Thesis for the Degree of Master of Fisheries Science

Effective RNA-silencing strategy of Lv-MSTN/GDF11 gene and its effects on the growth in shrimp, *Litopenaeus vannamei*

by

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KOICA-PKNU International Graduate Program of Fisheries Science

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Lv-MSTN/GDF11 유전자의 효과적인 RNA 간섭현상이 *Litopenaeus vannamei* 의 성장 및 탈피에 미치는 영향에 관한 연구

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by

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Abstract

Myostatin (MSTN), also known as GDF8, is of interest for its potential economic benefit to the livestock and aquaculture industries due to its inhibitory effects on muscle growth and development. Recently, Lv-MSTN/GDF11, the primitive isoform of MSTN and GDF11, was identified from the shrimp *Litopenaeus vannamei*. The major production site for Lv-MSTN/GDF11 is in the heart, not the tail muscle, which differs from MSTNs in mammals. Among the three injected RNAs, long dsRNA was the most effective for Lv-MSTN/GDF11 knockdown and transcripts of Lv-MSTN/GDF11 decreased in both the heart (88.85 %) and skeletal muscle (43.36 %) 72 h after injection of 10 μM of long dsRNA. We also found that higher doses of dsRNA did not lead to greater decreases in Lv-MSTN/GDF11 transcripts for amounts between 1 μM and 100 μM. Injection of Lv-MSTN/GDF11 dsRNA did not affect the upregulation of the skeletal actin gene (Lv-ACTINSK) in the tail muscle, but the expression of cytoplasmic and cardiac actins were

upregulated in both the heart and tail muscle. Over the course of 8 weeks of dsRNA injection, considerably higher mortality (~71%) was seen in the dsRNA-injected group compared to the control group (40%). Surviving shrimp in the dsRNA injected group had a lower growth rate due to the adverse effects of Lv-MSTN/GDF11 knockdown. Lv-MSTN/GDF11 appears to be involved in muscular or neuronal development, but not in doubling muscle fibers, as is the case with mammalian MSTN. These results suggest that Lv-MSTN/GDF11 may not be a suitable target gene for enhancing productivity in shrimp aquaculture.

Introduction

Proteins belonging to the transforming growth factor β (TGF-β) superfamily control cell growth, proliferation, and homeostasis. Growth and differentiation factor 8 (GDF8), also known as myostatin (MSTN), and growth differentiation factor 11 (GDF11) are thought to be the result of gene duplication from an ancestral gene and share primary structure, signaling pathways, and some functional features in mammals (Ge et al., 2005; Xu et al., 2003). The biological functions of these two genes have clearly changed and diverged. GDF 11 is involved in development of the axial skeleton, regulation of anterior/posterior patterning, and negative autoregulation of neurogenesis in the olfactory epithelium (McPherron et al., 1999; Wu et al., 2003). MSTN-knockout mice had significantly larger muscles compared with normal mice, and this gene is of interest for its potential economic benefit to the livestock industry (Kota et al., 2009; McPherron et al., 1997).

Facilitated muscle growth is beneficial to the aquaculture and livestock industries and MSTN and GDF11 homologs have been identified in several invertebrates, including arthropods (Covi et al., 2008; De Santis et

al., 2011; Qian et al., 2013)molluscs(Kim et al., 2004), and Cephalochordata(Xing et al., 2007). In contrast to mammals, only a single ancestral ortholog has been identified in invertebrates. MSTN/GDF11 genes were isolated and characterized in several decapod crustaceans, including brachyurans (Covi et al., 2008; Kim et al., 2009b), astacurans(MacLea et al., 2010), and penaeids(De Santis et al., 2011; Qian et al., 2013). Despite the available sequence information, its biological role is still not clearly established in decapod crustaceans mainly due to the lack of genetic tools for introducing or silencing the expression of specific genes. It is known that the major production sites for the MSTN/GDF11 gene are the muscular tissues including the heart, thoracic muscle, and claw muscle. Its expression is also related to the molting cycle (Covi et al., 2008; Kim et al., 2009b)as skeletal muscle shows a high degree of plasticity and its growth is tightly linked to the molting cycle (Mykles, 1997; Mykles and Skinner, 1981).

Since its first development in 1998 (Fire et al., 1998), RNA interference (RNAi) has been an effective and convenient strategy for decreasing the expression of target genes in most metazoan species. Application of RNAi to crustacean species may be an alternative means ofincreasing our limited knowledge of crustacean physiology, which could

be used for commercial development (Sagi et al., 2013). In fact, dozens of publications have shown that RNAi is an effective method for understanding growth and development (De Santis et al., 2011; Glazer et al., 2010; Soñanez-Organis et al., 2010), immunity (Robalino et al., 2007), and reproduction (Nagaraju et al., 2011; Sathapondecha et al., 2011; Treerattrakool et al., 2011). However, the size, dose, and type of RNA used vary and the functional mechanism within cells and tissues is not clearly understood. In this study, we tested three RNAs at various doses to evaluate their effectiveness in the interference of the Lv-MSTN/GDF11 gene. We also determined whether RNAi of the Lv-MSTN/GDF11 gene affected the transcription of the actin fiber genes. Finally, we measured molt frequency, body weight, and survival rate over eight weeks of RNA injection to determine the chronic effects of Lv-MSTN/GDF11 knockdown.

Materials and Methods

Shrimp and experimental treatment

Shrimp of similar size (23.59 ± 0.38 mm in carapace length) and body weight (7.12 ± 0.36 g) were purchased from a local aquaculture farm. Before the experiments each shrimp was acclimatized in a circulating aerated seawater tank (10 L) for 7 days at 27°C. The photoperiod was maintained at 12L:12D and shrimp were fed diced squid and polychaetes (5% of body weight). Salinity (34 ± 2‰) was maintained by daily adding deionized water and 20% of the total volume was changed weekly with fresh seawater. Octopus Diablo DC 170 skimmer (Reef Octopus, China) was used to eliminate nitrogenous waste from the shrimp culture. Before RNA injection, individual body weight and carapace length were measured. After the experiment, shrimp were sacrificed and dissected. The isolated tissues were directly frozen in liquid nitrogen and stored at -80°C before use for total RNA extraction.

Quantitative analysis of MSTN/GDF11 and three actin transcripts

qPCR was performed to measure Lv-MSTN/GDF11, Lv-ACTINCT (β-actin), Lv-ACTINSK (α-actin), and Lv-ACTINHT (cardiac muscle actin) expression. The nucleotide sequences for Lv-MSTN/GDF11 (GenBank number: JQ045427), Lv-ACTINCT (GenBank number: AF300705), and Lv-ACTINSK (GenBank number: AY646096) were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). The full-length Lv-ACTINHT cDNA sequence was obtained using the typical PCR-based cloning strategy using degenerate primers (Table 1) according to previous studies (Kim et al., 2009a; Uddowla et al., 2013). All PCR primers in this experiment were designed using the OligoAnalyzer 3.1 software (http://sg.idtdna.com/analyzer/Applications/OligoAnalyzer/) and were purchased from Bioneer Co. (Daejeon, Korea) (Table 1). All four sequences (Lv-MSTN/GDF11, Lv-ACTINCT, Lv-ACTINSK, and Lv-ACTINHT) were amplified by designed primers (Table 1) from the heart or tail muscle. Amplicons of expected sizes were purified using an AccuPrep Gel Purification Kit (Bioneer Co., Korea), ligated into the TA plasmid vector using the pGEM-T Easy Cloning Kit (Promega, USA), and transformed into

E. coli DH5-alpha competent cells. The cDNAs were sequenced with an automated DNA sequencer (ABI Biosystems, USA).

Because Lv-MSTN/GDF11 is expressed predominantly in muscle tissue (Qian et al., 2013), qPCR was performed using cDNAs from the heart and tail muscle. Total RNA was isolated using TRIzol Reagent according to the manufacturer's instructions (Invitrogen, USA). The integrity of isolated RNA was determined by 1% agarose gel electrophoresis and quantified by spectrophotometry (Nanodrop Technologies, Inc., USA). Qualified RNAs were aliquoted and stored at -80°C until used for cDNA synthesis. cDNA was synthesized as described previously (Lee et al., 2011). qPCR was performed using the DNA Engine Chromo 4 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SYBR Green premix Ex Taq II (Takara Bio Inc.). Reliability of the sequence-specific primers for Lv-MSTN/GDF11 was confirmed by constructing a standard curve as described previously (Kim et al., 2005b). The reactions (20 μL) contained 1 μL of cDNA (100ng/μL), 2 μL of sequence-specific primers (4 pM), 0.1 μL of SYBR Green premix Ex Taq II (Takara Bio Inc., Shiga, Japan), 2 μL of dNTPs (2.5 mM each), and 2 μL of 10× buffer. The PCR conditions were 1 min at 94°C followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and

72°C for 30 s. Copy numbers of Lv-MSTN/GDF11 were normalized to those of 18S rRNA, as described previously (Jeon et al., 2010). The statistical significance of changes in Lv-MSTN/GDF11 expression was analyzed by Student's *t*-test using the Excel software (Microsoft, ver. 2007). Differences were considered significant at $P < 0.05$.

Preparation of the three Lv- MSTN/GDF11 RNAs

The target sequence for Lv-MSTN/GDF11 RNAi was determined using the SciToolsRNAi Design software (http://sg.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx?source=menu). To select the most effective sequence for RNAi, three different RNAs were synthesized (Fig. 1). Two siRNAs were synthesized commercially (Integrated DNA Technologies, USA) as designed using the SCiToolsRNAi software. RNA (2) was a canonical siRNA (25-mer) with two bases overhanging on each strand, and RNA (3) was the dicer substrate (DsiRNA), which is longer (27-mer) and contains two replaced DNA residues on one of the strands (Fig. 1). ① RNA (1) was a long dsRNA (352 bp) synthesized using the mMESSAGEmMACHINE Kit according to the manufacturer's instructions (Ambion Inc., USA). Briefly, Lv-MSTN/GDF11 sequence-

specific forward and reverse primers with a T7 promoter extension at the 5' end were designed (Table 1). RT-PCR was performed using the primers and the expected PCR product (412 bp) was cloned into the pGEM-T Easy vector (Promega, USA) and transformed into *Escherichia coli* DH5-alpha competent cells. After confirmation of the obtained RNA sequence, the template for *invitro* transcription was prepared by PCR using the diluted plasmid as a template. The PCR product (20 μL) was purified using a PCR purification kit (Bioneer Co, Korea) and long dsRNA was synthesized using the mMESSAGEmMACHINE Kit (AmbionInc, USA). After in vitro transcription, synthesized RNA was cleaned up using the RNeasy Mini Kit (Qiagen Inc., USA) according to the manufacturer's instructions. For precise annealing of the synthesized dsRNA, RNA samples were subjected to the following conditions: denaturation at 95°C for 5 min and annealing by gradually lowering the temperature (1°C every 30 s) from 95°C to 25°C. The integrity and quantity of newly synthesized dsRNA were determined by 1% agarose/EtBr gel electrophoresis and by spectrophotometry. RNA stock solutions were aliquoted and stored at -80°C before use.

Injection of dsRNAand measurement of growth and molting

Injection of each RNA was performed using a syringe (0.3-mm gauge and 8-mm length) into the tail muscle of each shrimp. Since previous studies showed that the expression of Lv-MSTN/GDF11 changes during the molting cycle (De Santis et al., 2011; Qian et al., 2013), RNA was injected 48 h after molting. Individuals in the control group were injected with RNase-free water. Seventy-two hours after injection, body weight was measured and shrimp were dissected for transcriptional analysis.

To examine the chronic effects of long dsRNA injection on growth and molting, 10 μM of Lv-MSTN/GDF11 dsRNA were injected into the tail muscle once per week for 2 months. Individuals in the control group were injected with RNase-free water. The mortality and molting frequency were recorded daily. Body weight was measured prior to the weekly injection. Individual ecdysis was observed twice per day during the experimental period. The statistical significance of the data was determined by Student's *t*-test using the Excel software (Microsoft, ver. 2007).

Table 1. Primers for Lv-MSTN/GDF11 study

Names	Sequence(5'-3')	Application
Lv-MSTN F	CCAGTGCACTACCAGCCAGATC	qPCR for Lv-MSTN/GDF11
Lv-MSTN R	ACGATCTACTACCATGTCTTGGATGG	qPCR for Lv-MSTN/GDF11
Lv-ACTINHT Deg F1	ATGTGYGAYGANGANG	Cloning for Lv-ACTINHT
Lv-ACTINHT Deg F2	ATGTGYGAYGAYGAYGCNAC	Cloning for Lv-ACTINHT
Lv-ACTINHT Deg R1	AARCAYTTNCKRTGNAC	Cloning for Lv-ACTINHT
Lv-ACTINHT Deg R2	RCAYTTNCKRTGNACDAT	Cloning for Lv-ACTINHT
Lv-ACTINHT F	GAGACTTAACCGATTATCTCATGCG	qPCR for Lv-ACTINHT
Lv-ACTINHT R	GATAGACAGTTTCATGAATACCTGAA	qPCR for Lv-ACTINHT
Lv-ACTINCT F	CGGCATCCACGAGACCAC	qPCR for Lv-ACTINCT
Lv-ACTINCT R	CTGCTTGCTGATCCACATCTG	qPCR for Lv-ACTINCT
Lv-ACTINSK F	TCATGTGTGACGACGAAGACT	qPCR for Lv-ACTINSK
L-vACTINSK R	GGAGCCTCAGTGAGAAGTGT	qPCR for Lv-ACTINSK
Lv18S rRNA F	CTGCGACGCTAGAGGTGAAATTC	qPCR for 18S rRNA
Lv18S rRNA R	CCTTTAAGTTTCAGCTTTGCAACC	qPCR for 18S rRNA
M13F(-20)	GTAAAACGACGGCCAGT	DNA sequencing
M13R(-20)	GGAAACAGCTATGACCATG	DNA sequencing
Lv-MSTN RNA F	GAATTTAATACGACTCACTATAGGGCCA CCCCTCAGACAACCTCGGACTGG	Long dsRNA synthesis
Lv-MSTN RNA R	GAATTTAATACGACTCACTATAGGGCCA CCGCGCTGGTGCTATTCATCTTC	Long dsRNA synthesis

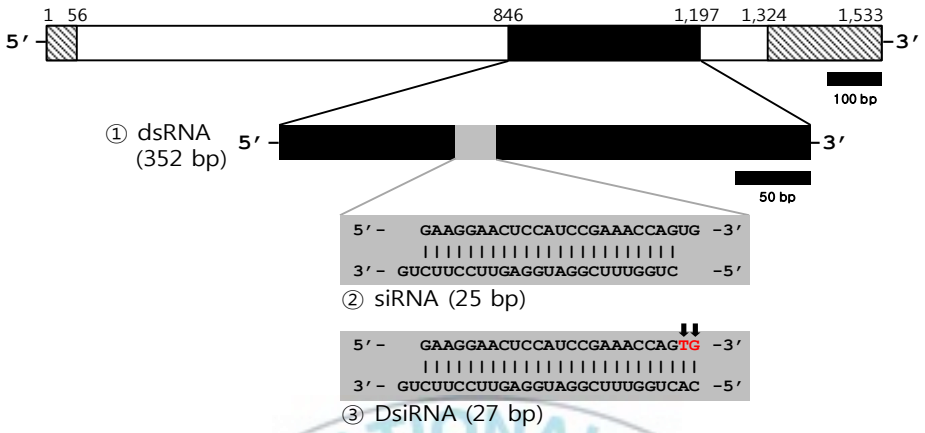

Fig. 1. Region of three different RNAs in myostatin from *L. vannamei*.

The ORF is shown as white box from 56 to 1,324, while diagonal lines indicate the 5'- and 3'-UTRs, respectively. Region of long dsRNA (①) is shown as black box from 846 to 1,197 (352bp). siRNA (②) is 25 base duplexs with 2 bases overhanged at each strand. Sense of dicer substrate siRNA (③) contained two DNA and 25 base duplexs, while antisense is 27 base duplexs. Arrows indicate 'TG' DNA molecules of dicer substrate siRNA.

Results

Long dsRNA silences the Lv-MSTN/GDF11 gene

After end-point RT-PCR, the greatest number of PCR products was detected in the heart, followed by the tail muscle and intestine. qPCR results also showed that expression of Lv-MSTN/GDF11 was 20.6-fold higher in the heart than in the tail muscle indicating that the major production site is the heart (Fig. 2). This differs from a previous study in which Lv-MSTN/GDF11 was highly expressed at similar levels in both the tail muscle and heart (Qian et al., 2013). Considering these results, we measured the amount of Lv-MSTN/GDF11 transcript in the two tissues after RNA injection (Fig. 2).

To determine the most effective RNAi strategy, three different RNAs were prepared: long dsRNA, canonical siRNA, and DsiRNA (Fig. 1). Following injection of the three RNAs (10 μM), strong interference with Lv-MSTN/GDF11 expression was identified in shrimp injected with long dsRNA (Fig. 2). Three days after 10 μM long dsRNA injection, there were 88.85% and 43.36% decreases in LvMSTN/GDF11 transcript levels in the

heart and tail muscle, respectively. However, the decrease was statistically significant only in the heart (Fig. 2B). In contrast, no significant reduction was identified in shrimp injected with the same amount of either canonical siRNA or Dicer substrate RNA.

To determine the dose-dependent effects of RNAi, three concentrations of long dsRNA (1, 10, and 100 μM) were injected (Fig. 3). In the heart, strong interfering activity was present in all three injected groups with 80.17–88.08% reductions in Lv-MSTN/GDF11 transcript levels. However, there were no significant differences among the three groups according to dsRNA concentration (Fig. 3B). In the tail muscle, individual variation was greater than the silencing effects and there was no significant difference between the 10 μM and 100 μM doses (Fig. 3A). However, Lv-MSTN/GDF11 transcription was significantly inhibited (48%, $p = 0.02$) in the three dsRNA-injected groups (1, 10, and 100 μM) compared to the control group. In conclusion, silencing of the Lv-MSTN/GDF11 gene was not dose dependent, but was tissue- or organ-specific, regardless of the injected dsRNA concentration (Fig. 3).

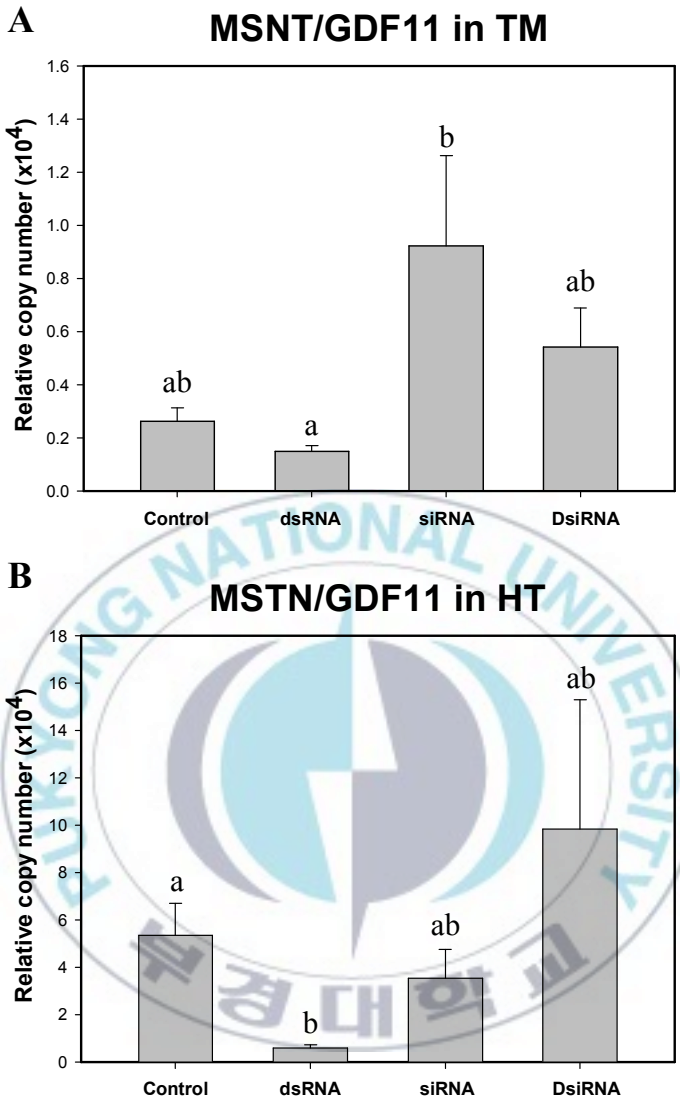

Fig. 2. Relative copy number of Lv-MSTN/GDF11 in tail muscle (A) and heart (B) tissues after injecting in 72 hours with three different RNAs.

Shrimp were injected with three different RNAs which are long dsRNA, siRNA and dicer substrate siRNA. The relative copy numbers were normalized against the copy number of 18S rRNA. Student t-test was used to determine significant effects. Bars with dissimilar letters are significantly different ($P<0.05$).

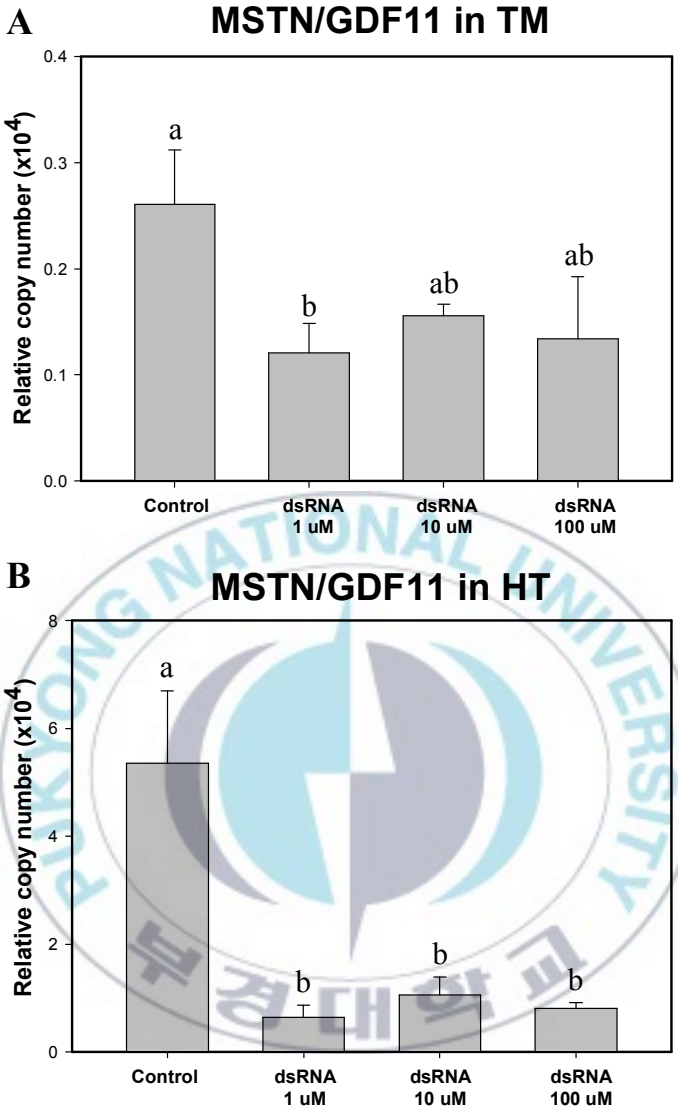

Fig. 3. Relative copy number of Lv-MSTN/GDF11 in tail muscle (A) and heart (B) tissues after injecting in 72 hours with three different doses of long dsRNA.

Shrimp were injected with three different doses of long dsRNAs (1 μM, 10 μM, and 100 μM). The relative copy numbers were normalized against the copy number of 18S rRNA. Student t-test was used to determine significant effects. Bars with dissimilar letters are significantly different ($P<0.05$).

Effect of dsRNA injection on the expression of three actin genes

To determine the effects of decreased Lv-MSTN/GDF11 expression on the transcription of muscle fiber genes, the levels of three actin transcripts were measured after dsRNA injection (Fig. 4). As described previously (Kim et al., 2009a; Uddowla et al., 2013), three types of actin genes are present in decapod crustaceans: cytoplasmic actins (ACTINCTs), skeletal muscle actins (ACTINSKs), and cardiac muscle actins (ACTINHTs). RT-PCR results confirmed two known actins: Lv-ACTINSK and Lv-ACTINCT. Actin from the heart, Lv-ACTINHT (GenBank number: KJ775802) was amplified by RT-PCR using degenerate primers designed in a previous study (Kim et al., 2009a). Deduced amino acid sequence comparison and phylogenetic analysis of the three actin cDNAs confirmed the presence of all three actin genes in the shrimp *L. vannamei*. Seventy-two hours after Lv-MSTN/GDF11 dsRNA injection, both Lv-ACTINCT and Lv-ACTINHT were significantly up-regulated in the heart, regardless of the concentration used (1–100 μM). In the heart, Lv-ACTINHT and Lv-ACTINCT transcript levels increased by 2.94- to 2.83- and 4.28- to 2.6- fold, respectively, compared to the control group (Fig. 4A). In the tail muscle, Lv-ACTINCT transcript levels increased significantly from 4.83- to 6.36-

fold compared to the control group (Fig. 5A). In contrast, expression of Lv-ACTINSK, skeletal muscle actin, was unchanged by Lv-MSTN/GDF11 dsRNA injection (Fig. 5B). As seen with Lv-MSTN/GDF11, there was no dose-dependent effect on the transcription level of all actin genes examined (Figs. 4 and 5). Therefore, the expression levels of cytoplasmic and cardiac actins were increased, whereas that of skeletal muscle actin was unaffected by Lv-MSTN/GDF11 dsRNA injection.

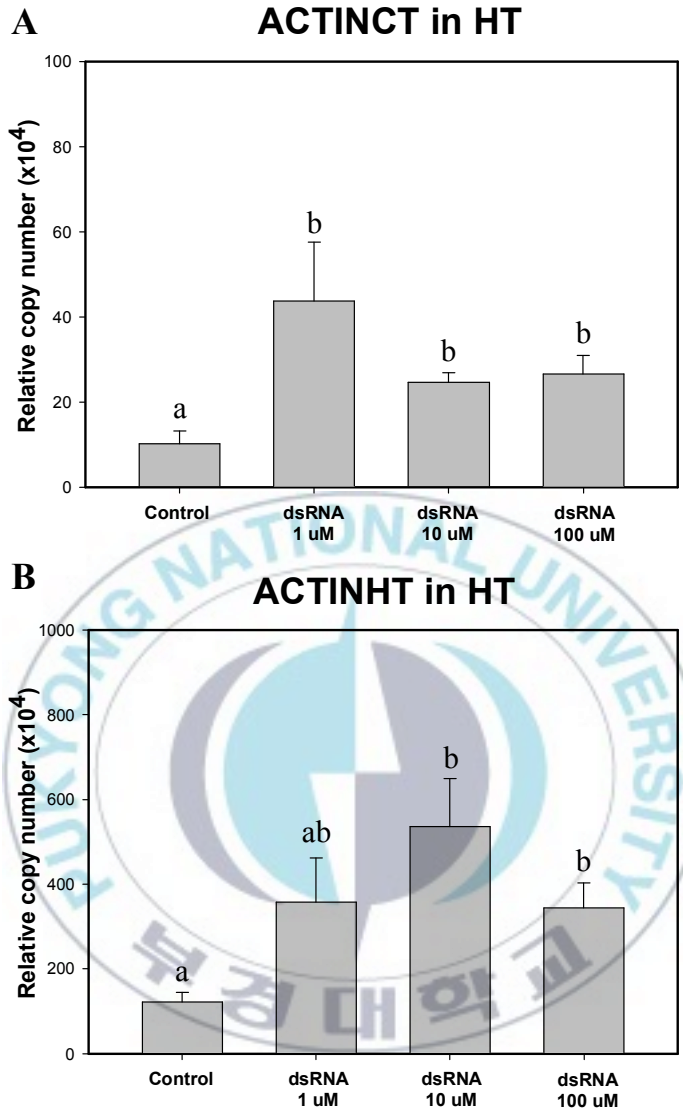

Fig. 4. Relative copy number of Lv-ACTINCT (A) and Lv-ACTINHT (B) in heart tissues after injecting in 72 hours with three different doses of long dsRNA.

Shrimp were injected with three different doses of long dsRNAs (1 μM, 10 μM, and 100 μM). The relative copy numbers were normalized against the copy number of 18S rRNA. Student t-test was used to determine significant effects. Bars with dissimilar letters are significantly different ($P<0.05$).

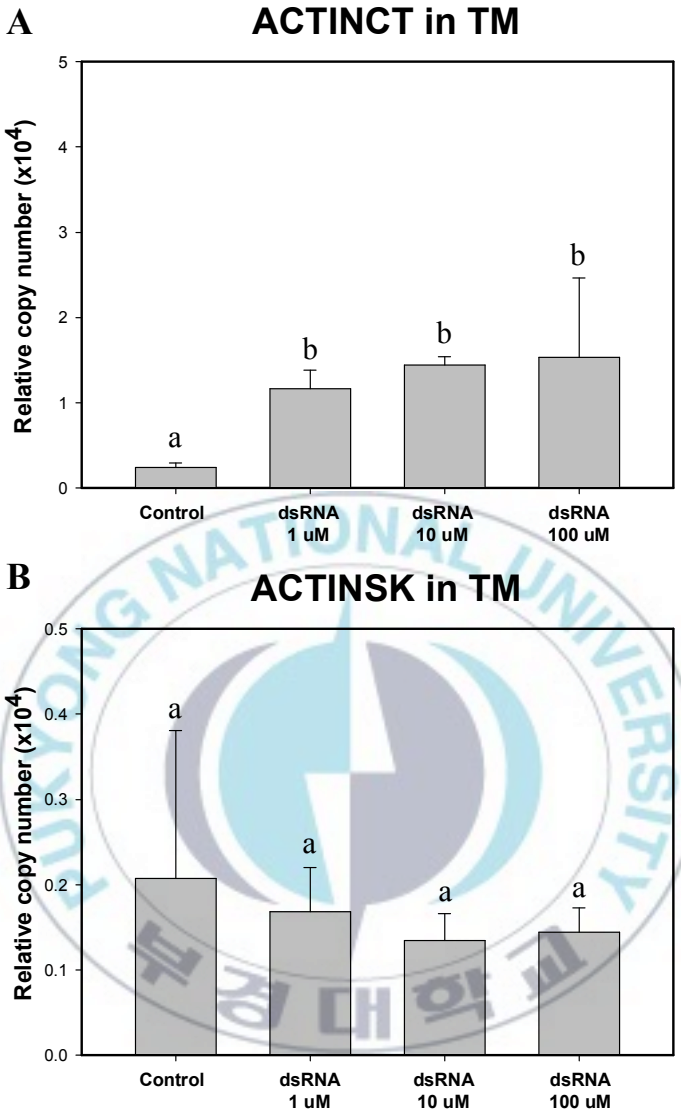

Fig. 5.Relative copy number of Lv-ACTINCT (A) and Lv-ACTINSK (B) in tail muscle tissues after injecting in 72 hours with three different doses of long dsRNA.

Shrimp were injected with three different doses of long dsRNAs (1 μM, 10 μM, and 100 μM). The relative copy numbers were normalized against the copy number of 18S rRNA. Student t-test was used to determine significant effects. Bars with dissimilar letters are significantly different ($P<0.05$).

Long-term effects of Lv-MSTN/GDF11 dsRNA injection

To determine the chronic effects of Lv-MSTN/GDF11 knockdown, 10 μMdsRNA were injected every 7 days. Significantly higher mortality (71%) was seen in dsRNA-injected shrimp. Over the 8 weeks of the experiment, 10 of 14 shrimp in the dsRNA-injected group died, compared to 4 of 10 (40%) in the control group (Table 2). Average survival days after dsRNA injection were measured to determine if stress from the injection was the major cause of mortality (Table 2). There was no significant difference in the survival days after injection between the control (5.25 ± 1.03 days) and dsRNA-injected (4.00 ± 0.58 days) groups. However, number of survival days post-ecdysisdiffered significantly between the two groups (Table 2). All shrimp in the control group died premolt stage (14.25 days ± 1.55), whereas most shrimp in the dsRNA-injected group died at the intermolt stage (5.89 days ± 1.79). This suggested that the cause of death differed between the two groups; therefore, the decreased Lv-MSTN/GDF11 levels during the postmolt stage might have been the cause of death in the dsRNA-injected group.

After 8 weeks of Lv-MSTN/GDF11 dsRNA injection, growth rate was higher in the control group, which showed a higher average (0.66g ±

0.18 g) gain in body weight (Fig. 6). In contrast, an average weight gain of only 0.20 ± 0.14 g was observed in the surviving individuals in the dsRNA injection group (Fig. 6). The molting period was also compared to determine whether Lv-MSTN/GDF11 dsRNA injection affected the molting cycle (Table 2). Compared with the control group (12.6 ± 0.66 days), a 1.8-day slower molting cycle was observed in the dsRNA-injected group but failed to identify the statistical significance (14.4 ± 0.75 days, $p = 0.089$).

Table 2. Comparison of mortality and molting periods between Lv-MSTN/GDF11 dsRNA-injected and control groups

Group	Total	Mortality	Mortality rate (%)	Average survival period post Ecdysis (days)	Average survival period post injection (days)	Molting period (days)
Control	10	4	40	14.25 ± 1.55	5.25 ± 1.03	12.6 ± 0.66
dsRNA	14	10	71	5.89 ± 1.79	4.00 ± 0.58	14.4 ± 0.75

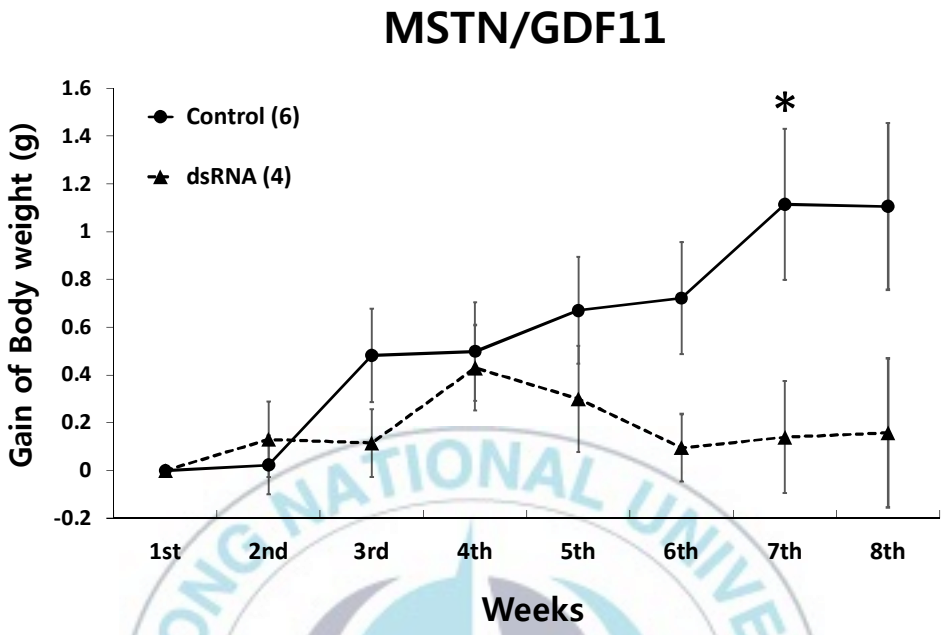

Fig. 6. Gain of body weight of *L. vannamei* in control and treatment groups during eight weeks.

Gain of body weight was calculated as 'weight of each week - initial weight (first week)' with surviving animals. The numbers in brackets indicate total number of shrimp. Student t-test was used to determine significant effects. 7th P-value is 0.05 (*).

Discussion

Expression patterns of Lv-MSTN/GDF11 differ from myostatins in mammals

As it is a negative regulator of muscle differentiation and growth in vertebrates, the MSTN gene is a potential target for use in the livestock industry. Its orthologs were identified in several invertebrates, mostly from economically important species, including decapod crustaceans and bivalves (De Santis et al., 2011; Kim et al., 2004; Qian et al., 2013). In contrast to the higher vertebrates, only a single putative ancestral ortholog was identified in invertebrates, which suggests that MSTN and GDF11 in vertebrates evolved from a single ancestral gene in invertebrates (Xu et al., 2003). Like other invertebrates, only a single Lv-MSTN/GDF11 gene was identified in decapod crustaceans (Kim et al., 2009b; MacLea et al., 2010; Qian et al., 2013). Because GDF11 and MSTN have different biological functions in mammals, it is not clear if the gene in decapod crustaceans is involved in facilitated muscle fiber growth as are mammalian MSTNs.

To determine the biological functions of Lv-MSTN/GDF11, we analyzed its tissue distribution using both end-point PCR and qPCR. In contrast to previous reports (Qian et al., 2013), considerably higher copy numbers of the Lv-MSTN/GDF11 transcript (20.55-fold) were identified in the heart muscle compared to the tail muscle (Figs. 2 and 3). Qian et al. (2013) showed that the Lv-MSTN/GDF11 expression level was similar in both heart and tail muscle when normalized to the Lv-ACTINCT gene. That result was based on the assumption that the expression of the β-actin gene, as a housekeeping gene, is similar in all tissues. In fact, it is now widely accepted that the expression of β-actin, a cytosolic actin gene, is no longer a reliable internal control for qPCR(Ruan and Lai, 2007; Selvey et al., 2001). We showed that the expression of the Lv-ACTINCT gene in the heart is 42.47-fold higher than in the tail muscle, which may explain the difference between the previous experiment and our results (Figs. 4 and 5). Similarly, the major production site for pmMstn/Gdf11, the ortholog of Lv-MSTN/GDF11 in *Penaeus monodon*, is the heart, where the expression level is 100-fold higher than in muscle tissue (De Santis et al., 2011). In *Pandalopsis japonica*, the highest level of MSTN/GDF11 expression was also in the heart (Kim et al., 2010). In brachyurans, MSTN/GDF11 was

expressed mainly in the claw, cephalothorax, and heart (Covi et al., 2008; Kim et al., 2009b). As the skeletal muscles of the cephalothorax and claw in *L. vannamei* are not as well developed as in brachyurans, we did not evaluate the expression of the Lv-MSTN/GDF11 gene in these two tissues. Collectively, the major production sites for MSTN/GDF11 include the heart, cephalothorax, and claws in decapod crustaceans, while the tail muscle in shrimp does not appear to be a major production site. This result suggests that its role in tail muscle growth and development may differ from that in other shrimp muscular tissues.

Lv-MSTN/GDF11 long dsRNA is the most effective method for knockdown of Lv-MSTN/GDF11 in shrimp

Since its first discovery (Fire et al., 1998), RNA interference (RNAi) has been an effective and convenient strategy to silence target genes in most metazoan species. The active components, small interfering RNAs (siRNAs), are generated endogenously by the RNase-III class endoribonuclease Dicer from long dsRNA and small hairpin RNAs (shRNAs) (Bernstein et al., 2001). siRNAs then enter into the RNA-induced silencing complex (RISC) serving as a sequence-specific locator of the

target RNAs, which are then degraded (Agrawal et al., 2003). Canonical siRNAs are short, 21–23-base duplexes with phosphorylated 5' ends and hydroxylated 3' ends with two overhanging nucleotides (Myers et al., 2003). However, longer siRNAs (25–27 bases) are produced in the protozoan parasite *Giardia intestinalis*(Macrae et al., 2006). Recently, DsiRNA, which is structurally asymmetric (25/27 nt) and chemically modified from canonical siRNA, was shown to be more efficient in inducing RNAi than canonical siRNAs(Kim et al., 2005a).

In this study, two short RNAs did not induce effective silencing, but long dsRNA exhibited significant Lv-MSTN/GDF11 gene knockdown activity (Fig. 2). Similar results were reported in *L. vannamei*, in which only dsRNAs of longer than 50 bp induced a significant reduction in mRNA levels (Labreuche et al., 2010). In addition, long dsRNA injection was demonstrated to be the most successful strategy for gene-specific RNA knockdown in most decapod crustaceans (see review by Sagi et. al. 2013). These results suggest that short RNAs, including siRNA or its chemically modified DsiRNAs, may not be as effective in decapod crustaceans as in mammals. Although the minimum effective size has been determined, the maximum size has not been investigated extensively. In this study, we used

352 bp of dsRNA, which was highly effective in decreasing Lv-MSTN/GDF11 expression (Figs. 2 and 3). Since injection of dsRNA into the tail muscle was an effective strategy for sequence-specific silencing activity in distantly located organs, including the SG/XO complex, hepatopancreas, gonad, and heart, a mechanism for transport of the large RNA molecules likely exists in decapod crustaceans. Further study should aim to identify this mechanism.

Although the amounts of injected dsRNA varied, the degree of interfering activity ranged from 40% to 80% in most decapod crustaceans regardless of dose and length, which is similar to our results. We found that knockdown levels of the Lv-MSTN/GDF11 gene in the same organ were similar (88.85% in the heart and 43.36% in the tail muscle) regardless of use of dsRNA concentrations greater than 1 μM (Figs. 2 and 3). Although the mechanism that determines the level of RNAi in shrimp remains unclear, tissue- or organ- specific factor(s) may be involved. The decreases in levels of the CDP transcript in the gills of *L. vannamei* were similar in four groups of different dsRNA lengths (Labreuche et al., 2010). In addition, dsRNA injection (5 μg) of the MSTN/GDF11 gene in *P. monodon* reduced the transcript levels in the tail muscle by ~40% (De Santis et al., 2011), which is

similar to our data (Figs. 2 and 3). Further studies should be conducted to identify the mechanism that regulates the level of RNAi in crustaceans.

The biological function of Lv-MSTN/GDF11 may differ from mammalian MSTNs

The majority of shrimp (~70%) died during the 8 weeks of the Lv-MSTN/GDF11 RNAi experiment (Table 2). Previous RNAi experiments with the Pm-MSTN/GDF11 gene from *P. monodon* showed a 68% reduction in growth, but no mortality (De Santis et al., 2011). Aside from the high mortality in our study, the results of the experiments were similar in that the growth rate was significantly lowered in individuals injected with MSTN/GDF11 dsRNAs. This indicates that MSTN/GDF11 in shrimp does not exert an inhibitory function as does MSTN in mammals. Several factors might have contributed to the higher mortality in our experiment. First, it may have come from the physiological differences between the two species. Second, there may also have been differences in the handling of shrimp during injection and measuring, possibly causing more stress, as indicated by the 40% mortality in the control group. However, the average survival days post-ecdysis differed markedly between the dsRNA-injected and

control groups (Table 2). All shrimp in the control group died just before ecdysis or at the premolt stage (14.25 days), whereas most shrimp in the dsRNA-injected group died after molting (5.89 days). Because the expression of MSTN/GDF11 is highest during postmolt (A stage), knockdown of MSTN/GDF11 dsRNA may be the major reason for the mortality in the early intermolt stage (De Santis et al., 2011; Qian et al., 2013). We postulate that this may explain the difference in mortality between the control and dsRNA-injected groups. Another possible explanation is an impaired neuronal or muscular network in the heart due to suppressed Lv-MSTN/GDF11 expression. In the arthropod, *Drosophila melanogaster*, a loss-of-function mutation in the MSTN/GDF11 receptor gene resulted in a dramatic reduction of synaptic development at the neuromuscular junctions, suggesting that ancestral MSTN/GDF11 in arthropods may not be involved in inhibiting muscle fiber growth and development (Aberle et al., 2002; Lee-Hoeflich et al., 2005).

Conclusion

In summary, the effectiveness of the three Lv-MSTN/GDF11 RNAs in silencing of the transcript was evaluated. Injection of long dsRNA (352 bp) was the most effective method of reducing expression compared to either canonical siRNA or Dicer substrate RNA. Lv-MSTN/GDF11 knockdown had no effect on skeletal actin (Lv-ACTINSK), whereas the cardiac and cytosolic actin transcript levels increased significantly.

Finally, we also showed that knockdown of Lv-MSTN/GDF11 had adverse effects on shrimp growth and molting, which differs from its effect in mammals, suggesting that this gene may not be a suitable target for shrimp aquaculture.

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