The Journal of Experimental Biology 214, 2671-2677

doi:10.1242/jeb.056374

RESEARCH ARTICLE

Growing backwards: an inverted role for the shrimp ortholog of vertebrate myostatin and GDF11

Christian De Santis[1], Nicholas M. Wade[2], Dean R. Jerry[1], Nigel P. Preston[2], Brett D. Glencross[2] and Melony J. Sellars[2,*]

[1]Aquaculture Genetics Research Program, School of Marine and Tropical Biology, James Cook University, Townsville, QLD 4811, Australia and [2]CSIRO Food Futures Flagship, CSIRO Marine and Atmospheric Research, PO Box 120, Cleveland, QLD 4163, Australia

*Author for correspondence (melony.sellars@csiro.au)

Accepted 9 May 2011

SUMMARY

Myostatin (MSTN) and growth differentiation factor-11 (GDF11) are closely related proteins involved in muscle cell growth and differentiation as well as neurogenesis of vertebrates. Both MSTN and GDF11 negatively regulate their functions. Invertebrates possess a single ortholog of the MSTN/GDF11 family. In order to understand the role of MSTN/GDF11 in crustaceans, the gene ortholog was identified and characterized in the penaeid shrimp *Penaeus monodon*. The overall protein sequence and specific functional sites were highly conserved with other members of the MSTN/GDF11 family. Gene transcripts of *pmMstn/Gdf11*, assessed by real-time PCR, were detected in a variety of tissue types and were actively regulated in muscle across the moult cycle. To assess phenotypic function in shrimp, *pmMstn/Gdf11* gene expression was downregulated by tail-muscle injection of sequence-specific double-stranded RNA. Shrimp with reduced levels of *pmMstn/Gdf11* transcripts displayed a dramatic slowing in growth rate compared with control groups. Findings from this study place the MSTN/GDF11 gene at the centre of growth regulation in shrimp, but suggest that, compared with higher vertebrates, this gene has an opposite role in invertebrates such as shrimp, where levels of gene expression may positively regulate growth.

Key words: *Penaeus monodon*, crustacean, myostatin/GDF11, growth, RNAi.

INTRODUCTION

Cell growth, proliferation and homeostasis are controlled by a number of structurally related proteins belonging to the transforming growth factor-β (TGF-β) superfamily. Growth differentiation factor 8 (GDF-8), also known as myostatin (MSTN), and GDF11 are closely related members of this superfamily that are thought to originate from one ancestral gene following gene duplication (Xu et al., 2003). In higher vertebrates, both MSTN and GDF11 are inhibitors of specific cellular functions, the former principally controlling the growth of muscle cells (McPherron et al., 1997) and the latter regulating neurogenesis in the olfactory epithelium as well as the development of the axial skeleton (McPherron et al., 1999; Wu et al., 2003). Both of these potent negative regulators (MSTN in particular) have recently been under investigation to explain mechanisms underlying important human muscular diseases, as well as to select fast-growing breeding lines for the enhancement of livestock and aquaculture production (De-Santis and Jerry, 2007; Lee, 2004).

Genes of the *Mstn/Gdf11* family have recently been isolated and characterized from a number of invertebrate species. Despite being reported under a diverse range of names – such as myoglanin in *Drosophila melanogaster*; *Mstn*-like gene in *Argopecten irradians*, *Homarus americanus*, *Pandalopsis japonica* and *Gecarcinus lateralis*; *Mstn/Gdf11* in *Nematostella vectensis*; and *Gdf8/11* in *Branchiostoma belcheri* – invertebrates have only had one ortholog of vertebrate *Mstn* and *Gdf11* isolated to date (Covi et al., 2008; Kim et al., 2004; Kim et al., 2010; Lo and Frasch, 1999; MacLea et al., 2010; Saina and Technau, 2009; Xing et al., 2007). For clarity, we refer to the abovementioned proteins as the invertebrate MSTN/GDF11 (iMSTN/GDF11).

Although the roles of both MSTN and GDF11 have been explored by functional studies in vertebrates (Lee et al., 2009; McPherron et al., 1997; McPherron et al., 1999; Wu et al., 2003), the physiological functional role of iMSTN/GDF11 is still not entirely understood. Previous findings reported that the *iMstn/Gdf11* gene showed widespread tissue expression, suggesting that, unlike the mammalian orthologs, its physiological significance might not be restricted to specific tissues (Covi et al., 2008; Kim et al., 2004; MacLea et al., 2010; Saina and Technau, 2009).

In invertebrate muscle, however, *iMstn/Gdf11* is actively regulated and is thought to control functions such as growth. In decapod crustaceans for example, mass gain is a discontinuous, moult-dependent process that may require a drastic and reversible reduction of muscle size in order to undergo exuviation of body parts such as claws from the old exoskeleton. In *G. lateralis* and *H. americanus*, this process of moult-induced rearrangement of the muscle structure is accompanied by a significant change in *Mstn/Gdf11* abundance (Covi et al., 2010; MacLea et al., 2010). In addition, the *Mstn/Gdf11* transcriptional changes strongly correlate with the level of circulating steroid hormones (ecdysteroids) that are known to regulate *Mstn* expression *in vitro* and *in vivo* in vertebrates (Ma et al., 2001; Ma et al., 2003). Evidence therefore suggests that, similar to vertebrate MSTN, iMSTN/GDF11 is involved in the protein cascade that regulates muscle growth in invertebrate species.

In this study, we isolated the complete coding sequence of *Mstn/Gdf11* from the penaeid shrimp *Penaeus monodon* Fabricius 1798, a commercially important decapod crustacean chosen as an experimental model because of its availability and high growth rate. We report on the differential expression pattern of *pmMstn/Gdf11* across various tissue types as well as its transcriptional regulation. To infer the specific function of MSTN/GDF11 on the regulation of growth in a crustacean, we measured the phenotypic changes induced by targeted downregulation of the gene in *P. monodon* using RNA interference (RNAi).

MATERIALS AND METHODS

Sequence isolation and analysis

A partial fragment of the *pmMstn/Gdf11* gene sequence was isolated using a primer pair (pmMSTN_CSIRO_F and pmMSTN_CSIRO_R; Table 1) designed on a region conserved across crustaceans [*Litopenaeus vannamei* (Lve, ESTs FE172407 and FE046767), *G. lateralis* (Gla, EU432218) and *Eriocheir sinensis* (Esi, EU650662)]. Mixed muscle cDNA was prepared as described below for differential tissue expression profiling, and a single PCR amplification product of ~330 bp was obtained, cloned into a pGEM-T Easy (Promega, Sydney, NSW, Australia) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Mulgrave, VIC, Australia). The full-length 3′ end was isolated using the GeneRacer advanced RACE kit (Invitrogen, Mulgrave, VIC, Australia) and gene-specific primers (RACE; Table 1). Attempts to amplify the full-length 5′ end of the transcript using the RACE kit and gene-specific nested primers (RACE; Table 1) yielded only a partial fragment (291 bp). Difficulties to RACE the 5′ end of the *Mstn/Gdf11* gene have been previously reported for crustaceans (MacLea et al., 2010). The complete 5′ open reading frame (ORF) was isolated by PCR amplification of muscle cDNA using a 5′ primer designed on a conserved region between *L. vannamei* and *G. lateralis Mstn/Gdf11* containing the ATG start codon and reverse primer designed on the known *pmMstn/Gdf11* sequence (5′ ORF discovery; Table 1).

To confirm the identity of the putative pmMSTN/GDF11 translated peptide sequence, evolutionary relationships were inferred between MSTN/GDF11 ortholog proteins (invertebrates and vertebrates), TGF-β1 and Inhibin (INH), which are the closest relatives of the MSTN/GDF11 sub-family (Herpin et al., 2004). An alignment was generated on the entire amino acid sequence using CLUSTALW (Thompson et al., 1994) and manually adjusted using MEGA 4.0 (Tamura et al., 2007). Evolutionary history was inferred using the neighbour joining method using MEGA 4.0 with bootstrap support based on 1000 replicates and the Jones–Taylor–Thornton (+G) model. Finally, the full-length protein sequence was analyzed for putative functional sites using the web-based server ELM (Puntervoll et al., 2003).

pmMstn/Gdf11 differential tissue expression and expression across the moult cycle

Expression profiles of *pmMstn/Gdf11* were analyzed in muscle, gill, hepatopancreas, eyestalk, heart and stomach. Each tissue was dissected from nine *P. monodon* and pooled into groups of three individuals per tissue type. Expression of *pmMstn/Gdf11* across the moult cycle was assessed in muscle tissue only from five *P. monodon* at each of the moult stages A, B, C, D_0, D_1, D_2 and D_3/D_4 [as determined according to setal staging and epidermal withdrawal in uropods (Smith and Dall, 1985)]. All tissues were immediately stored in RNAlater® (Applied Biosystems).

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and precipitated by adding 0.5 volumes of isopropyl alcohol and 0.5 volumes of RNA precipitation solution for purity improvement (Sambrook et al., 1989). Total RNA was DNase digested with the Turbo DNA-free kit (Applied Biosystems) and quality and quantity were assessed by gel electrophoresis and on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All RNA samples were diluted to 200 ng μl^{-1} using an epMotion 5070 (Eppendorf, North Ryde, NSW, Australia). Reverse transcription was performed on 1 μg total RNA using Superscript III (Invitrogen) with 25 μM oligo$(dT)_{20}$ and 25 μM random hexamers (Resuehr and Spiess, 2003). Expression of *pmMstn/Gdf11* in all samples was analyzed by real-time PCR as described below.

Real-time PCR primers specific to *pmMstn/Gdf11* (Table 1) were designed with PerlPrimer v1.1.17 (Marshall, 2004). Real-time PCR amplification reactions were carried out using 1× SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μmol l^{-1} of each primer and the equivalent of 7.5 ng reverse-transcribed RNA. Amplification

Table 1. Primer sequences used in the present study and their annealing temperature, position on the sequence relative to the GenBank submission and application

Primer name	Primer sequence (5′–3′)	Annealing temperature (°C)	Position	Application
pmMSTN_CSIRO_F	ATGCGCCGTTGCTGGAGAT	55	209	Gene discovery
pmMSTN_CSIRO_R	TCGTAATACAGCATCTTCATCGG	55	532	Gene discovery
pmMSTN_Csiro_3′RACE_F	ACCAATGCACTACCAGCCAGATCGAGTC	70	278	RACE
pmMSTN_Csiro_5′RACE_R	CGACAGCAGCGGGACTCGATCTG	70	317	RACE
pmMSTN_Csiro_5′RACE_R_(nest)	GGTAGTGCATTGGTTTCGGCTGGAGTTC	68	291	RACE
pmMSTN_Csiro_5′ORF_F	CAGACAGACATGCAGTGG	52	0	5′ORF discovery
pmMSTN_Csiro_5′ORF_R	GGATTGTTCAGCCATTCTTG	52	1058	5′ORF discovery
pmMSTN_Csiro_qPCR_F	GAAACCAATGCACTACCAGC	60	275	Real-time PCR
pmMSTN_Csiro_qPCR_R	TGTGGGCGTATAGATAAGGG	60	427	Real-time PCR
pmMSTN_Csiro_dsRNA_F	**GAATTTAATACGACTCACTATAGGGATC**CCAGAGGATTACATCATAC	48*	4	dsRNA synthesis
pmMSTN_Csiro_dsRNA_R	**GAATTTAATACGACTCACTATAGGGATC**AGCTTCATATACCTTAGG	48*	387	dsRNA synthesis
LUC_Csiro_dsRNA_F	**GAATTTAATACGACTCACTATAGGGATC**GCGCCATTCTATCCTCTA	55*	34	dsRNA synthesis
LUC_Csiro_dsRNA_R	**GAATTTAATACGACTCACTATAGGGATC**TACATCGACTGAAATCCCT	55*	495	dsRNA synthesis
pmACT_Csiro_dsRNA_F	**GAATTTAATACGACTCACTATAGGGATC**ATCATGTTCGAGACGTTC	50*	14	dsRNA synthesis
pmACT_Csiro_dsRNA_R	**GAATTTAATACGACTCACTATAGGGATC**CTCCTGCTTGCTGATCCA	50*	727	dsRNA synthesis

Boldface and underlined sections indicate the T7 promoter (to differentiate from the gene-specific sequence).
Asterisks denote gene-specific annealing temperature only (see Materials and methods for further details).

cycle conditions were 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 40 s at 60°C. Amplicon specificity was verified by cloning and sequencing and a dissociation melt curve analysis was routinely performed. Reactions were set up using the epMotion 5070 (Eppendorf) and run in quadruplicate on a 7500 real-time PCR system (Applied Biosystems). Verification of gDNA contamination was carried out by PCR amplification of DNAse-treated RNA samples using gene-specific primers. Normalization was performed using the ΔC_q method (where C_q is the quantification cycle), as it was considered the least biased approach (De Santis et al., 2011; Zhong et al., 2008).

Downregulation of *pmMstn/Gdf11*

Sequence-specific dsRNAs were synthesized from verified gene-specific PCR products using the MEGAscript RNAi kit (Applied Biosystems) according to the manufacturer's instructions. To synthesise double-stranded RNA (dsRNA), primers were designed to amplify the target genes *Luciferase* (*Luc*) (accession number EU754723), *pmMstn/Gdf11* (HQ221765) and β-*actin* (Sellars et al., 2011) (Table 1). dsRNA synthesis primers contained a gene-specific portion and a modified T7 promoter extension (boldface and underlined in Table 1) reported to increase transcription efficiency (Milligan et al., 1987; Tang et al., 2005). Integrity and size of newly synthesized dsRNA were quantified by 1.5% agarose/EtBr gel electrophoresis. *Luc*-dsRNA was used as an exogenous downregulation control and β-*actin*-dsRNA was used as an experimental technique control.

Penaeus monodon were collected from a commercial farm and maintained at CSIRO Marine and Atmospheric Research Laboratories, Cleveland, Australia, for 1 month in a bare-bottom 10 tonne tank prior to being stocked into the downregulation experiment. The holding tank received 1.6 l min^{-1} 28±2°C filtered (60 μmol l^{-1}) seawater, was fitted with a 10 mm bronze polycarbonate Polygal® sheet (Brendale, QLD, Australia) to reduce daytime light, and was subjected to a natural light cycle. Shrimp were fed a commercial feed (Lucky Star, Taiwan Hung Kuo Industrial Co., Su-Ao, I-Lan County, Taiwan) to apparent satiety twice per day at 09:00 and 17:00 h. On the day of stocking, shrimp were weighed, sexed and allocated randomly to tanks at a density of four shrimp per tank (two males and two females). Shrimp (20 per treatment) were tail-muscle injected with the four experimental treatments: saline solution, *Luc*-dsRNA (5 μg), *pmMstn/Gdf11*-dsRNA (5 μg) and β-*actin*-dsRNA (5 μg). Animals were weighed and re-injected with the assigned treatment every 10–11 days for the 45 day duration of the experiment. *pmMstn/Gdf11* transcript abundance was determined in pleopod (day 7) and tail muscle (day 45) of shrimp in experimental treatments.

Tanks received 1.6 l min^{-1}, 28±1°C, 34±2 ppt salinity seawater and had opaque white lids to reduce light intensity. Shrimp were fed a commercial feed (Lucky Star, Taiwan Hung Kuo Industrial Co.) to apparent satiety twice per day at 09:00 and 17:00 h. The number of shrimp alive in each tank were counted and recorded at 09:30 and 17:50 h every day for the 45 day experimental duration. Tanks were cleaned daily by siphoning and deaths removed.

Mean daily gain (MDG; g d^{-1}) [(final mass–initial mass)/growing period] and hepatosomatic index (HSI) [(hepatopancreas mass/body mass)×100] were calculated at the end of the experiment.

Statistical analysis

All statistical analyses were performed using SPSS (IBM, Somers, NY, USA). Assumptions for homogeneity of variance were tested using a Levene's test. Data that did not conform to the homogeneity of variance assumptions were log transformed. *pmMstn/Gdf11* expression (day 7 and day 45) and phenotypic (MDG and HSI) differences among treatments were tested using a two-way ANOVA, with treatment and sex as the independent variables. *pmMstn/Gdf11* expression across the moult cycle was analyzed using a one-way ANOVA. *Post hoc* multiple comparisons were made using Bonferroni tests. Differences were considered significant at $P<0.05$.

RESULTS

Sequence characterization and analysis

The complete coding sequence of the *pmMstn/Gdf11* gene (accession number HQ221765) shared the highest nucleotide identity (>70%) with *P. japonica*, *G. lateralis*, *E. sinensis* and *H. americanus* orthologs. The *pmMstn/Gdf11* ORF was 1260 bp in length, with a 66 bp 3′ untranslated region terminated by a classical polyadenylation signal. The translated protein was 419 amino acids (aa) in length (Fig. 1), shorter than that of other crustacean MSTN/GDF11 such as *G. lateralis* [497 aa (Covi et al., 2008)] and *E. sinensis* (468 aa). Three domains could be recognized, including a signal sequence (position 1–21) possibly cleaved at an N-Arg dibasic convertase cleavage site matching the motif ERK (position 22–24) and a C-terminal domain shared by members of the TGF-β superfamily. The furin/subtilisin-like cleavage site RXXR (RNRR, position 302–305) represented the putative cleavage site for the mature C-terminal peptide. The mature domain contained nine conserved cysteines, shown to be essential for the formation of interchain disulphide bonds involved in the final step of maturation of the protein (dimerization) (Daopin et al., 1992) (Fig. 1). A general motif for N-glycosylation Asp-X-Ser/Thr (PNMTG, residues 108–113) was also conserved in other vertebrate and invertebrate MSTN/GDF11 (Fig. 1).

All crustacean MSTN/GDF11 orthologs, including pmMSTN/GDF11, formed a highly supported single clade (Fig. 2). Outside this crustacean clade were other invertebrate orthologs of the MSTN/GDF11 family, including those from insects, molluscs and cnidarians (Fig. 2). Invertebrate MSTN/GDF11 clustered with vertebrate members of the MSTN/GDF11 subfamily (bootstrap value 95%) and was clearly distinguished from the closest relatives to this subfamily, TGF-β1 and INH. The overall topology of the MSTN/GDF11 tree was consistent with the hypothesis that invertebrates contain a single MSTN/GDF11 gene, and that a duplication event separated the GDF11 and MSTN paralogs in vertebrates (Fig. 2).

pmMstn/Gdf11 differential tissue expression and expression across the moult cycle

The *pmMstn/Gdf11* gene was widely expressed across all tissues under investigation including muscle, hepatopancreas, eyestalk, heart, gill and stomach. The highest transcriptional activity was observed in heart (C_q~13), where *pmMstn/Gdf11* mRNA copies were at least 100-fold higher than the remaining tissues (Fig. 3). Muscle, gill, eyestalk and stomach transcript abundance was altogether lower than that observed in heart and appeared similarly expressed in each of these tissues (C_q~17) (Fig. 3). *pmMstn/Gdf11* was detected at low levels in the hepatopancreas, where the mRNA copy number was 100-fold lower than all other tissues (C_q~25) (Fig. 3).

Muscle expression of *pmMstn/Gdf11* was regulated during the natural moult cycle (Fig. 4). A sharp peak of expression was detected immediately after exuviation (stage A, 0–9 h post-ecdysis) and was followed by a 10-fold drop in the next phases (late post-moult/inter-moult stages B–C, 9 to 48 h post-ecdysis; $P<0.01$) (Fig. 4). The pre-moult stages (D_0–D_4) displayed an overall higher

pmMstn/Gdf11 abundance than B–C stages ($P<0.05$) but did not statistically differ from the early post-moult stage (Fig. 4). During all stages of pre-moult, the expression levels of *pmMstn/Gdf11* remained relatively stable until the following exuviation (Fig. 4).

Phenotypic response to *pmMstn/Gdf11* downregulation

The *pmMstn/Gdf11* gene was downregulated by tail-muscle injection of sequence-specific dsRNAs (Fig. 5). Assessment of *pmMstn/Gdf11* expression among treatments revealed approximately 40% downregulation ($P<0.05$) of endogenous gene expression, which was evident at day 7 (7 days post-injection) and day 45 (3 days post-injection) (Fig. 5). As anticipated, positive control animals (N=12) injected with β-*actin*-dsRNA died within 2–4 days post-injection, indicating that endogenous gene silencing using tail-muscle injection of dsRNA was successful. Major growth differences were observed between the *pmMstn/Gdf11*

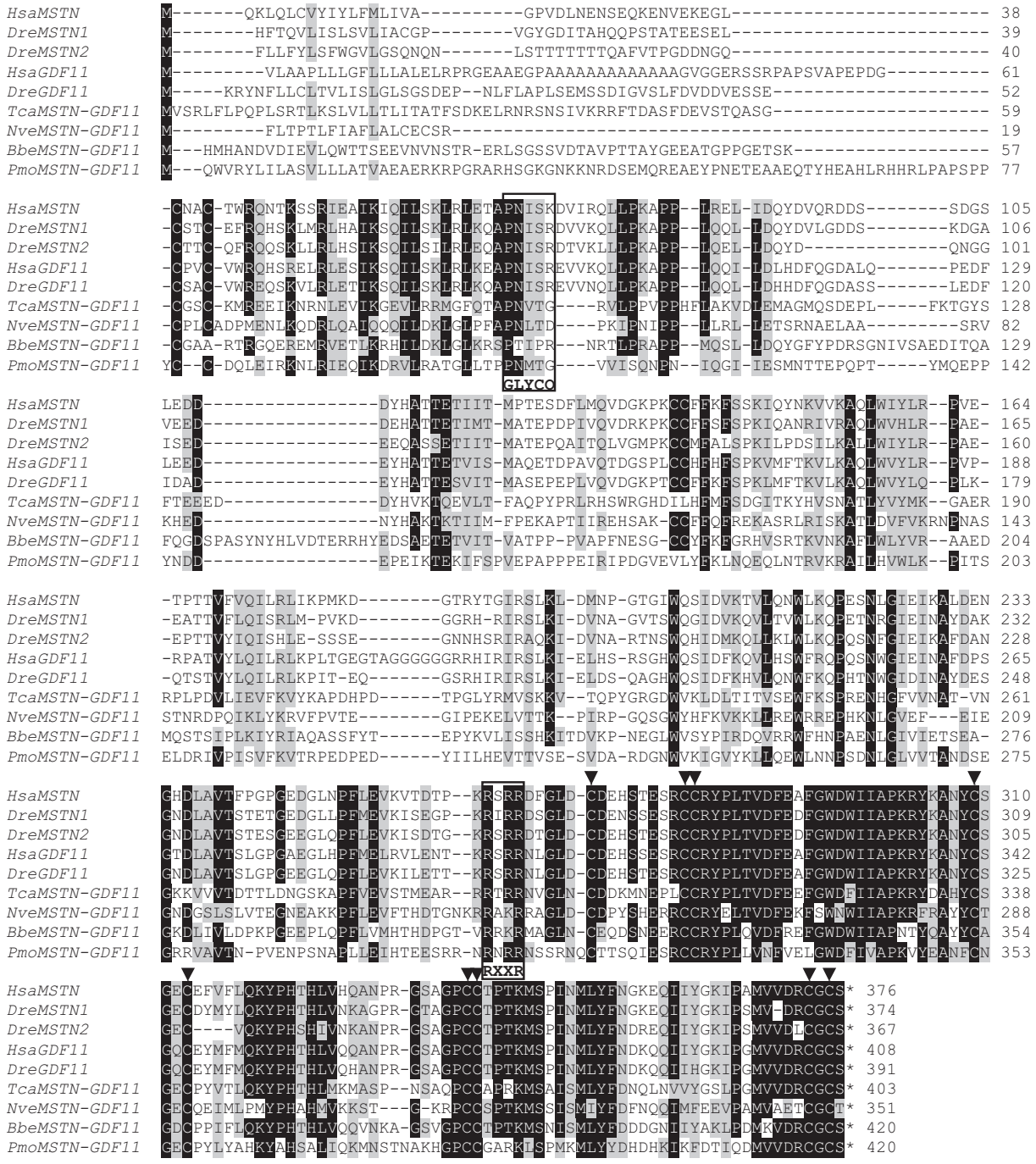

Fig. 1. Alignment of the translated amino acid sequence of pmMSTN/GDF11 (GenBank accession number HQ221765) with other representative members of the MSTN/GDF11 gene family. The putative conserved glycosylation site (GLYCO) and the furin/subsitilin-like cleavage site (RXXR) are boxed. The conserved cysteines are marked with triangles and the STOP codon is marked with an asterisk. Species compared in this figure include *Homo sapiens* (Hsa, AAH74757, NP_005802), *Danio rerio* (Dre, AAP85526, AAT95431 and NP_998140), *Tribolium castaneum* (Tca, XP_966819), *Nematostella vectensis* (Nve, XP_001641598) and *Branchiostoma belcheri* (Bbe, EF634365).

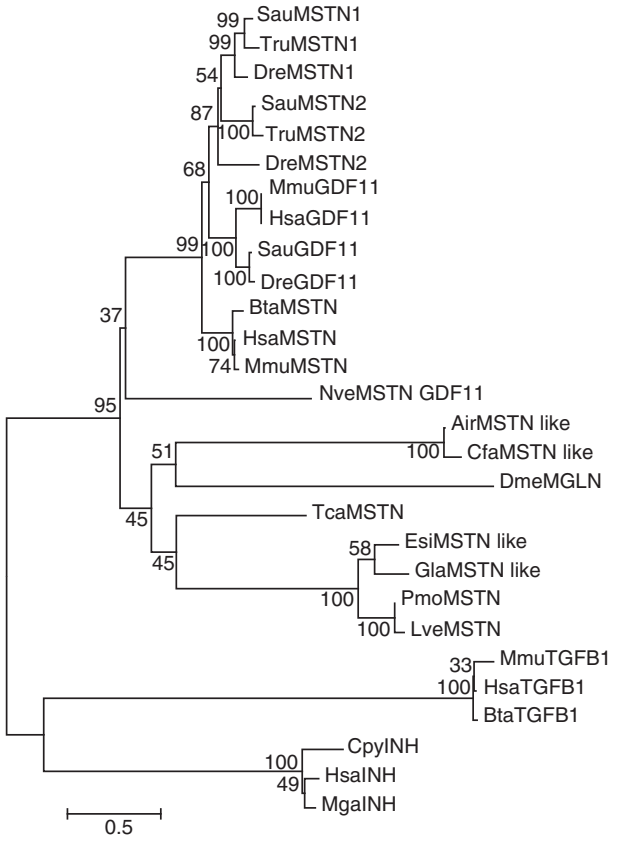

Fig. 2. Evolutionary relationships among vertebrate and invertebrate MSTN/GDF11, TGF-β1 and INH proteins. Species used to reconstruct the MSTN/GDF11 evolutionary relationship included *Sparus aurata* (Sau, AF258448_1 and AAL05943), *Takifugu rubripes* (Tru, AAR88255 and AAR88254), *Mus musculus* (Mmu, NP_035707, AAI03679 and NP_034402), *Homo sapiens* (Hsa, NP_000651 and NP_002183), *Bos taurus* (Bta, NP_001159540 and AAB86687), *Argopecten irradians* (Air, AAT36326), *Litopenaeus vannamei* (Lve, ESTs FE172407 and FE046767), *Gecarcinus lateralis* (Gla, ACB98643), *Eriocheir sinensis* (Esi, ACF40953), *Chlamys farreri* (Cfa, ACB87200), *Drosophila melanogaster* (Dme, AF132814_1), *Meleagris gallopavo* (Mga, AF336338_1) and *Cynops pyrrhogaster* (Cpy, BAA12693). The tree is drawn to scale, with branch lengths measured in the number of substitution per site. Values at the tree nodes indicate bootstrap support values.

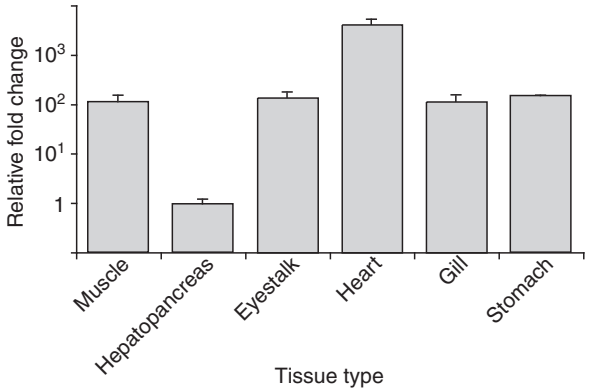

Fig. 3. Relative tissue-specific expression of the *pmMstn/Gdf11* gene. Values are reported relative to expression in the hepatopancreas. Bars indicate s.e.m. (*N*=3).

DISCUSSION

Vertebrate MSTN is a negative regulator of growth, whereby reducing *Mstn* expression induces increases in body mass (Acosta et al., 2005; Lee et al., 2009; McPherron et al., 1997). Loss-of-function mutations of the MSTN gene in vertebrates result in heavily muscled phenotypes (McPherron and Lee, 1997), whereas increasing circulating levels of MSTN induces muscle atrophy (Ma et al., 2003; Shao et al., 2007; Wehling et al., 2000). Recently, invertebrate orthologs of genes similar to *Mstn* have been reported in a number of species, including decapod crustaceans (Covi et al., 2008; Covi et al., 2010; Kim et al., 2010; MacLea et al., 2010); however, the function of these gene orthologs has not been specifically investigated.

In this study we isolated a previously unknown ortholog of *iMstn/Gdf11* named *pmMstn/Gdf11* from the shrimp *P. monodon*. The pmMSTN/GDF11 amino acid sequence contained all the principal functional sites common to all members of the MSTN/GDF11 family, including the nine cysteines, a subtilisin proteolytic site and a glycosidation site. Conservation of functional sites and evidence of three-dimensional structure similarity between vertebrate and crustacean MSTN/GDF11 (MacLea et al., 2010) suggests that this gene family may have maintained similar physiological functions throughout the animal kingdom.

Tail-muscle injection of a sequence-specific dsRNA against the isolated *pmMstn/Gdf11* resulted in a 68% reduction in *P. monodon* growth at the end of the experimental period. Interestingly, this response is opposite to that seen in higher vertebrates, suggesting that this gene is a positive growth regulator in this invertebrate species. In support of this theory, endogenous muscle expression of *Mstn/Gdf11* peaked immediately after moulting, a time where growth is not under the limitation of a hard exoskeleton and treatment and controls, where shrimp injected with *pmMstn/Gdf11*-dsRNA showed a notably slower overall growth rate compared with *Luc*-dsRNA- and saline-injected controls (Fig. 6; Table 2). Initially evident as early as 11 days (Fig. 6) after the first injection, by day 45, shrimp that received *pmMstn/Gdf11*-dsRNA had only grown by 32%, compared with control animals that had more than doubled their initial size. It is worth noting that despite the slightly smaller initial mass of *Luc*-dsRNA shrimp compared with saline shrimp, their mass gain patterns were similar (Fig. 6). MDG of 0.06 g d^{-1} for those receiving the *pmMstn/Gdf11*-dsRNA, was significantly slower than that of the *Luc* and saline controls at 0.23 and 0.18 g d^{-1}, respectively (P<0.01; Table 2). The small but significant difference of 0.05 g d^{-1} in MDG of *Luc* and saline controls is thought to be due to the variation in initial starting mass. No significant differences in HSI were observed, indicating a similar general health status of shrimp across all treatments and controls (Table 2), and there were no recorded deaths during the entire experimental period.

Table 2. Mean (±s.e.m.) daily gain and hepatosomatic index of shrimp injected with saline solution, *Luc*-dsRNA and *pmMSTN/GDF11*-dsRNA

	Saline	*Luc*-dsRNA	*pmMSTN/GDF11*-dsRNA
Mean daily gain (g d^{-1})	0.23±0.011^{a}	0.18±0.013^{b}	0.06±0.005^{c}
Hepatosomatic index	3.81±0.12	3.69±0.14	3.58±0.15

Within rows, values with different superscripts are significantly different (P<0.05).

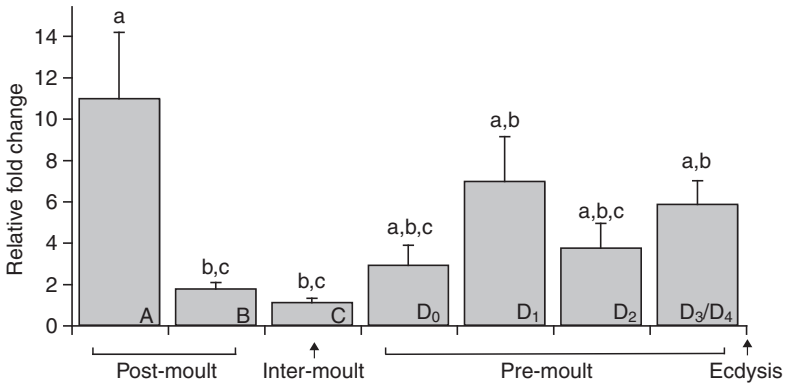

Fig. 4. Relative *pmMstn/Gdf11* expression across the molt cycle. Values are reported relative to the C stage (inter-moult). Error bars indicate s.e.m. (*N*=5). Columns denoted with the same letter are not significantly different (*P*>0.05).

significant expansion of the animal occurs. During this time, potential induction of muscle growth factors are required to promote growth into the larger shell. This notion is supported by previous work in crustaceans, where *Mstn/Gdf11* abundance gradually decreased in the claws of *G. lateralis* during premoult stages (D_0–D_4) as the muscle underwent severe atrophy to allow the withdrawal of the claws from the old exoskeleton during ecdysis (Covi et al., 2010). This again provides a consistent positive relationship between *iMSTN/GDF11* expression and muscle size.

Vertebrate *MSTN* and *GDF11* are thought to have separated from a single gene, the archetypal *Mstn/Gdf11* gene (Xing et al., 2007; Xu et al., 2003). The function of this gene is presumed to resemble that of vertebrate *Mstn* (i.e. growth and differentiation of muscle cells) and *Gdf11* (i.e. neurogenesis). It stands to reason that a combined functional role of the *iMstn/Gdf11* gene may be reflected in a broader tissue gene expression profile, one that is less specialized than that of the vertebrate *Mstn* or *Gdf11* paralogs. Indeed, transcripts of the *pmMstn/Gdf11* gene were expressed in a wider variety of shrimp tissues, including muscle, gills, heart, eyestalk and hepatopancreas, similar to the expression observed in other crustaceans such as *P. japonica*, *G. lateralis* and *H. americanus* (Covi et al., 2008; Kim et al., 2010; MacLea et al., 2010).

Evidence also exists for a role for *iMstn/Gdf11* in neurogenesis in *Drosophila melanogaster*, whereby mutant flies carrying a loss-of-function mutation at the gene coding for the MSTN/GDF11 receptor displayed a dramatic reduction of synaptic development at neuromuscular junctions (Aberle et al., 2002; Lee-Hoeflich et al., 2005). Interestingly, this functional role in insect synapses was also opposite to that observed in vertebrate systems, suggesting that there may have been an inversion of the entire functional role for the ancestral *iMstn/Gdf11* gene. At this point, it is not possible to define where this inversion may have occurred, either specifically within arthropods or elsewhere between arthropods and higher vertebrates. The precise functional role for *iMstn/Gdf11* in other crustacean tissues also requires further clarification, as this study specifically sought to investigate the role of *iMstn/Gdf11* in crustacean muscle and other phenotypes were not investigated.

In summary, here we characterized the *pmMstn/Gdf11* gene in the shrimp *P. monodon*, including the tissue expression profile, regulation in muscle across the moult cycle and biological function. We reconciled this gene and other invertebrate genes as an ortholog of the vertebrate MSTN/GDF11 family and provided clear evidence

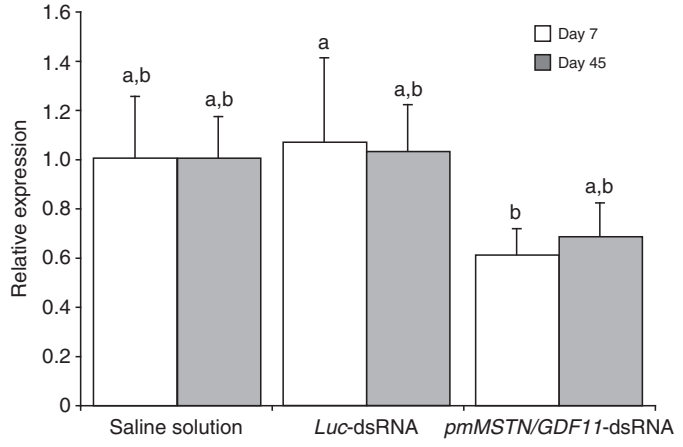

Fig. 5. Relative expression of the *pmMstn/Gdf11* gene in *Penaeus monodon* injected with saline solution, *Luc*-dsRNA and *pmMstn/Gdf11*-dsRNA. Values are reported relative to control shrimp (saline solution). White columns denote pleopod samples taken 7 days after the first injection; grey columns denote muscle samples taken at day 45 (3 days after the last injection). At both 7 and 45 days, a marginal reduction (~40%, *P*<0.05) of *pmMstn/Gdf11* mRNA abundance was observed in shrimp tail-muscle injected with *pmMstn/Gdf11*-dsRNA. Error bars indicate s.e.m. (*N*=20). Columns denoted with the same letter are not significantly different (*P*<0.05).

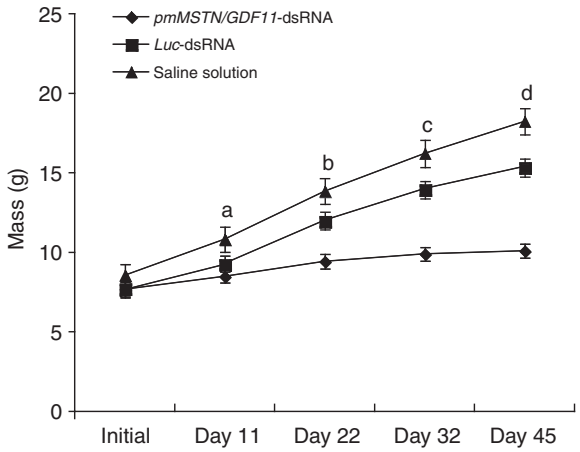

Fig. 6. Mass measurements of *P. monodon* in experimental treatments (injection of saline solution, *Luc*-dsRNA and *pmMstn/Gdf11*-dsRNA) recorded periodically along the 45 day duration of the experiment. Error bars indicate s.e.m. (*N*=20). Statistical significance of differences: 'a' denotes Sal>*pmMstn/Gdf11* (*P*<0.05); 'b' denotes Sal>*pmMstn/Gdf11* (*P*<0.01) and *Luc*>*pmMstn/Gdf11* (*P*<0.01); 'c' denotes Sal>*Luc* (*P*<0.05), Sal>*pmMstn/Gdf11* (*P*<0.01) and *Luc*>*pmMstn/Gdf11* (*P*<0.01); 'd' denotes Sal>*Luc*>*pmMstn/Gdf11* (*P*<0.01).

of a conserved growth regulation function of *iMstn/Gdf11* in muscle. We demonstrated a dramatic slowing of the overall growth rate in response to downregulation of the *iMstn/Gdf11* gene. This phenotypic effect in shrimp, in stark contrast to other vertebrate systems, appears to be positive rather than negative. As such, mechanisms that increase *pmMSTN/GDF11* gene expression may be valid targets to boost growth performance and improve shrimp production.

ACKNOWLEDGEMENTS

The authors thank Ekaterina Nowak for technical assistance and the Australian Network of Marine Science (ANiMS) for financial support.

REFERENCES

Aberle, H., Haghighi, A. P., Fetter, R. D., McCabe, B. D., Magalhaes, T. R. and Goodman, C. S. (2002). *wishful thinking* encodes a BMP type II receptor that regulates synaptic growth in *Drosophila*. *Neuron* **33**, 545-558.

Acosta, J., Carpio, Y., Borroto, I., Gonzalez, O. and Estrada, M. P. (2005). Myostatin gene silenced by RNAi show a zebrafish giant phenotype. *J. Biotechnol.* **119**, 324-331.

Covi, J. A., Kim, H. W. and Mykles, D. L. (2008). Expression of alternatively spliced transcripts for a myostatin-like protein in the blackback land crab, *Gecarcinus lateralis*. *Comp. Biochem. Physiol.* **150A**, 423-430.

Covi, J. A., Bader, B. D., Chang, E. S. and Mykles, D. L. (2010). Molt cycle regulation of protein synthesis in skeletal muscle of the blackback land crab, *Gecarcinus lateralis*, and the differential expression of a myostatin-like factor during atrophy induced by molting or unweighting. *J. Exp. Biol.* **213**, 172-183.

Daopin, S., Piez, K. A., Ogawa, Y. and Davies, D. R. (1992). Crystal-structure of transforming growth-factor-beta-2 – an unusual fold for the superfamily. *Science* **257**, 369-373.

De Santis, C. and Jerry, D. R. (2007). Candidate growth genes in finfish – where should we be looking? *Aquaculture* **272**, 22-38.

De Santis, C., Smith-Keune, C. and Jerry, D. R. (2011). Normalizing RT-qPCR data: are we getting the right answers? An appraisal of normalization approaches and internal reference genes from a case study in the finfish *Lates calcarifer*. *Mar. Biotechnol.* **13**, 170-180.

Herpin, A., Lelong, C. and Favrel, P. (2004). Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. *Dev. Comp. Immunol.* **28**, 461-485.

Kim, H. W., Mykles, D. L., Goetz, F. W. and Roberts, S. B. (2004). Characterization of a myostatin-like gene from the bay scallop, *Argopecten irradians*. *Biochim. Biophys. Acta* **1679**, 174-179.

Kim, K. S., Kim, Y. J., Jeon, J. M., Kang, Y. S., Kang, Y. S., Oh, C. W. and Kim, H. W. (2010). Molecular characterization of myostatin-like genes expressed highly in the muscle tissue from Morotoge shrimp, *Pandalopsis japonica*. *Aquacult. Res.* **41**, e862-e871.

Lee, C. Y., Hu, S. Y., Gong, H. Y., Chen, M. H. C., Lu, J. K. and Wu, J. L. (2009). Suppression of myostatin with vector-based RNA interference causes a double-muscle effect in transgenic zebrafish. *Biochem. Biophys. Res. Commun.* **387**, 766-771.

Lee, S. J. (2004). Regulation of muscle mass by myostatin. *Annu. Rev. Cell Dev. Biol.* **20**, 61-86.

Lee-Hoeflich, S. T., Zhao, X., Mehra, A. and Attisano, L. (2005). The *Drosophila* type II receptor, Wishful thinking, binds BMP and myoglianin to activate multiple TGF beta family signaling pathways. *FEBS Lett.* **579**, 4615-4621.

Lo, P. C. H. and Frasch, M. (1999). Sequence and expression of myoglianin, a novel *Drosophila* gene of the TGF-beta superfamily. *Mech. Dev.* **86**, 171-175.

Ma, K., Mallidis, C., Artaza, J., Taylor, W., Gonzalez-Cadavid, N. and Bhasin, S. (2001). Characterization of 5′-regulatory region of human myostatin gene: regulation by dexamethasone *in vitro*. *Am. J. Physiol. Endocrinol. Metab.* **281**, 1128-1136.

Ma, K., Mallidis, C., Bhasin, S., Mahabadi, V., Artaza, J., Gonzalez-Cadavid, N., Arias, J. and Salehian, B. (2003). Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. *Am. J. Physiol. Endocrinol. Metab.* **285**, 363-371.

MacLea, K. S., Covi, J. A., Kim, H. W., Chao, E., Medler, S., Chang, E. S. and Mykles, D. L. (2010). Myostatin from the American lobster, *Homarus americanus*: cloning and effects of molting on expression in skeletal muscles. *Comp. Biochem. Physiol.* **157A**, 328-337.

Marshall, O. (2004). PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* **20**, 2471-2472.

McPherron, A. C. and Lee, S. J. (1997). Double muscling in cattle due to mutations in the myostatin gene. *Proc. Natl. Acad. Sci. USA* **94**, 12457-12461.

McPherron, A. C., Lawler, A. M. and Lee, S. J. (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83-90.

McPherron, A. C., Lawler, A. M. and Lee, S. J. (1999). Regulation of anterior posterior patterning of the axial skeleton by growth differentiation factor 11. *Nat. Genet.* **22**, 260-264.

Milligan, J. F., Groebe, D. R., Witherell, G. W. and Uhlenbeck, O. C. (1987). Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15**, 8783-8798.

Puntervoll, P., Linding, R., Gemund, C., Chabanis-Davidson, S., Mattingsdal, M., Cameron, S., Martin, D. M. A., Ausiello, G., Brannetti, B., Costantini, A. et al. (2003). ELM server: a new resource for investigating short functional sites in modular eukaryotic proteins. *Nucleic Acids Res.* **31**, 3625-3630.

Resuehr, D. and Spiess, A. N. (2003). A real-time polymerase chain reaction-based evaluation of cDNA synthesis priming methods. *Anal. Biochem.* **322**, 287-291.

Saina, M. and Technau, U. (2009). Characterization of myostatin/gdf8/11 in the starlet sea anemone *Nematostella vectensis*. *J. Exp. Zool. B Mol. Dev. Evol.* **312**, 780-788.

Sambrook, J., Fritsch, E. and Maniatus, T. (1989). *Molecular Cloning: a Laboratory Manual* (2nd edn). New York: Cold Spring Harbor Laboratory Press.

Sellars, M. J., Rao, M., Arnold, S. J., Wade, N. and Cowley, J. (2011). *Penaeus monodon* is protected against gill-associated virus by muscle injection but not oral delivery of bacterially expressed dsRNAs. *Dis. Aquat. Org.* **95**, 19-30.

Shao, C. X., Liu, M., Wu, X. and Ding, F. (2007). Time-dependent expression of myostatin RNA transcript and protein in gastrocnemius muscle of mice after sciatic nerve resection. *Microsurgery* **27**, 487-493.

Smith, D. M. and Dall, W. (1985). Moult staging the tiger prawn *Penaeus esculentus*. In *Second Australian National Prawn Seminar, NPS2* (ed. P. C. Rothlisberg, B. J. Hill and D. J. Staples), pp. 85-93. Cleveland, Australia: CSIRO.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596-1599.

Tang, G. Q., Bandwar, R. P. and Patel, S. S. (2005). Extended upstream A-T sequence increases T7 promoter strength. *J. Biol. Chem.* **280**, 40707-40713.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). Clustal-W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Wehling, M., Cai, B. Y. and Tidball, J. G. (2000). Modulation of myostatin expression during modified muscle use. *FASEB J.* **14**, 103-110.

Wu, H. H., Ivkovic, S., Murray, R. C., Jaramillo, S., Lyons, K. M., Johnson, J. E. and Calof, A. L. (2003). Autoregulation of neurogenesis by GDF11. *Neuron* **37**, 197-207.

Xing, F., Tan, X. G., Zhang, P. J., Ma, J. K., Zhang, Y. Q., Xu, P. and Xu, Y. L. (2007). Characterization of amphioxus GDF8/11 gene, an archetype of vertebrate MSTN and GDF11. *Dev. Genes Evol.* **217**, 549-554.

Xu, C., Wu, G., Zohar, Y. and Du, S. J. (2003). Analysis of myostatin gene structure, expression and function in zebrafish. *J. Exp. Biol.* **206**, 4067-4079.

Zhong, Q. W., Zhang, Q. Q., Wang, Z. G., Qi, J., Chen, Y. J., Li, S., Sun, Y. Y., Li, C. M. and Lan, X. (2008). Expression profiling and validation of potential reference genes during *Paralichthys olivaceus* embryogenesis. *Mar. Biotechnol.* **10**, 310-318.