DISCOVERY AND CHARACTERIZATION OF A NOVEL MYOSTATIN IN ZEBRAFISH

By

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To the Faculty of Washington State University:

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Abstract

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The myostatin-null phenotype in mammals is characterized by extreme gains in skeletal muscle mass or "double muscling" as the cytokine negatively regulates skeletal muscle growth. Recent attempts, however, to reproduce a comparable phenotype in zebrafish have failed. Several aspects of myostatin biology in the fishes differ significantly from those in mammals and at least two distinct paralogues have been identified in some species, which possibly suggests functional divergence between the different vertebrate classes or between fish paralogues. Therefore, phylogenetic analysis was conducted on the entire myostatin gene sub-family. Maximum likelihood, Bayesian inference and bootstrap analyses indicated a monophyletic distribution of all myostatin genes with two distinct fish clades: myostatin-1 and myostatin-2. These analyses further indicated that all Salmonid genes described are actually myostatin-1 orthologues and that additional myostatin-2 paralogues may be present in most, if not all, teleosts. An additional zebrafish homologue was identified by BLAST searches of the zebrafish HTGS database and was subsequently cloned. Comparative sequence analysis of both genes (zfMSTN-1 & -2) revealed many differences, primarily within the latency

associated peptide regions, but also within the bioactive domains. The 2 kb promoter region of zfMSTN-2 contained many putative *cis* regulatory elements that are active during myogenesis, but are lacking in the zfMSTN-1 promoter. In fact, zfMSTN-2 expression was limited to the early stages of somitogenesis, while zfMSTN-1 was expressed throughout embryogenesis. These data suggest that zfMSTN-2 may be more closely associated with skeletal muscle growth and development. They also resolve the previous ambiguity in classification of fish myostatin genes.

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CHAPTER ONE

INTRODUCTION TO THE MYOSTATIN FAMILY OF GENES

Identifying Myostatin and Myostatin's Mutant Phenotypes

In 1997, a discovery was made which described the first negative self-regulator for skeletal muscle. McPherron *et al*. discovered a novel TGF-β family member, the eighth member of the growth/differentiation factor sub-family (GDF-8) (1). Targeted ablation of this gene produced extreme gains in musculature and thus, it was appropriately named myostatin. The homozygous myostatin-null mice resulted in a 135% increase in body weight compared to wild-type, while the mass of the specific muscle fibers increased by 200-262% due to both hyperplastic and hypertrophic muscle growth (Figure 1). There was an 86% increase in fiber cell number with a 14-49% increase in fiber diameter. (1). The homozygous mutants had the greatest gain in mass, although heterozygous mice were affected to a lesser extent in females (1). Therefore, the growth inhibiting effects *in vivo* of myostatin appear to be dose dependent. Despite these extreme gains in skeletal muscle mass, fiber morphology was normal in all animals as were other tissues and organs. These results indicate that myostatin is a potent negative regulator of skeletal muscle growth and development.

The double muscled phenotype of the myostatin-null "mighty mouse" is not unique and has been generated by artificial selection in many domestic breeds of cattle including the Piedmontese, Belgian Blue, Marchigiana, Blonde Aquitaine, Limousine, Parthenaise, and Rubea Gillya (2-6), in domestic sheep (7, 8), and in the compact mouse (9-11). Each of these animals posses a mutation within the myostatin coding frame,

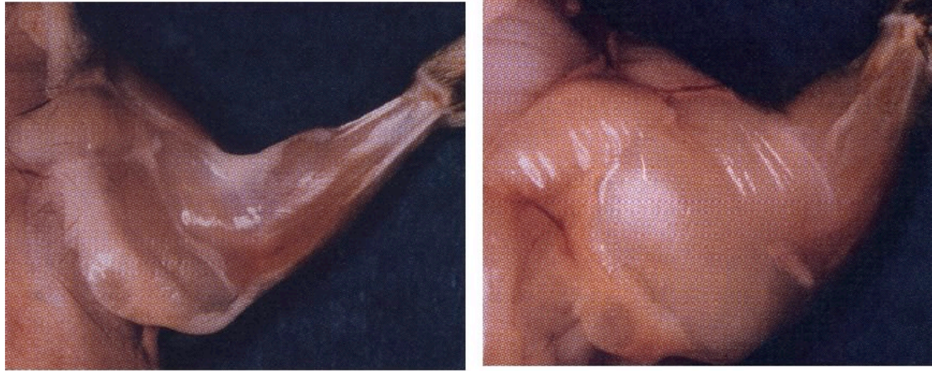


Figure 1. Double muscle phenotypes. Forearm musculature of wild-type (left) and myostatin -/- (right) mice. Similar phenotypes were reported for all four limbs, along with other muscle groups. Published by McPherron et al. Nature 387:83-90, 1997.

although different mutations have been identified (6, 12-15). Within the Belgian Blue, Blonde d'Aquitaine, Limousine and others, there is an 11 bp deletion that produces a frame-shift and introduces a premature stop codon. Therefore, the mature myostatin protein is never produced in these breeds. The myostatin gene of Piedmontese breeds have a critical amino acid substitution of an important and highly conserved cysteine residue that results from a G to A transition mutation, again within the conserved bioactive domain (5, 15). The hypermuscular phenotype of the Compact mouse results from a 12bp deletion within the propeptide domain, which segregated with the Compact trait leading to an over all increase in the musculature of the mouse (10, 11) The homozygous compact mice did not completely lose myostatin activity. The 12bp mutation in the latent associated protein region could possibly encode a misfolded protein or perhaps the mutation is within the region of the protein that associates with the bioactive domain leading to a decrease in binding.

These studies focused on defining a myostatin-null phenotype to prove function. To further test the hypothesis, myostatin was overexpressed in mice with the assumption that muscle atrophy would result. As predicted, skeletal muscle mass was greatly reduced in nude mice harboring CHO cell pseudo-tumors that expressed myostatin under an inducible promoter (16). There was a 33% loss in body weight along with a 25% decrease in fiber diameter. A similar study incorporating a muscle specific promoter (creatine kinase) to over express myostatin in transgenic mice reported a significant decrease in overall body weight and muscle mass in male, but not in female mice (17). Male mice had a significant decrease (20-22%) in fiber mass, along with a 10% decrease in total body mass compared to wild-type and an 18% decrease in fiber diameter. Muscle

mass in female mice was also reduced, although this difference was not significant (17). Both of these studies additionally support myostatin's role as a negative regulator of muscle growth.

McPherron *et al*. (18) additionally noticed a decrease in fat accumulation within myostatin null mice. Studies focusing on adipose tissue and myostatin all reported that upon knockout of the myostatin gene, mice had a decrease in adipogenesis and fat accumulation over time (18-21). McPherron *et al*. (18) reported that myostatin-null mice had a 70% decease in total body fat with age along with a decrease in adipose cell number and size. These results were further substantiated by Lin *et al*. (20) who reported a decrease in PPARγ and other fat markers in myostatin-null mice as well as in total fat mass. These studies together suggest that the loss of myostatin is associated with reduced adipogenesis and thus may play a role in regulation, however, the exact type and mechanism of regulation is still unknown (20).

Myostatin's actions may not be limited to muscle and fat as a few studies indicate that bone density may also be influenced by this cytokine (22). Hamrick *et al*. examined femoral bone mineral densities (BMD) in female myostatin null mice and reported a significant increase (23, 24). Similar results were obtained upon examining humerus BMD and with spinal disc thickness (22-25). These studies suggest that the double muscling phenotype associated with myostatin ablation is also associated with an increase in bone mass/density, which may be due to the increased physical demands of double muscling rather than to direct actions of the cytokine itself (22). Other bone studies that examined the genes expressed during bone fracture healing reported that myostatin was an up-regulated early response gene (26). Its role in regulating bone

density may or may not be a secondary effect, however, it is important to note that myostatin is involved in more than muscle growth and that any clinical or agricultural applications must consider its potential to affect other systems.

Myostatin Structure, Secretion and Receptor Signaling Regulation

As a member of the TGF-β family, myostatin shares the similar protein hallmarks as other members including TGF-β1 and growth differentiation factor 11 (GDF-11) (1, 27, 28). Shared similarities between all TGF-β family members include 9 common cysteine residues, a proteolytic processing site that separates the N-terminal latency associated protein (LAP) from the C-terminal bioactive domain (1, 29). These shared similarities are seen in all TGFβ members including those of different species as seen between myostatin-2 in zebrafish, TGF-β 5 in xenopus, bone morphogenic protein (BMP) in the florida lancelet, and GDF-11 in humans (29) (Figure 2). Conservation of the bioactive domain for any particular protein between different species suggests that function is equally conserved, although this has yet to be confirmed for myostatin in non-mammalian vertebrates.

Like other TGF-β family members, myostatin is secreted from the cell and influences muscle cells directly as an autocrine/paracrine factor. However, its presence in the circulation suggests that it may possess true endocrine actions as well (16). Regulation of myostatin secretion is not well understood but a few studies have reported

```
                          1                                                                        75
       TGFβ Xenopus   1 ---------------MEVLWMLLVLLVLHLSSLAMSLSTCKAVDMEEVRK----------------R--R-----
BMP Florida Lancelet   1 ------------------------------MIRAFESSLLNMFGLNERP--------------RP----RKNLVI
       GDF-11 Human   1 MVLAAPLLLGFLLLALELRPRGEAAEGPAAAAAAAAAAAAAGVGGERSSRPAPSVAPEPDGCPVCVWRQHSRELR
Myostatin-2 Zebrafish   1 ---------MFLLFYLSFWGVLGSQN-QNLSTTTTTTTQAFVTPGDDNG------Q-----CTTCQFRQQSKLLR
            Consensus   1           FLL  LEL  ML A     LSSIAAS SSA AV GED RK           C  C FRQRSKLLR

                          76                                                                      150
       TGFβ Xenopus  38 IEAIRGQILSKLKLDKTPDVDSEKMTVPSEAIFLYNSTLEVIREKATR----------EEEHVGHDQNIQDYYAK
BMP Florida Lancelet  28 PPYMLELYLSQTKDPENPSVNFNFAGKSTSTANTVRSFHHEEESEAG---------QPVWEGDDEIDRRLWFNTS
       GDF-11 Human  76 LESIKSQILSKLRLKEAPNISREVVKQLLPKAPPLQQILDLHDFQGDALQPEDFLEEDEYHATTETVISMAQETD
Myostatin-2 Zebrafish  55 LHSIKSQILSILRLEQAPNISRDTVKLLLPKAPPLQELLDQYDQNG-------GISEDEEQASSETIITMATEPQ
            Consensus  76 LESIKSQILSKLKLDEAPNISRE VKLLLPKAPPLQSILDLHD NA        I EDEEEASSETIISMAFET 

                          151                                                                     225
       TGFβ Xenopus 103 QVYRFESITELEDHEFKFKFNASHVRENVGMNSLLHHAELRMYKKQTDKNMDQRMELFWKYQENGTTHSRYLESK
BMP Florida Lancelet  94 AVPSVELIKAAELRLFREQIDVDHVQYGDSTDHHLYRVNVYEVMRPNSRTN-------------TDTITRLLDTK
       GDF-11 Human 151 PAVQTDGSPLCCHFHFSPKVMFTKVLKAQLWVYLRPVPRPATVYLQILRLKPLTGEGTAGGGGGGRRHIRIRSLK
Myostatin-2 Zebrafish 123 AITQLVGMPKCCMFALSPKILPDSILKALLWIYLRPAEEPTTVYIQISHLE---------SSSEGNNHSRIRAQK
            Consensus 151 AV QLEGIP CELF FSPKIL DHVLKALLWIYLRP  EP TVYLQISRL     E        G THSRIRESK

                          226                                                                     300
       TGFβ Xenopus 178 YITPVTDDEWMSFDVTKTVNEWLKRAEENEQFGLQPACKCPTPQAKDIDIEGFPALRGDLASLSSKENTKPYLMI
BMP Florida Lancelet 156 LVDVRNS-SWESFDVRSAVTKWKNSPERN--YGLEVEVVSPKRGALSNHHV-RLRRSTDMDDHSWQHRRPLLLTY
       GDF-11 Human 226 IELHSRSGHWQSIDFKQVLHSWFRQPQSN--WGIEINAFDPS--------------GTDLAVTSLGPGAEGLHPF
Myostatin-2 Zebrafish 189 IDVNARTNSWQHIDMKQLLKLWLKQPQSN--FGIEIKAFDAN--------------GNDLAVTSTESGEEGLQPF
            Consensus 226 IIL ARS SWQSIDVKQLL  WLKQPQSN  FGIEI AFDPS  A           GTDLAVTSS  G EGLLPF

                          301                                                                     375
       TGFβ Xenopus 253 TSMPAERIDTVTS-SRKKRGVGQEYCFGNNGPNCCVKPLYINFRKDLGWKWIHEPKGYEANYCLGNCPYIWS---
BMP Florida Lancelet 227 TDDGKGSSNSNRVASRQKRANGRKKQRRRLKANCRRHSLYVDFSDVGWNDWIVAPPGYQAYYCHGECPFPLADHL
       GDF-11 Human 285 MELRVLENTK-----RSRRNLGLDCDEHSSESRCCRYPLTVDFEAFGW-DWIIAPKRYKANYCSGQCEY------
Myostatin-2 Zebrafish 248 LEVKISDTGK-----RSRRDTGLDCDEHSTESRCCRYPLTVDFEDFGW-DWIIAPKRYKANYCSGEC--------
            Consensus 301 TELKI DS K    SRSKRALGLDCDEHSSESRCCRYPLYVDFEDFGW DWIIAPKRYKANYCSGECPY  A
                                                           *                            *   *

                          376                                                       436
       TGFβ Xenopus 324 MDTQYSKVLSLYNQNNPGASISPCCVPDVLEPLPIIYYVG-RTAKVEQLSNMVVRSCNCS-
BMP Florida Lancelet 302 NSTNHAIVQTLVNSVNPLAVPKACCVPTDLSPISMLYLNENDQVVLKNYQDMVVEGCGCR-
       GDF-11 Human 348 MFMQKYPHTHLVQQANPRGSAGPCCTPTKMSPINMLYFNDKQQIIYGKIPGMVVDRCGCS-
Myostatin-2 Zebrafish 309 --VQKYPHSHIVNKANPRGSAGPCCTPTKMSPINMLYFNDREQIIYGKIPSMVVDLCGCS-
            Consensus 376 M TQKYPVSHLVNQANPRASAGPCCVPTKLSPINMLYFNDKDQIIYGKIP MVVD CGCS
                                                 **                               * *
```

Figure 2. Amino acid sequence alignment of different TGFβ family members.

Each protein shares the conserved features of 7 cisteine residues highlighted in red with astrics beneath along with a “RXXR” proteolytic cleavage site boxed in red.

that certain proteins can act as inhibitors to secretion. Muscle cells express a protein called Titin-cap (T-cap), which normally associates with the large structural protein Titin and is thought to influence cytoskeleton organization (30). In multiple association studies, including yeast-two-hybrid (Y2H) screens, T-cap associated with myostatin in a way that prevented its secretion (31). It was also determined that the actual internal levels of myostatin were not affected and that the association was only between the full-length form of T-cap and the bioactive domain of myostatin. Nicholas *et al*. (31) found that when T-cap was overexpressed in C2C12 myoblasts, the cellular rate of proliferation increased suggesting that T-cap negatively controls myostatin secretion and thus myostatin serum levels. They also concluded that myostatin is found in the cytoplasm and that interaction may occur in either the golgi or cytoplasm (31). While this regulation does not affect the signaling pathway of myostatin, it does affect the amount of available extra-cellular myostatin, which in turn can regulate muscle cell growth.

Like all TGF-β family members, the primary translated product is heavily processed in the golgi before secretion (1, 32). The pre-propeptide is synthesized and then processed in two steps, with three forms similar to other family members (Figure 2). After cleavage of the signal peptide, the propeptide form is then proteolyzed at an internal proteolytic cleavage site that splits the propeptide into the latency associated protein (LAP) and the C-terminal bioactive domain (also referred to as myostatin/MSTN) (1). The exact processing enzymes have not been identified, however, the dibasic protealytic processing site is a known Furrin protease recognition sequence (32-35). The two separate LAP and MSTN homodimers stay associated and are believed to be

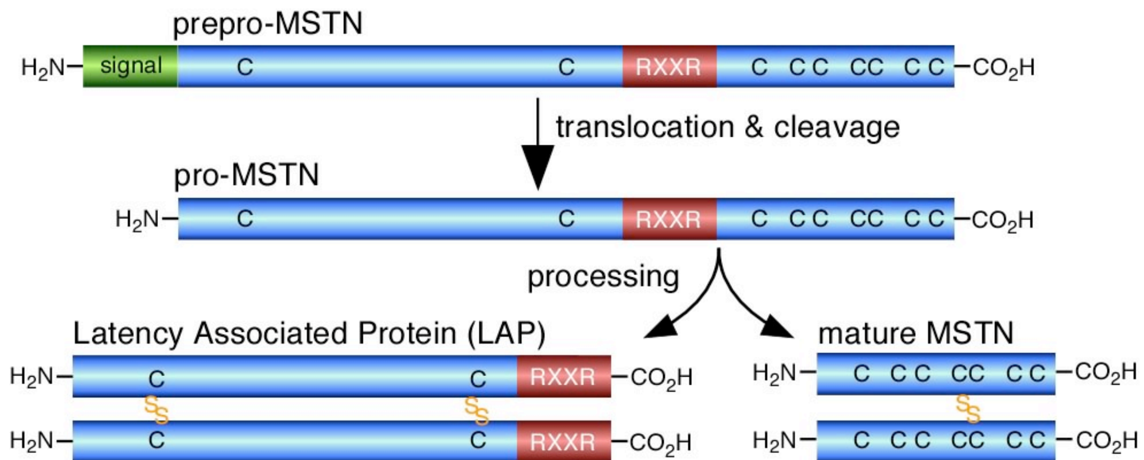


Figure 3. Myostatin (MSNT) Processing. Pro-MSTN is cleaved at a conserved RXXR epitope and the resulting peptides form disulfide –linked homodimers. Dominant-negative forms consist of LAP dimers alone, which sequester endogenously produced bioactive MSTN-dimers.

secreted together as a "small latent complex" through a non-covalent binding interaction. In turn, this complex is hypothesized to associate with a binding protein that attaches to the extra cellular matrix (ECM) in what is called the "large latent complex" (36-38). Thus, myostatin must first disassociate from LAP in order to have its known autocrine and paracrine functions (39). The association of myostatin to the cellular membrane in this function has not been proven, but is generally true for most TGFβ family members. (40-44). In this form, myostatin is not "free" to bind its receptor and is thus considered sequestered. In fact, the overexpression of LAP in transgenic mice produces similar gains in muscle mass as seen in myostatin-null animals (32).

Myostatin bioactivity is mediated, at least in part, by activation of the serine/threonine kinase receptors Activin Receptor II A/B (ActRIIA/B)(32). Competitive binding assays with radiolabeled myostatin and CHO cells overexpressing receptors for different TGF-β superfamily ligands determined that myostatin bound both ActRIIA and B receptors, although the binding affinity was significantly greater for ActRIIB (32). Binding was blocked by the addition of follistatin or by over expressing a dominant negative ActRIIB. When a TGFß family ligand binds a comparable RII receptor, a second RI receptor is recruited and signaling is initiated by the auto phosphorylation of the RII and RI receptors. In the case of myostatin, the exact RI receptor is not known, but it is thought to be either ALK4 (activin receptor-like kinase also known as ActRIB) or ALK5 (also known as TGFβRI) (45, 46) (Figure 3). Knowing that myostatin influences skeletal myogenesis and possibly adipogenesis, the type of ActRI receptor that is recruited may depend on the system/tissue. Although the precise tissue-specific receptor complements are currently unknown, a fundamental signaling cascade that predictably

involves the Smads has been defined (Figure. 3). Myostatin binding to ActRIIB ultimately stimulates the phosphorylation of the R-Smads (receptor activated smads) Smad2 and 3, which then form an active heterodimer (45, 46) that recruits the Co-Smad (co-activated smad) Smad4. This heterotrimer then translocates into the nucleus and activates gene transcription via direct interactions with *cis* regulatory elements (Figure 3e) (45, 46). Zhu *et al*. (45) determined that the I-Smad (inhibitory smad) Smad7, is upregulated as a result of this signaling, which in turn blocks the binding of Smad4 to Smad2/3 and prevents further translocation (45). Thus, the myostatin-regulated downstream activation of Smad7 gene expression acts as a feedback inhibitor of ActRIIB signaling (45) (See figure 3). Myostatin activation of Smad3 additionally results in the transcription factor's dimerization with MyoD, which prevents it from binding E proteins and presumably from stimulating gene transcription and myogenesis (47) (Figure 3g). Other proteins in addition to LAP are also capable of binding to myostatin and theoretically preventing ActRIIB activation. These include follistatin, follistatin-related gene (FLRG) and growth and differentiation factor-associated serum protein-1 (GASP-1) (32, 48-51). All of these proteins have follistatin like domains that are capable of binding the myostatin peptide and thus, preventing it from binding ActRIIB (Figure 3b) (32, 45, 51-53). Lee *et al*. (32) over expressed the follistatin protein in mice, which produced a double muscled phenotype that was more severe than the myostatin knockout. This was attributed to follistatin interference of other TGF-β family members in addition to

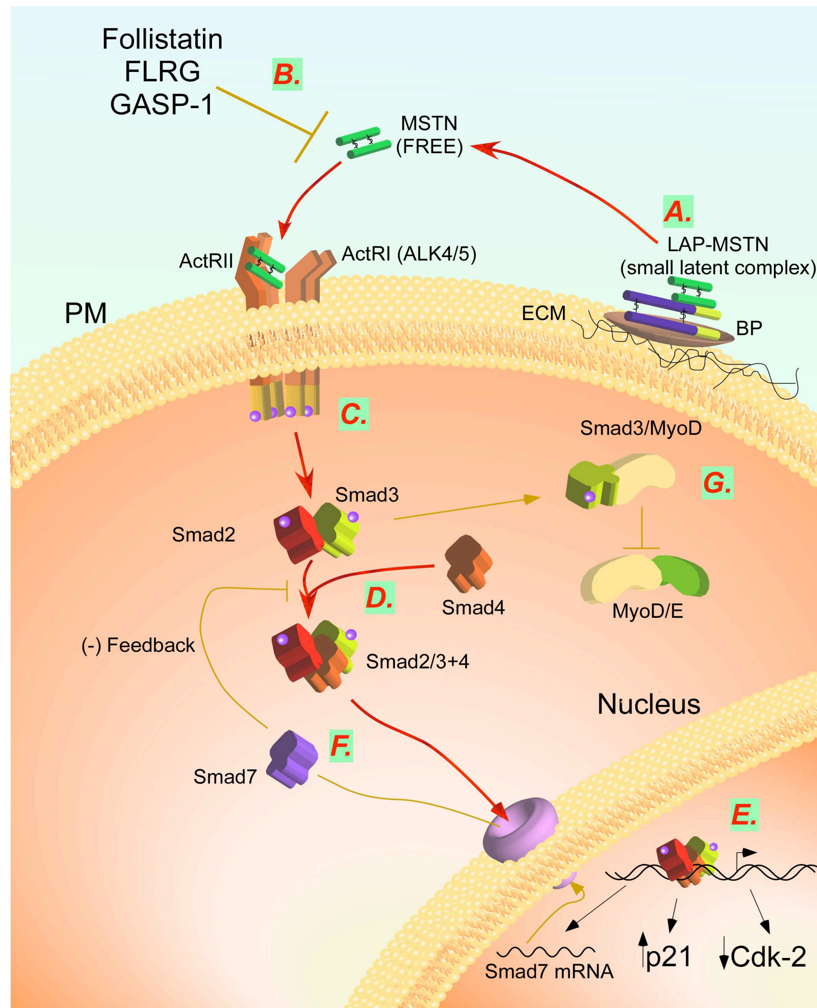


Figure 4: Myostatin Autocrine Signaling Cascade. A. LAP-MSTN (inactive) associates with the ECM. B. Inhibition by follistatin/follistatin domains. C. Binding to ActRII recruits ActRI and both autophosphorylate on serine/threonine residues. ActRII/I phosphorylates Smad2/3. D. Smad 4 binds with Smads 2/3 to form the active transcription factor recruiting others and turning on specific downstream targets. E. Downstream target genes (p21, Cdk-2, and smad7) are activated. F. Inhibitory Smad 7 binds Smads2/3 preventing binding of Smad4 thus shutting off the signal. G. Activated Smad 3 sequesters MyoD away from E proteins preventing transactivation of muscle regulatory genes.

myostatin. Activin is known to block skeletal muscle differentiation *in vivo*, thus the over expression of follistatin would block both activin and myostatin bioactivity (32).

Decreases in muscle mass and fiber diameter are the result of myostatin binding and causing a withdrawal from the cell cycle, through up-regulation of p21 and Cdk-2 (21, 32, 45, 47, 53-57). This causes a decrease in proliferation thus decreases the amount of myocytes that can fuse to create myotubes. This overall decrease leads to a lower muscle mass and fiber diameter in overexpressed systems. Myostatin prevents the proliferation of muscle cells by stimulating G_1 and G_2 cell cycle arrest (53, 57, 58), which is mediated by reduced levels and activity of Cdk-2 with a concomitant increase in its inhibitor, p21, and ultimately leads to the hypophosphorylation of retinoblastoma protein (Rb). This cell cycle withdrawal, however, is not a signal for apoptosis but rather for cellular quiescence; a state in which the cells are neither going through the cell cycle nor differentiating (55, 57, 59). This state is not uncommon to skeletal muscle and is rather well defined in myosatellite cells (60-62).

Myostatin Expression in Different Vertebrate Systems

Myostatin homologues have been characterized in many different vertebrate classes including different mammalian, avian, amphibian and boney fish species (28, 29, 63-75). In developing mouse embryos, myostatin expression first occurs in the myotome compartment of developing somites and continues to be expressed in the skeletal muscle of adults (1). This pattern is also seen in other mammals including pig, sheep, and cattle (76-80) and suggests that myostatin expression during development is similarly controlled in most mammals. In fact, the bovine, porcine, murine, and human myostatin

promoters all possess response elements for different muscle regulatory factors (MRF's) and other transcription factors involved in muscle development. Its expression has been found at low levels in other mammalian tissues, including cardiac muscle purkinje fibers and mammary glands (76, 80).

Although myostatin expression in mammals is limited primarily to skeletal muscle, its expression in fish is far less limited and occurs in a wide variety of tissues. Rodgers *et al*. (72) first described myostatin message in non-muscle tissues including the eye, gill, ovary, testi, gut, and brain, of the euryhaline teleost fish, the tilapia *Oreochromis mossambicus*. Since this initial discovery, others studies have similarly identified myostatin expression in these and other fish tissues from various species (29, 64, 65, 67-75, 81, 82). This diverse expression pattern is one of the major differences in myostatin biology between mammalian and fish systems and suggests that the biological actions of myostatin may not be restricted to skeletal muscle, but may additionally influence other fish tissues as well. An equally surprising difference between fish and mammalian systems is the presence of myostatin paralogues in some fish species that possess two distinct myostatin alleles, particularly the salmonids (69-72).

With the discovery of each unique salmonid myostatin, a name was assigned with no regard to a common nomenclature or to the true evolutionary relationships. Salmonids posses a tetraploid genome that arose from an early duplication event approximately 500,000,000 years ago (83) and may have four unique copies of myostatin. However, only two homologues have been discovered in each species to date, and the evolutionary relationships between each gene is still unknown as is the functional significance of having multiple unique copies of myostatin. It is possible that the various myostatins

specifically regulate different tissues with only one copy being the primary regulator of myogenesis. Indeed, two recent studies have attempted unsuccessfully to reproduce a double muscle phenotype in zebrafish by overexpressing a dominant-negative (75) or by disrupting myostatin production with anti-sense morpholinos (81). These results suggest that either the biological actions of myostatin are not necessarily well conserved in all vertebrates or that an additional and undescribed zebrafish paralogue is at least partially responsible for mediating the cytokine’s actions in muscle.

CHAPTER TWO

PHYLOGENETIC ANALYSIS OF THE MYOSTATIN GENE SUB-FAMILY AND THE DIFFERENTIAL EXPRESSION OF A NOVEL MEMBER IN ZEBRAFISH

Introduction

Several aspects of myostatin biology in the fishes are quite different from that in mammals. In order to better understand the evolutionary relationship between the different myostatin homologues, we conducted a phylogenetic analysis of all known vertebrate sequences. These studies ultimately helped to identify a novel zebrafish orthologue (zfMSTN-2) that is differentially expressed throughout embryogenesis when compared to the previously characterized myostatin (zfMSTN-1), and in a manner consistent with a functional role during myogenesis. These analyses additionally suggest that the vast majority of currently described fish myostatin genes are actually myostatin-1 orthologues, regardless of the current nomenclature. We therefore propose a standardized nomenclature for fish myostatin homologues that is based solely on the true phylogenetic relationship of each sub-family member.

Materials and Methods

Phylogenetic analyses

A single database consisting of almost all of the previously characterized cDNA sequences for myostatin was constructed using Vector NTI 9.0 for the Macintosh (Invitrogen, www.invitrogen.com). Growth/differentiating factor (GDF)-11 is structurally very similar to myostatin and thus, the known GDF-11 homologues (mouse exon1 accession # AF028335, mouse exon 2 AF028836, mouse exon3 AF 028337;

human, NM_005811; zebrafish, AF411599) were also included as were 2 TGFβ1 (zebrafish, NM_182873; rat, NM_021578) sequences, which served as an outgroup. Thirty seven myostatin sequences from 8 mammalian (baboon, AF019619; macaque, AY055750; human, AF019627; rat, NM_019151; mouse, U84005; dog, AY367768; bovine, AF019620; horse, AB033541), 6 avian (chicken, AY448007; turkey, AF019625; quail, AF407340; pigeon, AF440863; goose, AY448009; mallard duck, AF151692) and 16 fish (seabream, (a) AF258448 & (b) AF046314; shidrum, (a) AF316881& (b) AY059386; fugu, (1) AY445321 & (2) AY445322; zebrafish, AY258034; striped bass, AF290910; white perch, AF290911; white bass, AF197194; tilapia, AF197193; king mackerel, AF317667; little tunny, AF344158; Atlantic salmon, (1) AJ297267& (2) AJ344158; brook trout, (ov) AF313912 & (b/m) AF247650; rainbow trout, (1) AF273035 & (2) AF273036; coho salmon, (1) AY434465 & (2) AF394687; blue catfish, AY540992; channel catfish, AF396747) species were included in the original analysis that also included the TGFβ1 and GDF-11 sequences.

Blast Analysis

A low stringency nucleotide-nucleotide BLAST analysis of the human, mouse, rat and *Takifugu* assembled genome databases and of the zebrafish Hierarchical Tets Generation System (HTGS) database (all GenBank) was performed using the last 150 bp of the 3' end of the white bass myostatin open reading frame. Additional searches were performed using a comparable region of fugu myostatin-1 (formerly fugu 2). Several known myostatin and GDF-11 homologues were identified as well as previously uncharacterized myostatin and GDF-11 genes from zebrafish and fugu, respectively. The

second zebrafish myostatin (zfMSTN-2) was subsequently cloned and sequenced for verification (see below). These additional sequences were then included in a second phylogenetic analysis.

Amino acid and cDNA sequence alignments were performed using a two-step process. First, amino acid sequences were compiled and aligned using Clustal X 1.83 (84) with the Gonnet 250 cost matrix applied to pairwise alignments and the Gonnet series applied to the multiple alignments. The insertion and deletion (indel) events inferred from this alignment were then transferred to the appropriate locations in a matrix of the cDNA sequences using the program Se-Al 2.0a11 (85). This two-step process allows for the analysis of cDNA sequences that would be difficult to align using current software options by placing all inferred indels in frame within each corresponding cDNA sequence. Alignment results suggested that the 5' region of the gene was much less conserved than the 3' end among amino acid residues, with a significantly greater amount of inferred indel events in this region of the gene. In order to test the potential effect of this variable region on model choice and inferred tree topologies, two cDNA matrices were analyzed. The alignment of the entire coding region of study (hereafter referred to as the "complete" data matrix) was analyzed with 443 amino acid residues and 1332 aligned cDNA base pairs. The second matrix (hereafter referred to as the "3'" data matrix) excluded the base pairs associated with the first 84 amino acid residues of the complete matrix resulting in an alignment of 359 amino acids and 1080 base pairs.

Maximum likelihood (ML) analyses of the complete and 3' matrices were performed using PAUP* 4.0b10 (86). Heuristic searches were employed with the starting tree obtained via neighbor-joining (NJ) and using the tree-bisection-reconnection (TBR)

branch swapping algorithm. Clade support was estimated using 100 heuristic bootstrap replicates (10 random addition cycles and 100 total rearrangements per replicate, TBR branch swapping) (87). ML analyses of both the complete and 3' matrices employed the general time reversible (GTR) model with proportion of invariant sites (I) and gamma shape (G) parameters and empirical base frequencies (six substitution types: complete = A/C- 1.6975, A/G- 3.9020, A/T- 1.3550, C/G- 1.1273, C/T- 5.4876, G/T- 1.0000; I = 0.0980; G = 0.7399; A- 0.2353, C- 0.2991, G- 0.2836, T- 0.1820; 3' = A/C- 2.0452, A/G- 4.8605, A/T- 1.6102, C/G- 1.3310, C/T- 7.3185, G/T- 1.0000; I = 0.1152; G = 0.6469; A- 0.2476, C- 0.2993, G- 0.2727, T- 0.1804). These models and parameters were chosen based on the results of analyses using DT_ModSel (88). The DT_ModSel analysis uses a Bayesian information criterion to select a model using branch-length error as a performance measure in a decision theory framework that also includes a penalty for model overfitting.

Bayesian inference analysis was performed on the complete matrix using MrBayes v.3.0 (89), but not on the 3' matrix (see below). Ten million generations were run with four chains (Markov Chain Monte Carlo) and a tree was saved every 100 generations. Priors included a model with six substitution types allowed and rates following a gamma distribution and invariant sites. This model was chosen based on the results of analysis using DT_ModSel (see ML methods above; (88). In order to test for the occurrence of stationarity, convergence and mixing within ten million generations, multiple analyses were started from different random locations in tree space. The posterior probability distributions from these separate replicates were compared for convergence to the same posterior probabilities across branches. Majority rule consensus

trees of those sampled in Bayesian inference analyses yielded probabilities that the clades are monophyletic (90). The trees from the MrBayes analysis were loaded into PAUP*, discarding the trees generated within the first 2,000,000 generations or those sampled during the "burn in" of the chain (91) to only include trees after stationarity was established. Posterior probability values (pp) are presented on the single ML topology.

Cloning zfMSTN-2 cDNA and the in silico analysis of the zfMSTN-1 & -2 promoters

The putative zfMSTN-2 cDNA was constructed *in silico* from 3 predicted ORFs within the zebrafish HTGS scaffold BX548072.14 and gene-specific primers were then used to clone the zfMSTN-2 cDNA. Total RNA was extracted from adult zebrafish skeletal muscle using Trizol Reagent (Invitrogen, www.invitrogen.com) and cDNA was generated from 1 mg RNA with oligo-d(T) primers and Superscript III (Invitrogen) reverse transcriptase. Two separate polymerase chain reactions (PCR) were used to amplify zfMSTN-2 cDNA with primers that flanked the putative coding sequence (forward, 5'-ATG-TTT-CTC-CTT-TTT-TAT-CTG-AGC-3'; reverse, 5'-AGA-GCA-ACC-GCA-AAG-GTC-3') and with a high-fidelity polymerase, Platinum Taq-HIFI (Invitrogen). After an initial 4 min denaturation at 94°C, cDNA was amplified for 30 cycles with the following protocol: 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 60°C and a final extension cycle for 5 minutes at 72°C. Nothing was amplified after the first PCR so a second 30-cycle reaction was performed using 5 ml of the initial reaction product. The resulting 1133 bp amplicon was subsequently cloned into the Blunt-TOPO vector (Invitrogen) using the manufacturer's protocol, producing pCR4-zfMSTN2. zfMSTN-1 was similarly cloned and both myostatin cDNA were sequenced at the

university's Genomics Core facility. Several discrepancies in the HTGS database sequence were corrected and the complete cDNA sequence for zfMSTN-2 was deposited into GenBank (accession # AY687474). The 2 kb regions upstream of the first ORFs for both genes (zfMSTN-1, AY323521) were subjected to *in silico* subsequence promoter analysis using MatInspector (Genomatix, www.genomatix.de). Additional analyses were also performed with specific consensus searches using Vector NTI.

Analysis of zfMSTN-2 developmental expression

Total RNA was extracted from embryos/larvae pooled at different times or developmental stages (2.5 hpf, 5.5 hpf, 13 hpf, 19 hpf, hatch, YSA, juveniles and RT-PCR was then performed using PCR primers specific for zfMSTN-1 (forward, 5'-TCG-AAG-AGG-ACG-ATG-AAC-ATG-CC-3'; reverse, 5'-CCG-GTT-GTT-TTA-ACC-ACA-CC-3') or zfMSTN-2 (forward, 5'-ATG-TTT-CTC-CTT-TTT-TAT-CTG-AGC 3', reverse, 5'-CCG-TCT-GGA-TCG-CTT-CCC-TGT-G-3'). Primer specificity was previously verified by amplifying pBSSK-zfMSTN1 and pCR4-zfMSTN2 with both primer sets (data not shown). An equal amount of total RNA (1 mg) from each sample pool was reverse transcribed from a RT master mix and the cDNA was similarly amplified by PCR with gene-specific primers. A negative "RT-" control was also included by pooling equal amounts of RNA from each sample. The 30-cycle PCR protocol was identical to that above, but with a 60°C annealing temperature. The resulting amplicons were then separated on a 1% agarose gel and stained with GelStar (Cambrex, www.cambrex.com). The expression of zfMSTN-2 was also analyzed by *in situ* hybridization in 10, 13 and 24 hpf embryos and was compared to that of MyoD.

Dechorionated embryos were fixed in 4% paraformaldehyde, rehydrated by sequential washes with methanol/PBS+0.1% Tween-20 (PBST), digested with 10 mg/ml proteinase K and once again fixed in 4% paraformaldehyde. Embryos were then incubated in prehybridization buffer (50% formamide, 5X SSC, 0.1% Tween20, 50 mg/ml heparin, 500 mg/ml tRNA) for 2 h at 70°C and were then hybridized overnight in fresh buffer containing 200 ng of digoxigenin-labeled sense or antisense probes (~1 kb) generated from linearized pCR4-zfMSTN2 with T3 or T7, respectively. Embryos were blocked in 10% goat serum and incubated overnight at 4°C with an alkaline phosphatase-conjugated anti-digoxigenin monoclonal antibody (Boehringer-Mannheim, www.roche-applied-science.com) that was pre-adsorbed with a homogenate of previously fixed embryos. Positive hybridizations were then visualized by staining with NBT/BCIP.

Results

Maximum likelihood and Bayesian inference analyses of phylogenetic relationships

Two matrices (complete and 3') were analyzed with ML and each produced single trees (complete, -lnL = 18277.57143; 3', -lnL = 14473.69618) with identical topologies (Fig. 4, complete matrix tree shown). Further analyses were therefore performed on the complete matrix only. The ML bootstrap (MLB) and Bayesian inference posterior probability (PP) values were calculated as measures of branch support and are presented above or below each branch. Three independent Bayesian inference analyses resulted in nearly identical posterior probability values for all branches, with no posterior probability value deviating by more than one percent for any branch among analyses. This suggests that the individual analyses are sampling from the same posterior probability distribution

of trees and convergence and mixing are occurring. The values from a representative analysis are shown.

Overall, clade support for branches in the myostatin topology is good, as measured by both MLB and PP. Of the 40 resolved internal branches from the ML analysis, 23 have a PP ≥ 95% (57.5%), 10 have a MLB ≥ 95% (25%), and 22 have a MLB ≥ 70% (55%; Fig. 4). Within the myostatin gene sub-family, four major clades are found, representing mammalian myostatins, avian myostatins, and two copies of the myostatin gene in fish (myostatin-1 and myostatin-2; Figure. 4). These results suggest that there was an early duplication of the myostatin gene in the fish lineage, although copies of the myostatin-2 gene have yet to be found in a number of fish lineages. The myostatin-1 gene was additionally duplicated specifically within the salmonids producing myostatin-1a and myostatin-1b paralogues, which appears to have occurred more recently than the myostatin-1/2 duplication.

The single ML tree found in all analyses has several interesting topological characteristics. First, the root for the GDF-11 + myostatin gene sub-families is placed to create a paraphyletic GDF-11 gene family. This branching arrangement, however, is not well-supported statistically. An alternative branch arrangement was suggested by the MLB, which created a monophyletic fish GDF-11 clade and also placed the fish and mammal GDF-11 copies as monophyletic sister clades (Fig. 4A). Tests of alternate topologies did not find these branching arrangements to be significantly different statistically (data not shown). Given the level of sequence and amino acid divergence among the TGFβ, GDF-11 and myostatin gene sub-families, the inclusion of only two TGFβ gene copies may have influenced attachment of the outgroup branch. The

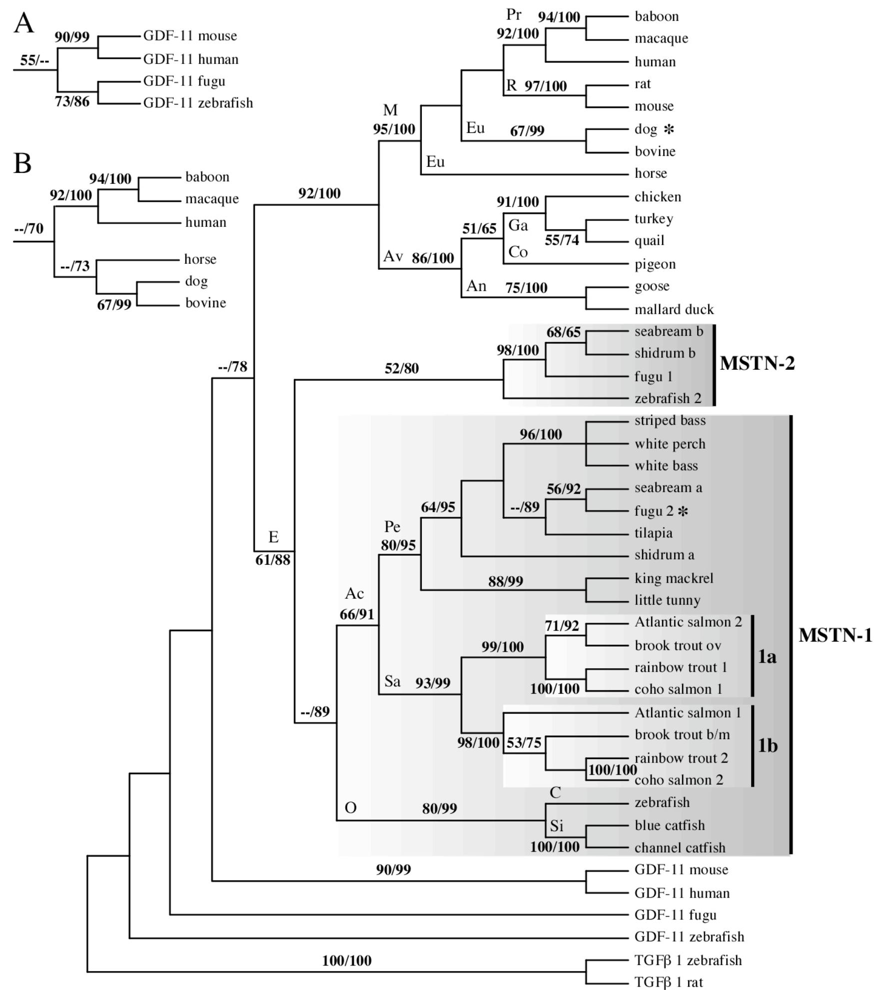


Figure 5. Phylogenetic Tree. Myostatin (MSTN)/GDF-11 gene sub-family maximum likelihood tree (-ln L = 18277.57143). Dark grey shaded clades refer to the two MSTN paralogs, 1 and 2. Light grey shaded clades refer to Salmonid MSTN-1 paralogs, a and b. Numbers above or below branches designate the maximum likelihood bootstrap and Bayesian posterior probability values, respectively, when greater than 50%. Major classification units are mapped onto branches using the following abbreviations: Ac, Acanthopterygii; An, Anseriformes; Av, Aves; C, Cyprinoformes; Co, Columbiformes; E, Euteleostei; Eu, Eutherians; Ga, Galliformes; M, Mammalia; O, Ostariophysi; Pe, Perciformes; Pr, Primates; R, Rodentia; Sa, Salmoniformes; Si, Siluriformes. Species not within the indicated group are denoted by an asterisk.

likelihood of this occurring is debatable, nevertheless, the exact relationships among the major sub-families of the TGFβ super-family need to be further explored. A second alternative topological arrangement was found in the Bayesian analysis and is associated with relationships among the mammalian gene copies (Fig. 4B). Specifically, the horse, dog, and bovine sequences form a separate sister clade (PP = 73%) to the primate clade (PP = 70%). As all of these branches have low support as measured by both MLB ($<$ 50%) and PP, the inferred relationships among these gene copies should be considered tenuous at best.

Branch arrangement is also well supported by the known phylogenies of the represented species (Fig. 4). This further suggests that the described phylogenetic distribution of each myostatin is accurate and that it does not reflect a biased arrangement due to convergence or divergence within the myostatin/GDF-11 sub-family *per se*. The only exceptions are the placement of dog myostatin within the Eutherians and fugu myostatin-1 (formerly "fugu 2") within the Perciformes. These anomalies are likely due to the small number of canine and Tetraodontoforme species included in our analyses (1 each) as their indicated placements are in fact, not statistically supported.

Comparative sequence analysis of zfMSTN-1 & -2 and genomic structure/organization

The phylogenetic analysis identified two distinct duplication events within the fishes. Therefore, low stringency BLAST searches of the human, mouse, rat and Takifugu genomes, using a highly conserved domain of the white bass myostatin-1 cDNA (see Methodology), were performed to identify potentially undescribed members of the Myostatin/GDF-11 subfamily. Several known homologues were identified (Table

2) including those for both genes as well as a previously undescribed fugu GDF-11 gene (CAAB01000063.1, the annotated ORFs are mislabeled within this scaffold). The identification of human, mouse and rat GDF-11 suggests that genomes for these species contain only a single myostatin gene as myostatin homologues are more similar to themselves than they are to those of GDF-11. Similar searches of the HTGS database, using the same parameters and sequences, identified a novel myostatin orthologue within scaffold BX548072.14 that was subsequently cloned and sequenced.

The cloned 1101 bp zfMSTN-2 cDNA sequence contained several discrepancies from the HTGS scaffold. These errors were corrected and the annotated cDNA sequence was submitted to GenBank (accession # AY687474). The predicted amino acid sequence of the novel homologue, zfMSTN-2, contains the structural motifs common to all myostatin proteins (Figure 5A) including the 9 invariant cysteine residues found in all TGFb superfamily members, the additional 2 cysteines found specifically in myostatin proteins and the RXXR proteolytic processing site that separates the LAP from the C-terminal bioactive domain (1, 29, 92). An amino acid alignment of the two proteins indicates that they are 61% identical and 71% similar overall. However, the degree of conservation differs throughout the proteins as the identity and similarity of the LAP and bioactive domains are 59/71% and 88/92%, respectively (Fig. 5B). Notable differences include a 6-residue motif (100GDDSKD105 in zfMSTN-1) not found within the LAP domain of zfMSTN-2 as well as a 4-residue motif (315DYMY318) lacking from its bioactive domain. The proteins also differ biochemically as zfMSTN-2 has 366 amino acids, a predicted mass of 41.3 kDa and a pI of 5.87 whereas zfMSTN-1 is larger with 8 additional amino acids, a predicted mass of 42.8 kDa and a pI of 6.55.

Table 1. BLAST Results* from Searching Different Vertebrate Genomes

database	accession #	score/E value	annotation
human genome	NT_029419.10	74/2e^{-11}	GDF-11
	NT_005403.14	68/2E^{-9}	GDF-8
mouse genome	NT_081856.1	70/4E^{-10}	GDF-11
	NT039170.2	60/3E^{-5}	GDF-8
rat genome	NW_0477773.1	66/6E^{-9}	GDF-11
	NW_047815.1	44/0.021	GDF-8
Takifugu genome	CAAB01000263.1	200/2E^{-50}	GDF-8 (MSTN-2)
	CAAB01000138.1	119/5E^{-26}	GDF-8 (MSTN-1)
	CAAB01000063.1	62/1E^{-8}	GDF-11

*Using 150 bp from the 3' end of the white bass MSTN-1 open reading frame. Annotations in parentheses were added and are not included in database.

A. zebrafish MSTN-2

```
      M  F  L  L  F  Y  L  S  F  W  G  V  L  G  S  Q  N  Q  N  L
   1 ATGTTTCTCCTTTTTTATCTGAGCTTTTGGGGTGTGTTGGGGTCACAAAATCAAAACCTG
      S  T  T  T  T  T  T  T  Q  A  F  V  T  P  G  D  D  N  G  Q
  61 AGCACAACAACGACAACAACGACGCAAGCATTTGTGACACCTGGAGACGACAACGGCCAG
      C  T  T  C  Q  F  R  Q  Q  S  K  L  L  R  L  H  S  I  K  S
 121 TGCACGACCTGCCAATTTAGACAGCAGAGTAAACTCTTGCGCCTGCATTCTATTAAGTCT
      Q  I  L  S  I  L  R  L  E  Q  A  P  N  I  S  R  D  T  V  K
 181 CAGATTTTGAGCATCCTGCGCCTAGAACAGGCTCCGAACATCAGCAGAGATACGGTCAAG
      L  L  L  P  K  A  P  P  L  Q  E  L  L  D  Q  Y  D  Q  N  G
 241 CTACTCTTACCCAAAGCACCTCCGCTGCAGGAGCTCCTGGATCAGTATGACCAAAACGGA
      G  I  S  E  D  E  E  Q  A  S  S  E  T  I  I  T  M  A  T  E
 301 GGCATTAGTGAGGATGAGGAACAAGCAAGCAGCGAGACCATCATTACTATGGCCACTGAA
      P  Q  A  I  T  Q  L  V  G  M  P  K  C  C  M  F  A  L  S  P
 361 CCTCAAGCCATCACCCAGCTTGTGGGAATGCCGAAGTGTTGCATGTTCGCACTGAGCCCA
      K  I  L  P  D  S  I  L  K  A  L  L  W  I  Y  L  R  P  A  E
 421 AAGATTCTGCCCGACAGCATCCTGAAGGCCCTGCTCTGGATCTACCTCCGACCAGCTGAG
      E  P  T  T  V  Y  I  Q  I  S  H  L  E  S  S  S  E  G  N  N
 481 GAGCCAACCACAGTCTACATCCAGATATCTCACCTGGAGTCTTCTTCTGAAGGGAACAAT
      H  S  R  I  R  A  Q  K  I  D  V  N  A  R  T  N  S  W  Q  H
 541 CACTCGAGAATACGTGCGCAAAAAATTGACGTGAATGCCCGGACAAATTCCTGGCAGCAC
      I  D  M  K  Q  L  L  K  L  W  L  K  Q  P  Q  S  N  F  G  I
 601 ATCGACATGAAGCAGCTGTTGAAACTCTGGCTCAAACAGCCCCAGAGTAACTTCGGGATA
      E  I  K  A  F  D  A  N  G  N  D  L  A  V  T  S  T  E  S  G
 661 GAAATCAAGGCCTTTGATGCAAATGGGAATGACCTGGCTGTGACCTCCACAGAATCTGGA
      E  E  G  L  Q  P  F  L  E  V  K  I  S  D  T  G  K  R  S  R
 721 GAAGAAGGACTGCAACCCTTCCTGGAGGTGAAAATATCAGACACAGGGAAGCGATCCAGA
      R  D  T  G  L  D  C  D  E  H  S  T  E  S  R  C  C  R  Y  P
 781 CGGGACACTGGCCTTGATTGTGATGAGCATTCCACCGAATCTCGCTGCTGCAGGTATCCA
      L  T  V  D  F  E  D  F  G  W  D  W  I  I  A  P  K  R  Y  K
 841 CTCACGGTTGACTTTGAGGACTTCGGGTGGGACTGGATAATTGCCCCCAAGCGCTACAAG
      A  N  Y  C  S  G  E  C  V  Q  K  Y  P  H  S  H  I  V  N  K
 901 GCCAATTACTGCTCTGGTGAATGTGTGCAGAAGTACCCCCACAGTCACATCGTCAACAAG
      A  N  P  R  G  S  A  G  P  C  C  T  P  T  K  M  S  P  I  N
 961 GCCAACCCAAGGGGCAGTGCAGGTCCCTGCTGCACTCCAACCAAGATGTCACCCATTAAC
      M  L  Y  F  N  D  R  E  Q  I  I  Y  G  K  I  P  S  M  V  V
1021 ATGCTGTATTTCAATGATCGTGAGCAGATAATCTACGGAAAAATCCCTTCGATGGTGGTA
      D  L  C  G  C  S  *
1081 GACCTTTGCGGTTGCTCTTGA
```

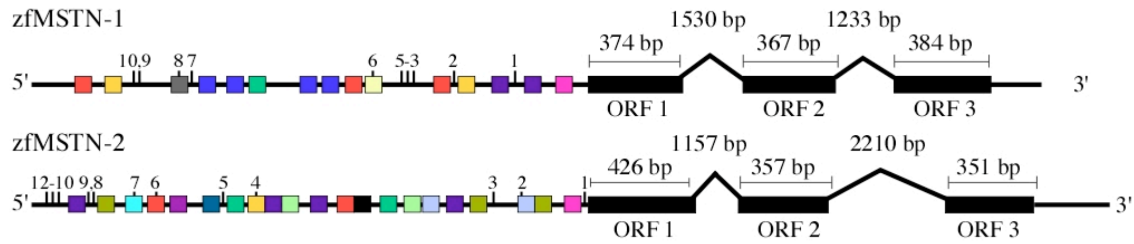
B. Amino acid alignment of zebrafish MSTN-1 & -2

```
MSTN-2   1 MFLLFYLSFW GVLGSQNQNL STTTTTTTQA FVTPGDDNGQ CTTCQFRQQS
MSTN-1   1 MHFTQVLISL SVLIACG.PV GYGDITAHQQ PSTATEESEL CSTCEFRQHS
           M     L     VL *    *       T Q   T  **    C*TC FRQ S

MSTN-2  51 KLLRLHSIKS QILSILRLEQ APNISRDTVK LLLPKAPPLQ ELLDQYDQN.
MSTN-1  51 KLMRLHAIKS QILSKLRLKQ APNISRDVVK QLLPKAPPLQ QLLDQYDVLG
           KL*RLH*IKS QILS LRL Q APNISRD VK  LLPKAPPLQ  LLDQYD

MSTN-2 101 .....GGISE DEEQASSETI ITMATEPQAI TQLVGMPKCC MFALSPKILP
MSTN-1 101 DDSKDGAVEE DDEHATTETI MTMATEPDPI VQVDRKPKCC FFSFSPKIQA
                G** E D*E A**ETI *TMATEP  I  Q*   PKCC  F* SPKI

MSTN-2 151 DSILKALLWI YLRPAEEPTT VYIQISHLES SSEGNNHSRI RAQKIDVNAR
MSTN-1 151 NRIVRAQLWV HLRPAEEATT VFLQISRLMP VKDGG.RHRI RSLKIDVNAG
             I**A LW* *LRPAEE TT V**QIS L     *G    RI R* KIDVNA

MSTN-2 201 TNSWQHIDMK QLLKLWLKQP QSNFGIEIKA FDANGNDLAV TSTESGEEGL
MSTN-1 201 VTSWQSIDVK QVLTVWLKQP ETNRGIEINA YDAKGNDLAV TSTETGEDGL
             SWQ ID*K Q*L *WLKQP  *N GIEI A *DA DNDLAV TSTE*GE*GL

MSTN-2 251 QPFLEVKISD TGKRSRRDTG LDCDEHSTES RCCRYPLTVD FEDFGWDWII
MSTN-1 251 LPFMEVKISE GPKRIRRDSG LDCDENSSES RCCRYPLTVD FEDFGWDWII
            PF*EVKIS*   KR RRD*G LDCDE S*ES RCCRYPLTVD FEDFGWDWII

MSTN-2 301 APKRYKANYC SGEC....VQ KYPHSHIVNK ANPRGSAGPC CTPTKMSPIN
MSTN-1 301 APKRYKANYC SGECDYMYLQ KYPHTHLVNK ASPRGTAGPC CTPTKMSPIN
           APKRYKANYC SGEC    *Q KYPH*HLVNK A PRG*AGPC CTPTKMSPIN

MSTN-2 351 MLYFNDREQI IYGKIPSMVV DLCGCS.
MSTN-1 351 MLYFNGKEQI IYGKIPSMVV DRCGCS.
           MLYFN *EQI IYGKIPSMVV D CGCS
```

Figure 6. Zebrafish myostatin-2. (A) Annotated cDNA sequence with numbered nucleotide positions. Cysteine residues conserved in all known myostatin sequences are circled and the proteolytic processing site is boxed. (B) Amino acid alignment of zfMSTN-1 and –2 proteins using Vector NTI 7 AlignX and the blosum62mt2 algorithm. Identities and similarities (*) are shown in the consensus while gaps (.) are indicated in the individual sequences.

The open reading frames of both zebrafish genes are similarly organized into three differentially sized and spaced exons (Figure 6A) indicating that the two genes are non-allelic. This is supported by BLAST analysis, which identified the genes on unique scaffolds (zfMSTN-1, BX323586; -2, BX548072). Subsequence analysis of the 2 kb region upstream of both genes identified appropriately placed TATA boxes as well as many other putative *cis* regulatory elements that are either muscle-specific or are at least activated during myogenesis. However, the *mstn-2* promoter contained many more putative binding sites for the myogenic regulatory factors (MRFs), transcription factors necessary for initiating and maintaining myogenesis, than did the *mstn-1* promoter (Fig. 6B,C). Many of these elements were found only in the zfMSTN-2 promoter and are also present (some proven to be functionally active) in the human, mouse and bovine myostatin promoters (93-95). These include 3 androgen response elements (ARE), 3 MyoD binding sites, 2 myogenin (MyoGN) sites, a myogenic enhancer factor (MEF)-3 binding site, a glucocorticoid response element (GRE) and a muscle initiator (MusIn) site. Of the muscle-specific elements found in both promoters, more were present in the zfMSTN-2 promoter including binding sites for MEF-2 (4 vs. 3) and the transcription enhancer factor (TEF)-1 (2 vs. 1) as well as several putative E-boxes (CAN(T/A)TG, 12 vs. 8).

A. genomic organization of zebrafish MSTN-1 & -2



B. zebrafish MSTN-2 promoter

```
   1 ATGCGCAGCT TGACAGGTCT CGAATCATGC ACACCAGTGG CGACAGCGGC
  51 GAGGAAAAAA CTTCACCAAT TGGCGAAAGT GAAGAGTTAA AAAACCTTGA
 101 CGGAAACCAG GCCCAGATGG GCATGACCAT TTCTCCAATG GCCAAACGTC
 151 TTGTGCAGAG CTGCAGTCTA GGCGCCAGAG GCTGGAGAAC CCTGAATGTC
 201 AGCGAAGAAG ACTCATTTGT CCCTGGAGCA TCACAGGAAT CTCAACTAAA
 251 ACACTAAGAG AGGGTTGGAC ATAGTGTAGA TCCACCCTTT TTTAAAATCA
 301 GCCAATAGCA ATTTGATTTA TCACTCCTCT GCCAGTGAGT GGTTGAGCTA
 351 AAGCGCATCA AATGAAAAGC AAATGAGAAG CCTCTCGAAA GGGGCGGGGC
 401 ATGTCAGATC CTATAAAGCA GGAGTGTCCA AACTCTGTCC TGAAGGGCCG
 451 GTGTCCAGCA AGTTTAGTT  CCAACATTAA TTAAACACAC CTGAACCAGC
 501 TAATCAAGCT CTTACTAGGT ATACTAGAAA TGTCCAGGCA GATGTGTTGA
 551 AGCAAGTTGG AGCTAAACTC CTAAACTGGA CCCTTTGAGC ATTTGATTGG
 601 TTATGATATG ATGAGGAACT TTATATGAGG TTATGAGAAT AAAATCGTTG
 651 ATCTATTCAG AATCAAGCGG AAGTGACAAA CTACTAGCTT TACATGCTTA
 701 TAACAGTTTT TTATCTTCTA AACATGGATT TTGTCACTGT TTTGGAGCAC
 751 ACTAGCAAAT AGATATCATA ACTGATGCTA ACATCTAAGA AAGTTTATTT
 801 TAATTTCATG GGACCTTTAA GAAGAGCGGT CAGTTTACAT TCAAAATCTC
 851 AAATGTGGGA TGGCATTCCT AGGGCAAGGG TAGGTAACCT ATGGCTCGGG
 901 AGCCACATGT GGCTCTTTGA CTAAAAATAT GTGGCTCTCC AGCTGTCTCT
 951 ATTAAATAAA ATTTTAAGTT TACAAAAAAA TATCTGTGAC TAGAAAAGTT
1001 TCCTGTTGAA ATACAATTTA ATTCCATTTT AATGTGTTTT ACAGCAAAAT
1051 TAACTCCTGT TACAATAAAA GTGCTGCTGT GGATTAACTT AAATCATGAC
1101 ACCAATAAAG TGCAAGCAAC TTATTATTAT GGTCACCTTG TGCACGTAAA
1151 CTCAAACAAC ATTCATGAGT GGTGATGGCG AAAGAGAAAA ATTCTCTAAA
1201 CTGACCTTTC TTCATTTATG GACTTGCTCA ACATGTGATG CTTCATATTT
1251 GTTTATTTTT GAGTTGTGCA GAGACATAAA TAAAGAAAGG ATATCATATA
1301 CACCTCAATG GCTCTTTAAA AAAAATGTAT TTGCCAAAAA ATCAGGAATG
1351 GCTTATTTTT GCTGAACAAA GGTTCCCATC TCCTGTCCTA GGGAAATGAA
1401 AATAAAATTC CCACATGAAG TACTAAACTG CTCCAAGGGT TAACTGCATC
1451 ACTCTGAATA TCCTATGGGT ACCACCCCCT TAGAAACCCT CTATTCTCCA
1501 TATAGAAACA CTAACAAAAT CTTTCAGAAC AACAATCTAG CAACTAAACT
1551 ACCCATAAAC TAACATAATC ATGGTCGAAA TTGATGCAAA AACCTCACAG
1601 GAACCATCTT GCAAAGTATT AAAGTAAAAA ACAAATCAGG GTGGCTACAC
1651 ATACGAGTGC AACAGATTAC AACAGTCTCC ATATTGAAGT TCTTATGATA
1701 TGGTAGTGTA CTGCATTCAT AAATACAACA AATCAAACAA CCCCCCCGCC
1751 CCTCTCCTGA AAATCCTCAA ATCCAAATGT CCTTTTTAGA ATCTACCCAA
1801 AAAGATGAAA ATCTCTGCGC GGGTTCATCT GGACTTGTTC TTATCAACCC
1851 AACCAATCAT AAGATATCTA ACAGCAGGGA ATCTTAAATA TAAAACCGCC
1901 TGGGAGGCTG CACATCTGTC CAGTGCTGTT TGGAGAGAGT GCGTAAAGGT
1951 GCTTGTCTGT TCCCAAATGA GAGAACGCCT ATAAAGGCTC ATCAGAAGAC
```

C. zebrafish MSTN-1 & -2 promoter elements

Name	location for MSTN-2 (bp)
ARE-1	436-441,1384-1389,1778-1783
COMP1	589-609,1076-1096
GRE	700-714
HAND2/E12	----
MEF2	285-307,914-936, 1037-1059,1307-1329
MEF2/E2F	----
MEF3	827-839
MyoD	936-950,1228-1242
MyoD/E47orE12	535-549
MyoGN	1246-1274,1774-1802
MtBF	----
MusIn	1096-1114
SRF	899-917
TATA Box	1889-1893
TEF-1	862-874,1158-1170

Figure 7. Genomic structure and organization of zfMSTN genes. (A) Map of zfMSTN-1 and -2 gene and putative skeletal muscle *cis* regulatory elements within each gene's promoter region. (B) Sequence of the 5' promoter region of the zfMSTN-2 gene with color-coded regulatory elements. Boxed are consensus sequences (CAN(T/A)TG) for E-boxes. (C) Key to color-coded promoter elements in A and B.

Developmental expression of zfMSTN-1 & -2

The expression of both genes was initially and qualitatively assessed by RT-PCR with gene-specific primers. Expression of zfMSTN-2 was difficult to detect in adult skeletal muscle even by RT-PCR; amplicons were present after 70 cycles of amplification, but not 40 (data not shown). There was also no expression of zfMSTN-2 during gastrulation (5.5 hpf) and late embryogenesis (hatch) (Figure 7). However, zfMSTN-2 message was sufficiently present in 2.5 and 13 hpf (somitogenesis) embryos and minimally detected at yolk-sac absorption and in juvenile fry. This is in stark contrast to the developmental expression pattern of zfMSTN-1, which was detected throughout embryogenesis and after hatching. Embryos were also assessed by whole mount *in situ* hybridization and although MyoD expression was readily detected in developing somites after 10, 13 and 24 hpf (Figure 8), zfMSTN-2 mRNA levels were beyond the detection limits of this assay. This is similar to the expression limits of zfMSTN-1 as its mRNA could not be localized by *in situ* hybridization either (75). These data together suggest that zfMSTN-2 expression coincides specifically with somitogenesis, which is in contrast to the continuous expression pattern of zfMSTN-1. However, additional experiments are required to define the zfMSTN-2 tissue-specific expression pattern.

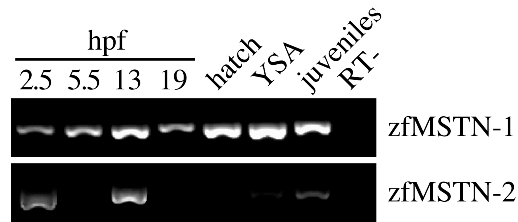


Figure 8. Differential expression of zfMSTN-1 & -2 throughout development. Levels of zfMSTN-1 and -2 mRNA were qualitatively assessed by RT-PCR using embryos 2.5, 5.5, 13 (8 somites) and 19 (21 somites) hours post-fertilization (hpf), at hatch, yolk-sac absorption and in juvenile (96 hp-YSA) fish ("RT-" = PCR of pooled total RNA w\ equal amounts from each sample). Primer specificity was validated functionally by amplifying plasmids and by sequencing amplicons from PCR using cDNA template.

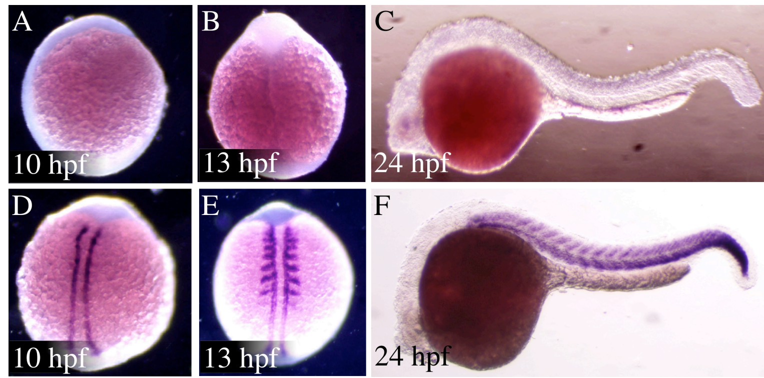


Figure 9. Developmental expression of zfMSTN-2 and MyoD. Fixed embryos were hybridized with zfMSTN-2 (A-C) or MyoD (D-F) digoxygenin-labeled riboprobes. Positive hybridization was identified using an alkaline phosphatase-conjugated anti-digoxigenin monoclonal antibody and NBT/BCIP. Embryos were sampled at 10, 13 and 24 hpf as indicated. Representative images are shown. (Photos provided by Jim Du)

Discussion

Phylogenetic relationships among the myostatin sub-family gene members within the TGFβ super-family. Three previous studies have constructed neighbor-joining trees to evaluate myostatin phylogenies using a limited number of sequences (65, 66, 72). These previous studies share some similarities in branching structure to the analyses presented herein and differ primarily in the number of species sampled and the rigor of the analyses presented. Of these previous studies, the most extensive (66) sampled 34 gene copies split into two separate neighbor-joining analyses of a Poisson-corrected distance matrix calculated from amino acid sequences. It is well known that Poisson-corrected distances are underestimated with increased sequence divergence (96). Thus, we chose a more rigorous model-based approach that analyzed nucleotide sequences aligned to an inferred amino acid alignment. The advantage to this approach is that the problems of sequence alignment are less pronounced by using amino acid homology as a means of assessing nucleotide alignments. Additionally, model-based analyses allow us to more accurately infer relationships given the level of divergence found in this gene family. The inclusion of more TGFβ super-family members has additionally allowed for a more detailed exploration of clade membership and timing of gene copy duplication.

The divergence of two myostatin sister clades (myostatin-1 vs myostatin-2) within the teleosts suggest that there was an early duplication in the fish lineage. This likely occurred before the divergence of teleosts or recently thereafter as myostatin-2 homologues have been identified in two separate teleost Superorders: Acanthopterygii (seabream, shi drum & fugu) and Ostariophysi (zebrafish). However, the exact timing of

duplication and the degree of allelic conservation within each order will only be determined once additional homologues are identified from non-teleost species, particularly from the other basal groups. The myostatin-1 gene was additionally duplicated within the salmonids producing myostatin-1a and 1b paralogues, which occurred more recently than the myostatin-1/2 duplication. Taken together, these analyses suggest that the vast majority of currently described fish myostatin genes are actually myostatin-1 orthologues. The current nomenclature is misleading and for the most part, is based on the order by which each gene product was identified within a particular species rather than its true phylogenetic relationship. We therefore propose a standardized nomenclature for all fish myostatin homologues that is based solely on the true phylogenetic relationship of each sub-family member.

The two zebrafish myostatin proteins differ significantly, especially within the LAP domains, but also within the bioactive domains. Such differences could presumably influence their comparative activities or more likely, their ability to interact with either LAP domain. Nevertheless, the bioactive domains of all fish myostatin homologues are extremely well conserved. Thus, functional differences between orthologues or paralogues of any given species are likely due to divergent regulation of gene expression rather than to protein structure. Subsequence analysis of their respective promoter regions revealed significant differences in the quantity and functional nature of the putative *cis* regulatory elements found within each region. Both promoters contained elements required for skeletal muscle expression, however, many more skeletal muscle-specific elements were found within the zfMSTN-2 promoter (Fig. 6). In fact, many of these elements were found only in the zfMSTN-2 promoter and are also present (some

proven to be functionally active) in the human, mouse and bovine myostatin promoters (93-95). Activation of muscle-specific gene expression by MEF-2/3 is dependent upon their association with other transcription factors or E-box binding proteins, including the MRFs, as they cannot initiate transcription alone. Although the zfMSTN-1 promoter contains three MEF-2 binding sites, only one is located within an appropriate context as the others are relatively isolated. By contrast, each MEF-2 and MEF-3 site within the zfMSTN-2 promoter is located next to an E-box or to another functional domain. In addition, many of the elements unique to the zfMSTN-2 promoter are functionally active during mammalian muscle development and contribute to the expression of genes necessary for myogenesis (97-104). If these domains are indeed legitimate transcription factor binding sites, zfMSTN-2 expression may be more closely associated with embryonic muscle development rather than with growth regulation *per se*. This is particularly noteworthy as zfMSTN-1, as well as other fish myostatin-1 orthologues, is expressed in several tissues in addition to skeletal muscle and most notably in the brain (67, 71, 72, 74). These data together suggest that zfMSTN-2 may play a more influential role in regulating skeletal muscle growth and development than does zfMSTN-1, whose actions may be as ubiquitous as its expression pattern in different tissues.

Vertebrate myogenic determination, the early stages of myogenesis, begins in the somite with the development of muscle precursor cells and the formation of the myotome compartment. The presence of so many putative myogenic regulatory elements within the zfMSTN-2 promoter suggests that its expression is likely to occur during myogenic determination and not in fully differentiated myofibers. In fact, we have determined that the two myostatins are differentially expressed from one another in both developing (Fig.

7) and adult (data not shown) zebrafish. The expression of zfMSTN-1 was detected at all stages and increased slowly throughout development, which is similar to the developmental expression pattern of other fish myostatin-1 paralogues as previously described (72). Although mRNA for both myostatins was detected at 2.5 hpf, this likely represents maternal expression, which again is common for myostatin-1 paralogues of other fish species as well (64, 71, 72, 74, 75). Significant zfMSTN-2 expression, however, occurred only at the 8-somite stage (13 hpf) and was not detected during gastrulation (5.5 hpf). Somitogenesis and myogenic determination would have initiated shortly before the 13 hpf sampling and would be nearly complete at 19 hpf. These data suggest that the limited expression of zfMSTN-2 coincides with the early stages of myogenesis whereas zfMSTN-1 expression occurs throughout development. They are also consistent with the predicted expression pattern based on the subsequence analysis of the zfMSTN-2 promoter.

Adult zebrafish express zfMSTN-1 in many different tissues, which could explain its continuous temporal expression pattern throughout development. However, the embryonic tissue-specific expression pattern has yet to be conclusively determined and may be beyond the detection limits of *in situ* hybridization (see below). We also attempted to identify zfMSTN-2 expression in developing embryos from many different stages using whole mount *in situ* hybridization. In addition to probing for zfMSTN-2, MyoD expression was also monitored as a positive control (Fig. 8) and although it was readily detected at different stages and in developing somites, zfMSTN-2 expression was not. This suggests that the mRNA levels for both myostatins are too low to be detected using this technique.

Two studies have recently attempted to explore the physiological and developmental actions of zfMSTN-1 and to reproduce the double muscle phenotype. Although Xu *et al*. (75) were unable to detect zfMSTN-1 expression by *in situ* hybridization in developing embryos, they determined that it was minimally expressed in embryonic skeletal muscle, forebrain and floorplate using transgenic fish that overexpressed a GFP reporter gene via the endogenous zfMSTN-1 promoter. They also demonstrated that the muscle-specific expression of the LAP domain alone, which is a known myostatin binding protein and inhibitor, produced only minor changes in hyperplastic muscle growth (12% increase in the number of fast fibers of female fish only, no change in slow fibers or fiber diameter) and did not alter the mRNA levels of different MRFs, as assessed by quantitative real-time PCR. These observations were considerably different from the double-muscled phenotype of LAP transgenic mice (32) and are in stark contrast to a recent study by Amali *et al*. (81) who, using *in situ* hybridization, reported ubiquitous expression of myostatin in virtually every tissue throughout embryonic development. These results are somewhat controversial as the sense strand controls were never shown and because they conflict with Xu *et al*. and to previous studies with mice (1). Using antisense morpholinos, Amali *et al*. purported to have also disrupted embryonic myostatin expression, which appeared to have multiple effects on somitogenesis and on whole embryo size. Levels of MyoD and myogenin mRNA were also reported to have increased with morpholino treatment, although this was assessed using non-quantitative PCR methods. Attempts to validate the efficiency of morpholino “knock-down” by western blotting for myostatin protein using human myostatin antiserum identified a single band of 27 kDa under reducing and denaturing

conditions. This again conflicts with Vianello *et al*. (74) who identified the expected 13 kDa processed and 42 kDa unprocessed proteins using two different antisera generated specifically against the zebrafish proteins. Therefore, it is difficult to determine whether morpholino treatment of embryos had any specific effects on myostatin production and thus, on embryonic development and MRF gene expression.

The discrepancy of data presented by Xu *et al*. and Amali *et al*. suggests a need for a more thorough analysis of myostatin function in this model organism. We have identified a second myostatin gene in zebrafish that appears to be more closely associated with skeletal muscle development than does zfMSTN-1. Many of the differences between these two zebrafish studies or between zebrafish and mammalian studies may be due to the combined influence of both zfMSTN-1 and zfMSTN-2 rather than to either gene *per se*. In fact, chronic stress reduces myostatin-1 mRNA levels in fish (72, 74) and increases myostatin expression in humans and rodents (94, 105, 106). This was originally interpreted as evidence of differential regulation of expression between fish and mammals. However, it may also be due to confusion between the different myostatin genes and proteins, which further suggests that the former studies with zfMSTN-1 (and other fish myostatin-1 orthologues) may not be physiologically or developmentally relevant to mammalian systems. By contrast, the temporal expression pattern of zfMSTN-2 and the presence of *cis* regulatory elements conserved within different mammalian promoters suggest that its biological role may be more consistent with that of the mammalian cytokines.

CHAPTER THREE

APPLIED ASPECTS OF MYOSTATIN BIOLOGY

Summary

Although the pathophysiological implications of a myostatin-null phenotype are beginning to be understood, the underlying developmental mechanisms have not. Considering its importance as a model for vertebrate development, it is surprising that the expression and function of myostatin in zebrafish is not as well understood or characterized as in other fish species. The identified second myostatin gene in zebrafish (zfMSTN-2) is differentially expressed in developing and adult fish and based on initial promoter studies, it is the best candidate as the muscle regulatory gene in zebrafish.

This suggests that myostatin-1, *or at least this particular paralogue in zebrafish,* is not as important to skeletal muscle development as it is in mammals. One of the best developmental models for applied molecular and genetic investigation is the zebrafish because of its rapid developmental growth rate and its embryonic transparency. The mouse is a great model organism for mammalian studies but is very limited in the cost and time it takes to characterize a mutant phenotype. For understanding development and myogenesis *in vivo,* the mouse model would be very difficult to use due to the obvious inability of visualizing embryogenesis during every stage. However, a better model organism for vertebrate systems is the zebrafish with its low cost and fast mutation capabilities. The zebrafish is completely transparent from the two-cell stage until hatch and would provide a literal window into embryonic development. Zebrafish also happen to be very popular tools for studying muscular dystrophies (107-110). Understanding and

characterizing the myostatin genes in this superior model will open up the possibilities to understanding this unique negative regulator of muscle growth and development. Due to the initial studies of myostatin's role during myogenesis in zebrafish, it was thought that this model may not be applicable. Our discovery of a second myostatin in zebrafish that appears to be more closely associated to myogenesis than its predecessor suggests a need for further studies using this model. Generating transgenic animals is an extremely powerful way to study genes. However, making mammalian transgenics is difficult and time consuming whereas transgenic zebrafish are easily made and screened. Incorporating this technology in studies with zebrafish will help us to better understand myostatin's role during myogenesis.

Clinical Applications

Controlling muscle fiber functionality and bulk is not a new therapeutic goal. The clinical and agricultural benefit of administering growth promoters (111-114) has recently proven successful, although their use has been limited in scope. However, not until the discovery of myostatin has a gene, or lack thereof, been reported to so drastically increase muscle bulk with little other side effects. Successfully manipulating myostatin production and/or bioactivity has great clinical potential especially in treating muscular dystrophies (115-119).

Since myostatin's first discovery, scientists have been trying to develop therapies for muscle wasting and muscular dystrophies. Myostatin was reported by Tseng *et al.* (118) to have a 25% decrease in mRNA levels in the regenerative muscle of *mdx* mice

compared to wild-type. This discovery could suggest a natural control to combat the wasting of muscle by the body. Studies using *mdx* mice, which contain a mutation in the dystrophin gene causing a Duchenne/Becker muscular dystrophy phenotype, compared wild-type *mdx* muscle to that of myostatin (-/-)/*mdx* crosses (119). The myostatin (-/-)/*mdx* mice were more muscular and larger than the wild-type *mdx* mice and had an approximately 25% increase in fiber diameter along with an increase in limb strength. Both mice had the typical abnormalities associated with the degeneration/regeneration cycle of Duchenne muscular dystrophy, however, the myostatin (-/-)/*mdx* mice had less fibrosis and fatty replacement in degenerated fibers. The enhanced muscle growth in these animals did not cure the primary disease, the loss of dystrophin itself, but it did increase the maintenance of muscle mass over time and substantially improved the phenotype (119). Using antibodies to target and knockdown myostatin, Bogdanovish *et al*. (115) improved muscle function in dystrophic mice, which increased creatine kinase (CK) levels, muscle force and twitch levels, and fiber size. Like the Wagner *et al*. (119), studies these results are promising for short-term treatment, but because they do not address the lack of dystrophin, a myostatin only treatment is unlikely.

The applications of this kind of gene therapy cannot be overestimated. It is known that satellite cells are responsible for regenerating damaged muscle in adults. It is not surprising therefore that current research has taken an interest in manipulating myostatin function in these cell types. If myostatin production or bioactivity could be attenuated in proliferating satellite cells of dystrophic patients, then a beneficial effect of increased fiber size and muscle could result. The limitation of disrupting myostatin, whether by direct ablation or by overexpressing a dominant negative (LAP), is that the fibers would

still be dystrophic. Therefore, the most beneficial use of manipulating myostatin in dystrophic patients would be its “booster” effects . If a functional dystrophin gene could be incorporated into the nucleus of satellite cells (120, 121), then all daughter cells from the parent satellite cell would have functional sarcolemas.

Incorporating a vector that would carry the functional dystrophin protein has been an on-going goal in muscular dystrophy research. Successful delivery of the gene has been the largest obstacle. Using stem cells as a way to deliver genetic material is one answer. Characterization and isolation of different cell populations within adult skeletal muscle has lead to the discovery of a subset of myogenic stem cells called muscle side populations (SP) (122). Comparable cells have been discovered in other tissues as well as bone and skin (123). In 2003, skin SP cells were successfully grafted into adult mouse muscle (124). One year later, the same research team successfully grafted adult muscle with muscle SP cells (125). In both studies, the SP cells carried functional dystrophin and were administered intravenously to adult *mdx* mice. Four to 10 weeks after treatment dystrophin could be found incorporated into the adult muscle tissue (125). This system of delivery of function genes has the limit however, of only being able to affect a very small percentage of diseased tissue.

The problem with SP therapies is with generating a significant amount of cells *in vivo*. Wagner *et al*. (119) reported that the loss of myostatin in dystrophic mice improved fiber regeneration, while Bogdanovich *et al*. (115) also reported a functional improvement by using antibodies to immunoneutralize myostatin. Therefore, genetically modified muscle SP cells overexpressing a functional dystrophin gene could be engineered to express the antagonist LAP domain as well. This would theoretically

stimulate proliferation and differentiation of modified myoblasts and subsequently their incorporation into mature fibers. One concern, however, with using stem cells to knock down myostatin is the potential negative long-term effect. It was questioned whether knocking down myostatin would have a lasting effect or an initial burst of growth then a noticeable decrease in regeneration. Long-term studies looked at the maintenance of increased muscle mass in *mdx* and wt adult mice that were myostatin-null. Neither mice had any negative side effects and both maintained myostatin-null phenotypes (126). This type of therapy would most likely be limited to muscular dystrophies where the muscle fiber alone is affected through genetic abnormalities, like Limb-Girdle, Becker, and Duchenne muscular dystrophies.

Studies looking at basal lamina associated muscular dystrophy have not shown positive results when myostatin was knocked-out (127). Li *et al*. (127) crossed dy mice, which are models for merosin-deficient congenital muscular dystrophy (MCMD), and myostatin (-/-) mice. MCMD is characterized by the lack of functional laminin $\alpha 2$, an important basal lamina component associated with the motility and attachment of muscle to the extra cellular matrix (ECM) (127). Myostatin ablation may not have had a functional effect in these mice because the deficiency was in the basal lamina and not the muscle fiber itself. Healthy or otherwise, muscle cannot properly attach to the ECM without laminin $\alpha 2$. Thus, increasing muscle size should not matter because none of the fibers would be functioning effectively.

Agricultural Applications

A mutation in the bovine myostatin gene was created by artificially selecting for the double muscle phenotype in different cattle breeds (2-6). These breeds have not been particularly popular due to the negative side effects of birthing overly muscular calves that require cesarean delivery, which is prohibitively costly. Meat quality in some of these breeds, but not in Piedmontese, is tough with very little fat. This suggests that the genetic background can influence the marketability of inducing muscle growth by blocking myostatin. Despite these negative effects, disrupting myostatin production or bioactivity could substantially enhance commercial animal production if it could be controlled post-birth. Currently, genetically modified (GM) fish have been created that carry extra copies of growth hormone, which create larger and faster growing fish (128). Another realistic implication would be to create a GM fish that overexpress the myostatin LAP domain. This would potentially create fish with more muscle per pound, similar to double muscle cattle breeds. Our studies suggest, however, that most if not all fish posses multiple copies of myostatin that are expressed ubiquitously. Therefore, blindly disrupting myostatin could have grave effects as the cytokine may have multiple functions. By understanding the expression and developmental impacts of myostatin-2, the proposed skeletal muscle regulator in fish, one could target the ‘correct’ myostatin only, thus affecting only skeletal muscle growth and development.

Future Myostatin Studies

LAP mutants and transgenic zebrafish

Future studies looking at transgenic fish overexpressing zfMSNT-2 LAP under the control of the zfMSNT-2 promoter would clarify the gene's developmental role. It has already been established that the overproduction of the LAP domain acts as an inhibitor to myostatin bioactivity. Embryos can be injected with a vector containing a mutated form of zfMSTN-2 that produces a truncated LAP version driven by the zfMSTN-2 promoter or by a heat-shock inducible promoter construct (these constructs have already been made, see figure 10c). Plasmids would be microinjected into zebrafish embryos during the 1-2 cell stage. Using the inducible promoter, LAP expression would be induced at the beginning of somitogenesis (10hpf), near the end (24 hpf) and in juvenile and adult fish. Muscle phenotypes would be examined by measuring muscle mass density and fiber size. Whole fish morphology would need to be measured and observed because in reality the exact role of zfMSTN-2 is hypothesized to be muscle specific but may indeed influence other tissues. The predicted phenotype of knocking down zfMSTN-2 during somitogenesis would be an overall gain in muscle mass with an increase in fiber number and diameter.

Promoter expression studies

Promoter studies would enhance any LAP mutation results along with understanding the general role of both zfMSTN-1 and -2. Because zfMSTN-1 and possibly zfMSTN-2 are expressed in a variety of tissues, overexpressing fluorescing reporter genes (GFP, RFP) driven by the zfMSTN-1 and –2 promoters in transgenic fish

will tell which myostatin is specific to which tissue (Figure 10a and 10b). Such studies would also determine which tissues co-express both genes. Currently the mRNA levels studied (zfMSTN-1) showed transient expression through embryo development and later into adult, while the protein levels were detectable in the adult stages consistently (74). *In situ* studies will not work to find myostatin expression in zebrafish because the current antibodies available cross react with GDF-11 therefore, promoter analysis is required to determine true myostatin localization.

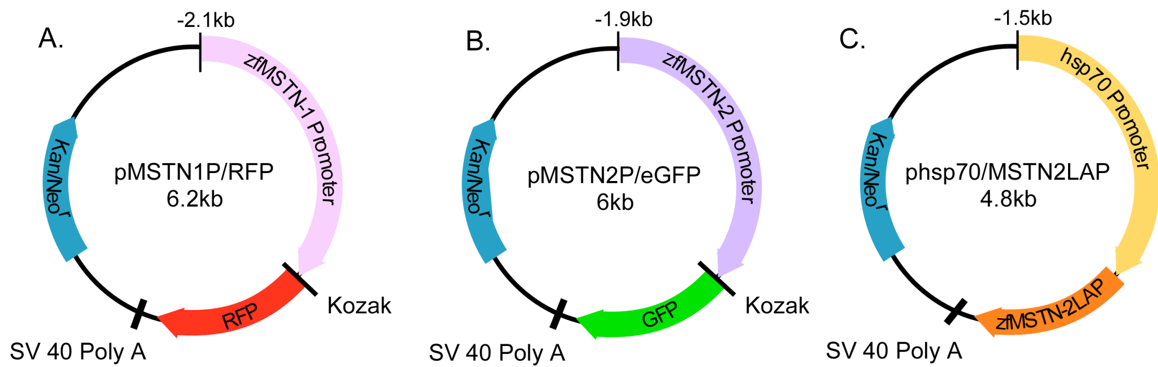


Figure 10. Plasmid constructs for injection into zebrafish. A. pDsRed vector (Invitrogen) with zebrafish myostatin-1 2.1 kb region upstream of the first open reading frame. B. peGFP (Invitrogen) vector with zebrafish myostatin-2 1.9kb region upstream of the first open reading frame. C. Heat shock protein 70 (hsp70) promoter driven vector with myostatin-2 LAP truncated cDNA.

CHAPTER FOUR

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