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Abundance of myostatin gene transcripts and their correlation with muscle hypertrophy during the development of barramundi, *Lates calcarifer*

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ABSTRACT

Myostatin (MSTN) is a pivotal protein that regulates vertebrate muscle growth and development. Teleost fish possess two MSTN paralogs (MSTN-1 and MSTN-2) whose respective physiological functions are still largely unclear. To clarify the role of each of these paralogs the transcript abundance of *Mstn-1* and *Mstn-2* was quantified during embryonic and larval development of the teleosts, barramundi, *Lates calcarifer*. Histological analyses of developing muscle fibers were also obtained to correlate *Mstn* paralog expression with muscle hypertrophy as larvae undergo metamorphosis. *Mstn-1* and *Mstn-2* transcripts were detected as early as immediately postfertilization of eggs, with the level of expression observed to increase during embryonic development and reach a peak near hatching. *Mstn-2* expression was thousands-fold higher than that observed for *Mstn-1*. Close to metamorphosis, the expression of *Mstn-1* was non-significantly, although positively associated, with muscle hypertrophy ($r = 0.384$, $p = 0.064$), while that of *Mstn-2* showed a highly significant negative correlation ($r = -0.691$, $p < 0.0001$) suggesting that this paralog may be responsible for muscle hypertrophy that occurs post-metamorphosis. Altogether, findings from this study support the hypothesis that *Mstn* paralogs are differentially regulated during various phases of fish development and that they may have evolved different functions in fish, particularly that related to muscle hypertrophy.

1. Introduction

Myostatin (MSTN), a member of the transforming growth factor-β (TGFβ) superfamily, is a key protein that regulates vertebrate muscle growth and development. In mammalian cells, where its function was first proposed, MSTN inhibits muscle growth and affects both fiber hyperplasia and hypertrophy by preventing myoblast cell cycle progression. MSTN has also been shown to influence myogenesis from early into embryonic development (Amali et al., 2008; Rodgers and Garikipati, 2008). Unlike mammals where only one *Mstn* gene is present and where expression is primarily restricted to muscle, teleosts possess at least two paralogs (*Mstn-1* and *Mstn-2*) that are independently regulated in a range of tissue types where they possibly have a diverse array of functions. Based solely on tissue expression patterns, several studies have speculated that MSTN-1 may be the protein responsible for inhibition of muscle growth, while MSTN-2 was thought to be primarily associated with neural functions as its expression is most abundant in the brain (Rodgers and Garikipati, 2008; De Santis and Jerry, 2011). Nevertheless, recent works have refuted this hypothesis and provided evidence that modification of the muscle structure also occurred in *Danio rerio* over-expressing the *Mstn-2* gene, hence indicating that MSTN-2 is at least capable of regulating myogenesis (Amali et al., 2008). Further confusion as to the role of MSTN proteins in teleosts arises from Garikipati and Rodgers (2012) in rainbow trout, whereby they showed *Mstn-1a* to be positively associated with myosatellite cell differentiation, contradictory to that seen in mammalian cell lines.

In the muscle of teleosts, the hypothesis that MSTN-1 and MSTN-2 are both involved in growth regulation is supported by several studies. However, whether these two proteins possess redundant functions, or regulate different physiological muscle processes, remains unclear. Rodgers et al. (2007) among others have suggested that perhaps our failure to understand the individual roles of MSTN-1 and MSTN-2 in regulating muscle growth may partially originate from methodological difficulties in distinguishing paralogs in experimental comparisons. Accordingly, a more attentive comparative analysis to unravel the functional evolution of these closely related proteins has been encouraged (Kerr et al., 2005; Helterline et al., 2007; Rodgers et al., 2007). Despite this encouragement, assessments of MSTN functions in regulating fish muscle growth have still to date been far from conclusive. For example, one approach adopted to clarify paralog role is that of RNA interference (RNAi). By injecting double-stranded RNA designed from tilapia *Mstn-1* into *D. rerio* embryos, Acosta et al. (2005) induced a "giant phenotype" with increased hyperplasia and hypertrophy; a phenotype similar to that

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observed in mice with disrupted MSTN (McPherron et al., 1997). A similar RNAi experiment, later conducted using *D. rerio*-specific *Mstn-2* double-stranded RNA, also measured increased muscle growth through hypertrophy, although growth through hyperplasia was not examined (Lee et al., 2009). Interestingly, Lee et al. (2009) reported that co-suppression of both *Mstn-1* and *Mstn-2* occurred even when only *Mstn-2* was targeted in a highly specific manner. This suggests that RNAi suppressed both genes in these experiments due to high similarity among paralogs, hence limiting the ability in this experiment to attribute specific roles to one paralog or the other. A more selective approach that employed targeted mutagenesis showed that decreasing only the abundance of *Mstn-1* did not affect overall growth in medaka (*Oryzias latipes*), although muscle hyperplasia was still induced (Sawatari et al., 2010). Finally, growth improvement was also achieved by blocking the activity of MSTN-1/MSTN-2 synergistically increasing circulating levels of follistatin, MSTN prodomain and the activin type 2 receptor (Carpio et al., 2009; Medeiros et al., 2009; Lee et al., 2010). Summarizing evidence arising from the aforementioned studies, it can be speculated that in teleosts MSTN-1 appears to primarily inhibit muscle hyperplasia, but perhaps not hypertrophy, and that growth through muscle hypertrophy is only attained when also the MSTN-2 is down-regulated. It is evident that more studies are needed to elucidate the specific roles of MSTN-1 and MSTN-2 in fish muscle.

Teleost *Mstn* genes, and particularly *Mstn-1*, are dynamically regulated from early stages of embryonic development where they may control the commitment of germ cells to muscle lineages, and/or regulate the proliferation and growth of myoblasts (Maccatrozzo et al., 2001; Xu et al., 2003; Roberts et al., 2004; Garikipati et al., 2006, 2007; Helterline et al., 2007; Zhong et al., 2008a). While several authors have reported detection of *Mstn-1* transcripts during early development, only in *D. rerio* has the distinct regulation of *Mstn-1* and *Mstn-2* genes been comparatively analyzed during embryogenesis (Helterline et al., 2007). During the development of *D. rerio*, *Mstn* paralogs exhibited individual relative expression profiles, confirming that the two genes are at least switched on/off by different trans-regulatory factors and suggesting that they may ultimately have evolved different physiological roles. Since *D. rerio* is the only fish species where the *Mstn-2* early developmental profile has been characterized in comparison with that of the *Mstn-1*, it is premature to conclude that this expression trend is representative of other teleosts. Lack of studies comparatively investigating the expression of *Mstn* paralogs in teleosts has even more significance in view of the fact that mechanisms of muscle growth are known to differ as early as during embryonic development in fish exhibiting determinant (i.e. *D. rerio*) and indefinite (most aquaculture species) growth (Johnston, 2006). If *Mstn* paralogs influence different aspects of fish muscle development it is very conceivable that their expression profiles might differ in fish which exhibit different mechanisms of muscle growth. Thereby, elucidating the regulation of *Mstn* paralogs during the development of other fishes with determinant and indefinite growth may help clarify our understanding of their function and evolution in fish.

To further clarify our understanding of how *Mstn* paralogs are regulated throughout teleost development, the present investigation assessed the relative abundance of *Mstn-1* and *Mstn-2* in the barramundi, *L. calcarifer*, throughout embryonic, larval and post-metamorphic growth. Barramundi (or Asian sea bass in south-east Asia) is a commercially important aquaculture species renowned for its very fast growth and large overall adult body size (up to 137 cm and 45 kg). Therefore this species is a good representative expression model for other teleosts possessing an indefinite pattern of growth. Furthermore, to examine if differential regulation of *Mstn* paralogs explain differences in muscle fiber growth histological cross-sections of juvenile barramundi muscle were sampled from fish of the same age, but from different size grades, and compared to levels of *Mstn-1* and *Mstn-2* transcript abundance.

2. Materials and methods

2.1. Sample collection, RNA preparation and cDNA synthesis

L. calcarifer used in this study were obtained from a commercial hatchery in North Queensland (Australia). Broodstock spawning was performed according to commercial procedures at 28 °C and fertilized eggs were collected immediately after first becoming visible in the water column using mesh nets. Further sampling of developing embryos and larvae occurred periodically and the 8-cell stage (CS) (1 h), 64-CS (3 h), blastula (5 h), gastrula (6.5 h), neurula (8.5 h), fully formed embryo (12 h), hatching (17 h), 3, 9, 13, 20, 30 h and 4, 15 and 21 days after hatching (time elapsed after eggs were first visible in the water column is provided in brackets). Stages of embryonic and larval development were visually confirmed under a light microscope (Tattanon and Tiensongrusmee, 1984; Tiensongrusmee et al., 1989). At 30 h post egg hatching, growing larvae were transferred from the hatchery to the James Cook University Marine and Aquaculture Facility and raised according to commercial farming procedures until completion of the experiment (Schipp et al., 2007). All samples collected were stored in RNA later (Ambion) until further processing. Where the size of individuals did not suffice for RNA extraction (before 30 h post-hatch), pools of approximately 50 embryos/larvae were processed as a single biological replicate. Consequently in pre-30 hour stages *Mstn* expression relates to total expression in the developing embryo/larvae. At 21 days after hatching fish were graded into four size-classes based on their breadth using a slatted grill with known gap size: small (<1.5 mm), medium (1.5 mm<1.8 mm), large (1.8 mm<2 mm) and extra-large (>2 mm). Cross-section of muscle samples from graded fish were obtained from the caudal region of fish, preserved in 10% formalin solution and later processed for muscle fiber histological analyses. RNA was extracted from the remaining part of the fish muscle.

Protocols for total RNA extraction, DNAse treatment, cDNA synthesis and quantification were as those described in De Santis et al. (2011). Briefly, RNA was extracted by homogenizing samples in Ultraspec RNA (Biotecx). Quality of RNA was verified on agarose gels by visual inspection of 18S and 28S ribosomal RNA bands and lack of visible genomic DNA contamination, as well as by $OD_{260/280}$ (range: 2.00–2.11; average 2.06) and $OD_{260/230}$ absorbance ratios (range: 1.65–2.14; average: 1.89) measured on a NanoDrop spectrophotometer (NanoDrop Technologies). A Turbo DNA-free kit (Ambion) was used for DNA removal. For verification of complete DNA removal, an aliquot of each sample's DNAse treated RNA was diluted to the same concentration as that used in the cDNA syntheses, this was later PCR amplified using *L. calcarifer Mstn* gene specific primers as a no-amplification control (NAC) ($C_{q\ (NAC\ control)} - C_{q\ (cDNA\ synthesis)} > 10$). First strand complementary DNA (cDNA) was synthesized from 3 μg of DNAse treated RNA using Superscript III first-strand synthesis supermix (Invitrogen) and purified using Nucway spin columns (Ambion). The RNA strand was digested using RNAse cocktail (Ambion) and cDNA then quantified in triplicate using a Quant-it Oligreen ssDNA kit (Invitrogen).

2.2. Real-time PCR quality control and data analysis

Intron-spanning primer pairs for real-time PCR amplification of *LcMstn-1* (accession number: EF672685) (LcMstn_F: ATGTAGTTATGGAGGAGGATG and LcMstn_R: CTTGGACGATGGACTCAG) and *LcMstn-2* (accession number: GU590863) (LcMstn2_qPCR_F: ACGACAGAGACCATCATCAC and LcMstn2_qPCR_R: TGAACAGACAACACAAGGAC) were previously designed and validated in De Santis et al. (2011) and De Santis and Jerry (2011), where reaction conditions are also reported. Primer specificity and cross-hybridization results were also reported in De Santis and Jerry (2011), using the approach suggested by Helterline et al. (2007). The efficiency (E) was calculated