A potential negative regulation of myostatin in muscle growth during the intermolt stage in *Exopalaemon carinicauda*

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Introduction:

Molting, the cyclic exchange of exoskeleton, is a growth-related phenomenon in crustaceans. The periodic molting is a special growth and development mode of crustaceans. Like most other crustaceans, the growth and development of shrimp primarily depend on muscle restoration and net muscle gain during molting. Abdominal muscle buildup occurs mainly during intermolt, a cell proliferating metabolic phase. Therefore, it is essential to explore the target genes regulating muscle buildup in crustaceans during intermolt. Myostatin (MSTN) is a member of the transforming growth factor-β (TGF-β) superfamily and is a potent negative regulator of skeletal muscle differentiation and growth. MSTN plays important roles in regulating embryonic development and maintaining tissue homeostasis in mammals, but until now solid evidence supporting a similar function of MSTN in invertebrates has been lacking. Therefore, it is crucial to clarify the function of the MSTN gene and its molecular mechanism of regulating growth traits in crustaceans.

The ridgetail white shrimp *Exopalaemon carinicauda*, belonging to the Palaemonidae family of crustaceans, is a major commercial mariculture species naturally distributed on the coasts of the Yellow Sea and Bohai Sea. Since MSTN in aquatic animals plays a vital role in regulating of growth traits and its manipulation may significantly impact aquaculture and domestication, we aimed to elucidate its function in *E. carinicauda*. The main purpose of the current study was to identify and characterize the MSTN gene from *E. carinicauda* and investigate the possible function of MSTN during the intermolt stage in *Exopalaemon carinicauda* and provide effective genetic markers from MSTN associated with growth traits for the genetic improvement of *E. carinicauda*.

Materials and Methods:

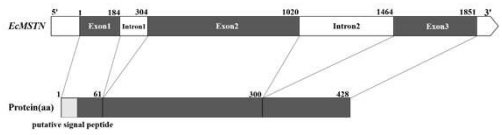
The healthy *E. carinicauda* adults (body length 44.15±3.02 mm, body weight 1.36±0.42 g) were obtained from the Yellow Sea near Rizhao City, Shandong Province, China. For tissue-specific expression determination, the abdominal muscle, stomach, eyestalks, heart, intestine, ovary, gill, hemocyte and hepatopancreas were collected and rapidly frozen in liquid nitrogen, then stored at -80℃ until RNA isolation. Incubatory shrimps were collected to obtain newly hatched larvae from one day. The six shrimps at each developmental stages of *E. carinicauda* were collected, including zoea I to zoea VI (zoea-1 to zoea-6), and postlarvae aged of one day (postlarvae-1).

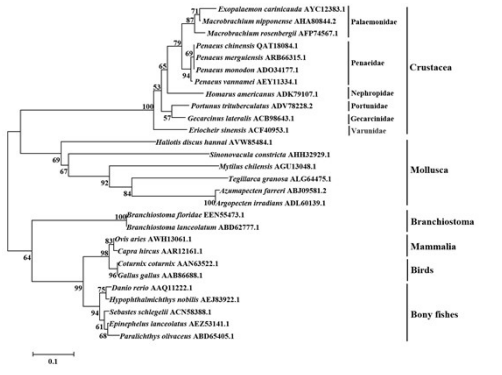
After four weeks, the post-larvae shrimps were transferred into 120 L aerated sand-filtered seawater with a temperature 25.0-26.0℃ and initial salinity 31±0.5 for rearing. We collected the heavier (H) and lighter (L) shrimps during intermolt stage and assessed the growth of the animals by recording the body weight, when the animals had reached post-larval ages of 60 days, 80 days, 100 days, and 120 days.

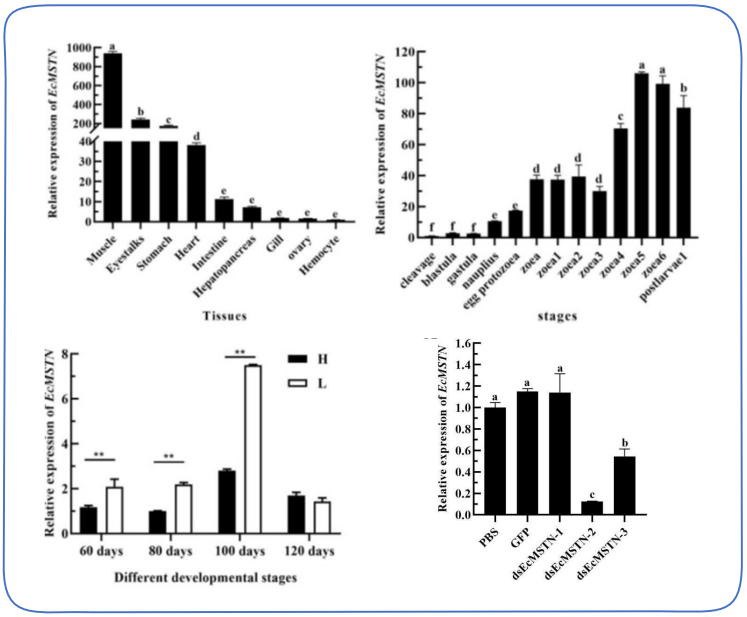
In dsRNA-mediated RNAi experiments, healthy shrimps (body weight 1.00~1.20 g) during intermolt stage were used for EcMSTN knock down. The total RNA from abdominal muscle of three shrimps in each group was extracted at 24 h and 48 h after dsRNA injection, and then the qRT-PCR was performed to analyze the RNAi efficiency of the three dsRNAs injection. The expression levels of growth-related genes including myosin heavy chain (MHC) and actin were detected in each group.

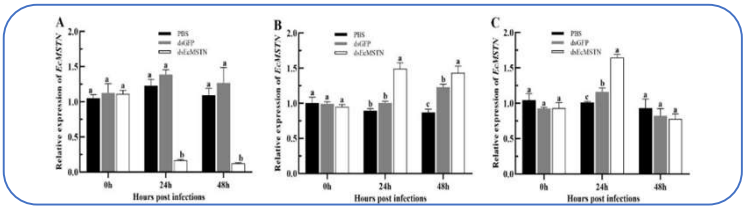
Meanwhile, the most efficient dsRNA was injected once per week at 1, 7, 14, 21, 28 and 35 days, and the body weight and body length were measured at 1, 7, 14, 21, 28, 35 and 42 days. Individual mortality was observed twice per day during the experimental period.

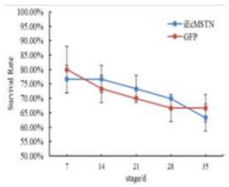
Results:











Groups	1 d	7 d	14 d	21 d	28 d	35 d
iEcMSTN	0.70±0.15	0.76±0.16	0.85±0.14	0.93±0.17	1.00±0.19	1.11±0.20
GFP	0.68±0.17	0.71±0.17	0.77±0.15	0.83±0.16	0.87±0.17*	0.93±0.16*

SNP name	Genotype	Frequency	Body length (mm)	Body weight (g)
g.Mstn220	AA	0.28	54.97 ± 2.12^{a}	2.54 ± 0.29^{a}
	GG	0.53	52.33 ± 1.75^{ab}	2.27 ± 0.27^{ab}
	AG	0.19	49.13 ± 3.87^{b}	1.79 ± 0.40^{b}
g.Mstn567	TT	0.16	49.68 ± 3.88^{b}	1.80 ± 0.44^{b}
	CT	0.59	50.48 ± 3.68^{b}	1.99 ± 0.43^{b}
	CC	0.25	54.65 ± 2.39^{a}	2.49 ± 0.36^{a}

The full-length cDNA of *EcMSTN* was 1,518 bp, and the genomic sequence was 1,851 bp, including three exons and two introns. *EcMSTN* was expressed in a wide range of tissues, but predominantly detected in the abdominal muscle. *EcMSTN* expression was negatively correlated with the growth traits. After *EcMSTN* knockdown using RNA interference, EcMSTN expression was down-regulated in the abdominal muscle and up-regulated the expression of growth-related genes, including myosin heavy chain and actin. After inhibiting *EcMSTN* for 5 weeks, the RNAi-treated shrimp with reduced *EcMSTN* levels exhibited a dramatically higher body weight compared with that of the control group. Association analysis revealed that two SNP loci g.Mstn220 and g.Mstn567 were markedly associated with both body weight and body length.

Conclusions:

In conclusion, we successfully identified and characterized *EcMSTN* from *E. carinicauda*. The tissue and embryonic developmental expression patterns suggested that it may be involved in muscle differentiation and growth. Further results of RNAi indicated its negative function in myogenesis and growth traits. Moreover, association analysis identified two SNP loci in *EcMSTN*. This study would contribute to clarify the negative role of MSTN in crustaceans, suggesting that it may have great potential and economic benefits for crustacean breeding programs.

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