSMVVDRCGCS	376
Larimichthys	IYYGKIPSMVVDRCGCS	376

Figure 3. Putative amino acid sequence alignment of MSTN between *Siniperca chuatsi* and other vertebrate species. The conserved RXXR proteolytic cleavage sites are boxed. The accession Nos. for these myostatin sequences are as follows: *Micropterus salmoides* (EF071854.2), *Lates niloticus*, *Siniperca chuatsi* (JF896453) (EF681885), *Umbrina cirrosa* (AF316882.1), *Larimichthys crocea* (Y842934.2).

Sequence alignment and phylogenetic analysis

Phylogenetic analysis of the MSTN gene was performed to analyze the evolutionary relationship between the Chinese perch and other species. We downloaded 16 MSTN gene protein sequences of other representative species to be aligned and constructed a phylogenetic tree for the Chinese perch (*Siniperca chuatsi*) (Figure 4). The results indicated that the MSTN genes were classified into two groups, the lower vertebrates (teleost fish) were clustered together into one group, while some higher mammals such as *Homo sapiens*, *Capra hircus*, *Bos taurus*, *Mus musculus*, and *Macaca mulatta* formed another group. This tree indicated that the species of the second group were the most distantly related to the Chinese perch (*Siniperca chuatsi*). Interestingly, we found that the first group had three branches and indicated that the Chinese perch was the most closely related with the three species (*Paralichthys olivaceus*-1, *Lateolabrax japonicus*, and *Epinephelus coioides*), and was in the cluster closest to species such as *Salmo Salar*-1a, *Salvelinus fontinalis*, *Hypophthalmichthys nobilis*, *Danio rerio*. Additionally, Chinese perch myostatin was closely related to the MSTN-1 of some fish. Thus, we speculated that the Chinese perch MSTN gene has two genetic subtypes.

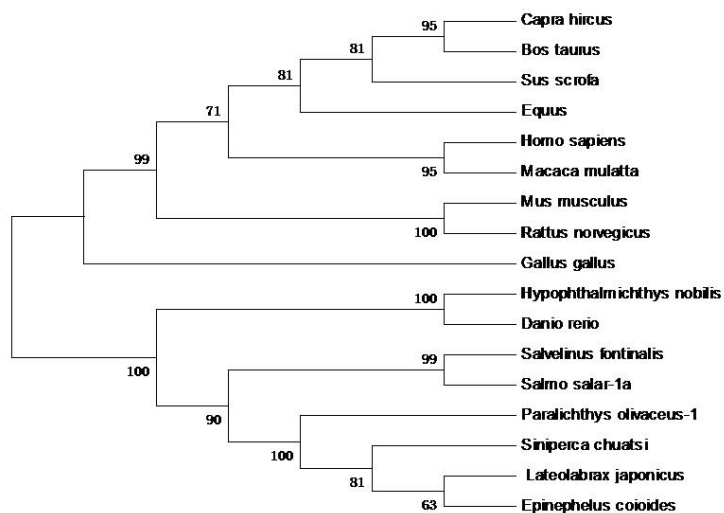


Figure 4. Phylogenetic relationship of the *Siniperca chuatsi* MSTN gene with other species. The phylogenetic tree was constructed using the neighbor-joining method. The bootstrap confidence values shown at the nodes of the tree are based on 1000 bootstrap replications. Note: *Siniperca chuatsi*: AEH16746; *Sus scrofa*: AAR18245; *Paralichthys olivaceus*-1: ABD65405; *Salvelinus fontinalis*: AAK08152; *Gallus gallus*: AAK18000; *Lateolabrax japonicus*: AAX82169; *Equus caballus*: BAB16046; *Capra hircus*: AFX59924; *Hypophthalmichthys nobilis*: AEJ83922; *Mus musculus*: AAO46885; *Bos taurus*: ACN65829; *Danio rerio*: AAP85526; *Homo sapiens*: ABI48419; *Epinephelus coioides*: AAW47740; *Salmo salar*-1a: NP_001117021; *Macaca mulatta*: ABI48518; *Rattus norvegicus*: AAB86691.

A comparison of amino acid sequences indicated that the Chinese perch MSTN gene was conserved throughout the vertebrates and was highly homologous with *Epinephelus coioides* (95%), *Paralichthys olivaceus*-1 (92%), *Lateolabrax japonicus* (90%), *Salmo Salar* (86%), and *Salvelinus fontinalis* (84%), respectively. However, it displayed a lower homology with mammals such as *Homo sapiens*, *Capra hircus*, *Bos taurus*, *Rattus norvegicus* and *Macaca mulatta* (homology ranged from 59 to 63%). All of these results suggest that, throughout evolution, there was a variety of genetic relationships between Chinese perch and other species.

Expression of MSTN mRNA in various tissues of *Siniperca chuatsi*

The MSTN mRNA expression levels of the seven tissues from the adult Chinese perch were measured using quantitative RT-qPCR. The results indicated that the MSTN gene expression levels varied across the various tissues. It exhibited a muscle-specific expression pattern, and as a result, the MSTN mRNA, was expressed at the highest levels in skeletal muscle and only moderately in the heart, brain, and intestines. Additionally, it was expressed in trace amounts in the kidney, spleen, and liver (Figure 5A). The gene expression varied greatly among the various tissues ($P < 0.05$).

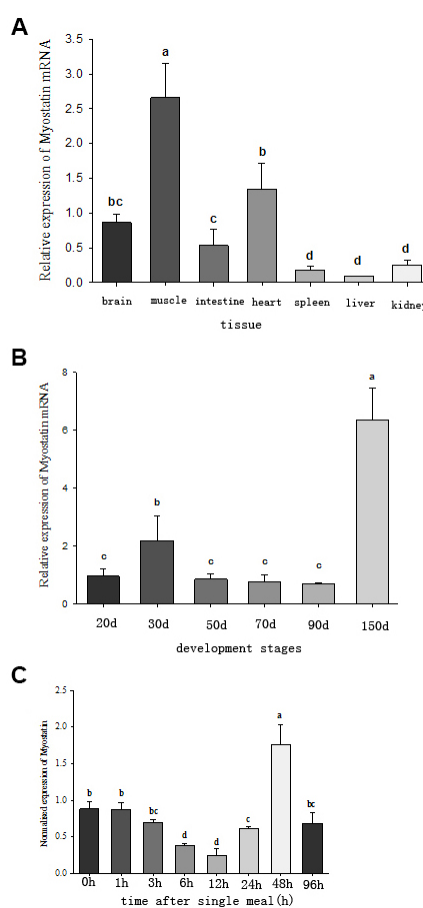


Figure 5. Quantitative real-time PCR analysis of the MSTN gene expression levels. **A.** mRNA expression profiles of myostatin in adults tissue of *Siniperca chuatsi*. RPL13 have been used as inner controls. The relative expression variance of the seven tissues is detected by qRT-PCR. Error bars represent the standard error of the mean (SEM). A Student's *t*-test was performed to determine the significant differences. Columns sharing different letters indicate significant differences ($P < 0.05$) with a, b, c, d superscripts. All experiments were repeated at least three times. **B.** mRNA expression profiles of myostatin in skeletal muscle at different stages of post-embryonic development in *Siniperca chuatsi*. Levels associated with histogram bars with the same letters do not differ significantly at $P < 0.05$. **C.** Effects of fasting and re-feeding on relative expression of the MSTN gene in skeletal muscle of *Siniperca chuatsi*. Levels associated with histogram bars with the same lowercase letters do not differ significantly at $P < 0.05$.

Expression profiles of *Chinese perch* MSTN mRNA at different ages

Moreover, we also measured and compared the temporal expressions throughout various stages of Chinese perch development (Figure 5B). We found that the expression levels at the early post-embryonic developmental stage were very low and there were no significant differences ($P > 0.05$) from 20 dph (weight = 5 ± 0.5 g) to 90 dph (weight = 150 ± 5 g). However, the ones at 30 dph (weight = 10 ± 0.8 g) had a high expression, up to the stage at 150 dph (weight = 150 ± 10 g), the MSTN mRNA levels increased significantly ($P < 0.05$) and reached their maximum.

Effects of fasting and refeeding on Chinese perch MSTN gene

To investigate the effect of fasting and refeeding on MSTN mRNA expression in the skeletal muscles, we analyzed the expression of the MSTN mRNA at various times throughout a single meal refeeding after a one-week-fasting. The results (Figure 5C) indicated that the MSTN gene transcript levels significantly decreased from 1 to 12 h ($P < 0.05$), reduced to the lowest at 12 h ($P < 0.05$), and in order to respond to refeeding, hereafter, increased significantly from 24 to 48 h ($P < 0.05$). It reached the highest levels at 48 h ($P < 0.05$), and then decreased, finally returning to the original level of MSTN gene transcript at 96 h ($P < 0.05$).

DISCUSSION

In our study, we isolated and cloned the full-length sequence of the Chinese perch MSTN gene for the first time, which was composed of 5' UTR, ORF, and 3' UTR. We then analyzed and characterized its gene structure, function, phylogenetic evolution, expression patterns in various tissues and at various developmental stages and the effect of fasting.

The results of an analysis of the promoter prediction demonstrated that the 5' UTR sequence was 863 bp, which contained three E-boxes, one I-box, and two CAAT-boxes. The roles of these domains could combine with muscle control factors, such as the MRFs gene, to complete the transcriptional regulation of the muscles. For example, the previous study showed that the E-boxes played a critical role in the regulation of muscle gene expression and were activated by myogenic regulatory factors (MRFs) such as MyoD, MyoG, Myf5, and MRF4 (Spiller et al., 2002). The CAAT-box had a distinct pattern of nucleotides with a consensus sequence of GGCCAATCT located ~80 bp upstream of the start codon of the eukaryote genes, and played an important role during transcription (Benoist et al., 1980). Afterwards, a research team found that the promoter of the MSTN gene served as a negative regulator of cell proliferation and differentiation (Dall'Olio, et al., 2014). These conclusions indicated that the MSTN gene transcription was controlled by these elements of the promoter.

In the present study, the deduced MSTN protein sequence of ORF, which encoded 376 amino acids (1131 bp), was highly homologous to those of previously studied mammal and fish species (Garikipati et al., 2007; Kanjanaworakul et al., 2015). The predicted protein had a molecular mass of 42.56 kDa, an isoelectric point of 5.56 at pH 7.00, and was strongly hydrophobic. The second and 3D structure showed the Chinese perch MSTN protein shared the characteristics of proteins in the TGF- β superfamily families including an N-linked glycosylation site and an O-linked glycosylation site, an N-terminal signal sequence for secretion, a conservatively hydrolytic site (RARR) (representing a putative proteolytic

proportion site to release the processed mature peptide), and a C-terminal domain of 113 amino acids. In the bioactive domain, the nine cysteine residues were essential for the dimerization of the two MSTN subunits (Hu et al., 1998). Initially, the protein peptide chain had no function, it had to be processed and modified by the signal peptide which was carried out by 22 amino acids. Once the task was finished, a second cleavage occurred at the RXXR site to generate the mature active peptide by removing the signal peptide. Previous evidence has indicated that mutations in the C-terminal section of the mature peptide leads to a loss function of the MSTN gene as it generates the double-muscling phenotype of the Piedmontese cattle breed when tyrosine is substituted for cysteine (Kambadur et al., 1997). Proof that the MSTN gene has been highly identifiable throughout the evolution of vertebrates can be confirmed in their conservative structure and function (De Santis and Jerry, 2011). Phylogenetic analysis indicated that the Chinese perch MSTN gene has a close relationship with teleostean fish genes but is distantly related to the mammalian gene, as it shared 84-95% identity with the former and only 59-63% identity with the latter. These results confirmed that the Chinese perch MSTN gene cloned by us was highly in accordance with other members of the TGF- β superfamily.

The MSTN gene presented diverse profiles in different teleosts and had complicated tissue-specific expression profiles. In tilapia, MSTN gene expression was observed in the brain and particularly in the optic lobe (Rodgers et al., 2001). In adult zebrafish, the MSTN gene was expressed highly in all tissues except the eye and gall bladder (Helterline et al., 2007). In the barramundi, it was also observed in various tissues including the skeletal muscles, gills, heart, blood, kidneys, and intestines (De Santis et al., 2008). Our study found that the MSTN gene was more highly expressed in the heart where it could affect the growth of the cardiac muscle (Morissette et al., 2006), and the brain, which illustrated that MSTN could have an endocrine function (Rodgers et al., 2001; Kanjanaworakul et al., 2015). However, there was also a very weak signal detected in the kidney, liver, spleen, and intestine (Maccatrozzo et al., 2001). Particularly, the MSTN gene in Chinese perch was mainly and significantly expressed in skeletal muscle tissues, which was in keeping with the ubiquitous expression observed in the previous studies ($P < 0.05$) (Garikipati et al., 2007). The results indicated that the MSTN gene was involved in many functions but its primary function was to inhibit skeletal muscle growth (McPherron and Lee, 1997).

During skeletal muscle development, the expression of MSTN was initially restricted to the myotome compartment of developing somites and continued to be limited to the myogenic lineage at later stages of development and in adult animals (Tripathi et al., 2011). Our results demonstrated that the MSTN mRNA expressions were higher in adult Chinese perch than in juveniles, which was consistent with the results observed in zebrafish and channel catfish (Kocabas et al., 2002; Xu et al., 2003). The lower levels of MSTN in juvenile fish indicated that there was less restriction of muscle growth than what occurred in adult fish (Rowlerson and Veggetti, 2001). Similarly, our results showed that there were remarkably low expressions from 20 to 90 dph (except at 30 dph), it then increased significantly and reached its maximum at 150 dph ($P < 0.05$). This indicated that the 90 dph stage of Chinese perch development could be a rapid growth stage due to its association with an intense myoblast proliferation and hypertrophy, which mainly contributed to skeletal muscle growth, but also controlled the number of muscle fibers by inhibiting myoblast proliferation in cattle embryos (Helterline et al., 2007) and tilapia, gilthead seabream, and post-hatch larvae of Chilean flounder (Delgado et al., 2008). A recent report indicated that the expression levels in 7-day

old larvae of Walking catfish were higher than those in 2-month old juveniles, unchanged from 2-month old juveniles to 6-month old juveniles, and then increased significantly in adult fish of 1-3 years of age (Kanjaworakul et al., 2015). The results indicate that the rapid growth of 2-month old Walking catfish juveniles was due to the low expression levels of the MSTN gene (Kocabas et al., 2002). The MSTN gene played a negative regulatory role in embryonic skeletal muscle growth and development (Kocamis et al., 1999). Consistent with this view, our results revealed that the downregulated expression levels would help to promote the rapid and highest growth rate of the skeletal muscles at the early stages of post-embryonic development (from 20 to 90 dph), while higher expression restrained the speed of muscle growth at 150 dph.

Nutrient availability was amongst the most important environmental factors that affected muscle growth (Valente et al., 2012). As the fish were subjected to harsh and disadvantageous environments, such as being re-fed after fasting, they would enable a mechanism of compensatory growth (a period of accelerated growth in response to nutritional restriction) (McCue, 2010). In teleostean fish, the fasting experiments were employed to investigate the molecular and genetic control mechanisms the muscle growth process (Preziosa et al., 2013). It was discovered that the expression levels did not decrease, on the contrary, the expression levels of other myogenic factors (MyoD, MRF4, Myf5) increased at first and then declined in the initial stages of the seven day fasting of *Salmo salar*, which were then re-feed a single meal to visual satiation (Valente et al., 2012). To study the MSTN genes response to nutrient availability with a relatively higher temporal resolution, the postprandial expressions of the MSTN gene in Chinese perch juvenile muscles were investigated by executing a single-meal refeeding experiment. Therefore, we conducted a fasting-refeeding experiment using 90 dph individuals to investigate the regulation of muscle growth during the transition from catabolic to anabolic states and explored the nutrient regulation of the MSTN gene in fast muscles. The results indicated that the MSTN gene expression levels decreased from 1 h to 12 h and then increased from 24 h to 48 h, and finally, ultimately recovered to a normal level after the single satiation meal. Similar results were reported in human muscles that went through an anabolic stimulus by essential amino acid (EAA) ingestion (Drummond et al., 2009). The results indicated that it provided an indirect mechanism of regulation on the MSTN gene as its abundance gradually decreased during refeeding after fasting, and congruently, the effect of food deprivation and refeeding resulted in attaining a full compensatory growth response by improving the feeding rate in the Chinese perch (Terova et al., 2006). As a result, when the growth of the fish was affected, the expressions of the MSTN gene were accordingly affected in the skeletal muscle.

In summary, we isolated and cloned the full-length sequence of the Chinese perch MSTN gene for the first time. It was composed of 5'UTR, ORF and 3'UTR. *In silico* analysis based on the sequence, the protein structure, and amino acid sequence confirmed that the Chinese perch MSTN gene shared the typical characteristics of the functional domains of the transforming growth factor the β (TGF- β) superfamily. We also demonstrated that the Chinese perch MSTN gene had different expression patterns in various tissues and at various development stages and was affected by fasting. Therefore, we speculate the MSTN gene acts as a negative factor in muscle growth and plays a role in nutritional compensation in fish. These results will help us establish strategies to improve the growth rate of mass-produced Chinese perch in the future.

Conflicts of interest

The authors declare no conflict of interest.

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