THE COMPARATIVE GENOMICS AND PHYSIOLOGY OF MYOSTATIN

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of DILIP KUMAR GARIKIPATI find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Myostatin is well described as a negative regulator of skeletal muscle growth in mammals and myostatin-null animals possess a "double muscle" phenotype. Recent attempts to produce a similar phenotype in fish have failed. Myostatin biology in fishes significantly differs from that in mammals and the presence of multiple myostatin genes suggests possible functional divergence. Two new rainbow trout myostatin genes were identified in addition to the available MSTN-1a and -1b genes. Phylogenetic analyses grouped the new genes into the myostatin-2 clade supporting previous analyses. The genomic structure of all four rainbow trout myostatin genes, rtMSTN-1a, -1b, -2a and -2b, was conserved with three exons, two relatively short introns and conserved exon/intron boundaries that for the most part were conserved among all vertebrate homologs. Subsequence analysis of the promoter regions identified several putative myogenic elements, although the structure of each promoter was unique and was reflected in the differential gene expression. The rtMSTN-2b gene contained two in-frame stop codons suggesting that it is a pseudogene. The embryonic expression of rtMSTN-1 genes was similar, however, rtMSTN-1a was highly expressed compared to rtMSTN-1b gene and rtMSTN-2a was not significantly expressed throughout

development. All the myostatin genes were expressed in various tissues of rainbow trout, although the expression level varied with gene and tissue. Alternative splicing was detected with both rtMSTN-2 transcripts and occurred in a manner that limited mature rtMSTN-2a transcripts to the brain and contributed to rtMSTN-2b pseudogenization. The tissue and gene specific processing, differential gene expression and pseudogenization of rtMSTN-2b likely represent compensatory and adaptive response to tetraploidization. The ubiquitous expression of at least two rtMSTN genes in all tissues surveyed suggests that myostatin's actions may be pleiotropic in fish. Recent studies, however, suggest that myostatin may also influence tissues other than skeletal muscle in mammals as well. Therefore, we determined the effect of myostatin on cardiomyoblasts. Myostatin suppressed both basal and stimulated proliferation of these cells and also inhibited differentiation. These data indicate that myostatin may play a wider role than previously believed as it similarly regulates skeletal and cardiac muscle in mammals. Knowledge from the studies with rainbow trout will help explain the underlying mechanisms of functional divergence while the cardiomyoblast studies may help design novel therapeutics for treating myocardial infarction. Together, these studies highlight the value of a comparative approach to understanding the evolution and physiology of this dynamic gene family.

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CHAPTER ONE

INTRODUCTION TO MYOSTATIN BIOLOGY

Summary of mammalian models

Myostatin also known as growth and differentiation factor (GDF)-8, is a member of the transforming growth factor (TGF)-β superfamily. Members of this family play an important role in the regulation of proliferation, differentiation and apoptosis in a wide variety of cells (1-3). Myostatin was discovered in 1997 by McPherron *et. al.* (4) where it was shown that its expression was restricted to the somitic myotome during early development and primarily to skeletal muscle in adult mice. Targeted ablation of MSTN in mice produced "Mighty Mice" (Fig. 1C,D) that are heavier with a wide spread increase in skeletal muscle mass compared to wild type animals. The muscle mass of specific muscles increased from 200-262% as a result of both hyperplasia and hypertrophy of the muscle fibers irrespective of sex (4). Homozygous mice gained the greatest in mass, though heterozygous mice were affected to a lesser extent. This suggests that the growth inhibiting effects of myostatin appear to be dose dependent. Despite the pronounced increase in skeletal muscle mass, the animals showed no gross muscular defects or pathological problems in other organs. A similar phenotype called "double muscling" (Fig. 1A,B) occurs in some cattle breeds including Belgian blue, Peidmontese, Limousin, Maine-Anjou, Gasconne, Marchigiana and Asturiana, as a result of mutations or deletions in the myostatin gene (5-7) that were generated from decades of artificial selection.

In Belgian blue cattle, a deletion of 11 bp in the coding region of myostatin led to frame shift resulting in a truncated protein due to premature stop codon (Fig. 1A) (6, 7).

The same 11 bp deletion is also responsible for double muscling in Asturiana (5). In Peidmontese and Gasconne, a missense mutation in exon 3 results in change of cysteine residue to tyrosine caused the double muscle phenotype (6). This is a result of non-functional myostatin protein incapable of forming the "cysteine knot" that is critically important for the proper folding of all TGF-β superfamily members (8). Similarly, in other double muscle cattle breeds there are other mutations in the myostatin gene that also results in hypermuscularity (5) while similar phenotypes and mutations have also been described in other mammals as well.

Texel sheep display an analogous phenotype to double muscled cattle and are known for hypermuscularity. However, there are no mutations in the coding region of the Texel myostatin gene. Instead, they possess a polymorphism in the 3' untranslated region that introduces a micro RNA docking site and results in reduced myostatin transcript stability (9). A myostatin mutation has also been described in a young child with extraordinary musculature, particularly in the thighs and upper arm (10) (Fig. 1E). The child is homozygous for a G-to-A transition in the 5' splice site of the first intron. This produces a misspliced transcript and a premature stop codon that is encoded by the intron and results in misspliced myostatin transcripts. The enhanced muscle growth was clearly evident at an early age as the 4.5 year old child was able to hold 3 kg weights with both arms extended (10). Recently, a two base pair deletion, which causes a frame shift and again introduces a premature stop codon, was linked to hypermuscularity in the Whippet dog breed (Fig 1F,G) (11). These results together suggest that the biological functions of myostatin are highly conserved, at least in mammals.

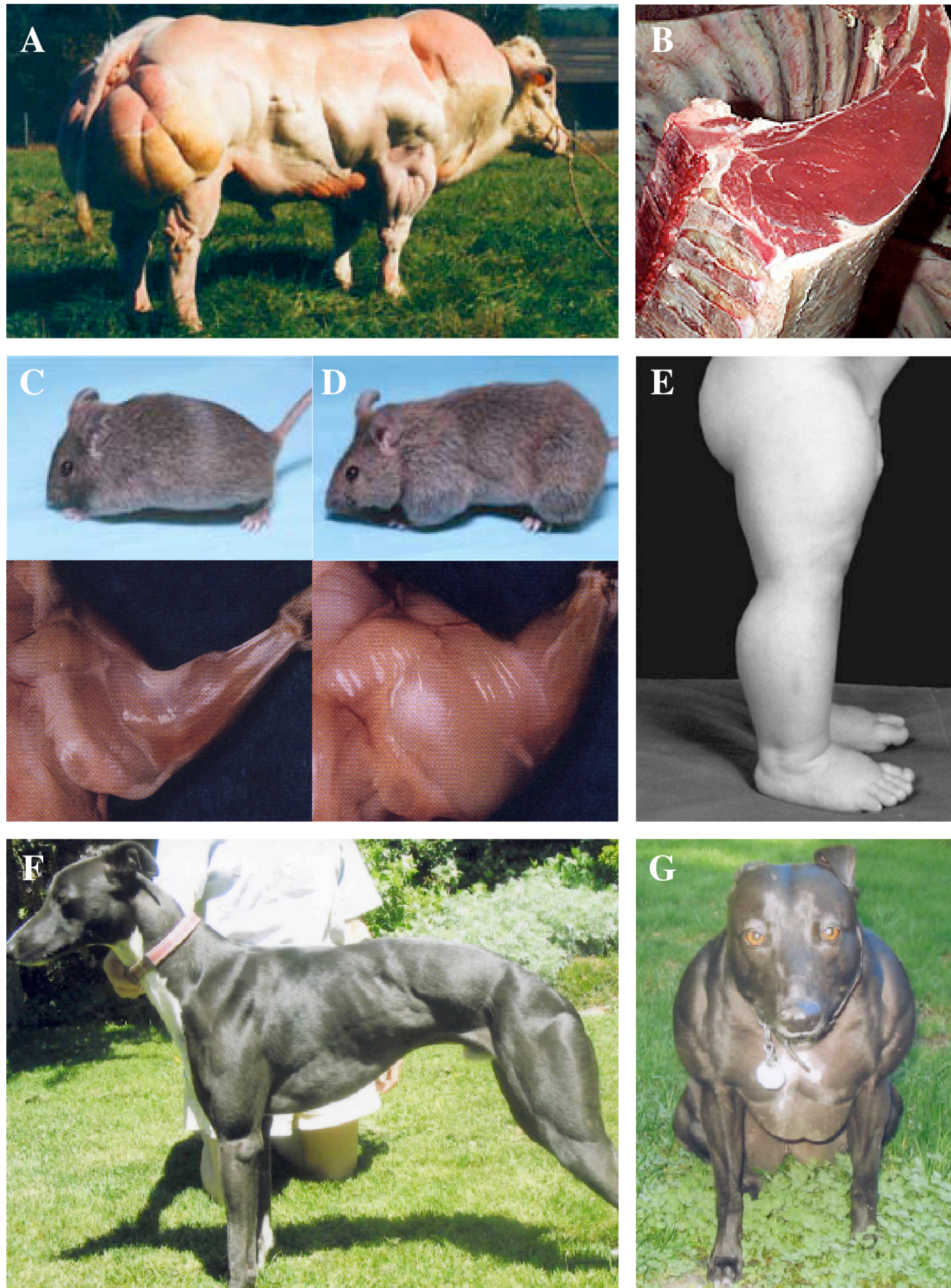

Figure 1. Myostatin null phenotypes. A. "Double-muscling" in Belgian blue cattle due to an 11 nucleotide deletion in the gene (7). B. Cross section of meat from Belgian blue cattle. C. Forelimb musculature of wild-type mouse. D. Forelimb musculature of myostatin "knockout" mouse (4). E. Leg musculature of a 7-month old infant boy with a null-mutation within the splice donor site of intron 1 of the myostatin gene (10). G. Whippet dog heterozygous for a myostatin null mutation (11). H. Whippet dog homozygous for a myostatin null mutation (11).

Protein processing and receptor signaling

Myostatin shares all of the structural characteristics common to the TGF-β super family. This includes nine conserved cysteine residues and the RXXR proteolytic site that separates the N-terminal latency associated protein (LAP) and the C-terminal bioactive domain (4). The protein is synthesized as a precursor protein and is proteolyzed twice before being secreted. The first proteolytic event cleaves the signal peptide while a second cleavage separates the C-terminal bioactive domain from LAP (Fig. 2). Both LAP and MSTN peptides form homodimers and are secreted together as a latent complex through non-covalent binding interactions (12). The LAP plays an important role in proper folding of myostatin and in the formation of the cysteine knot structure. It also regulates the activity of myostatin by binding non-covalently and preventing receptor activation (12). Myostatin bioactivity is mediated through the activation of activin type II receptors, specifically ActRIIa and ActRIIb. Cross-linking studies and radioactive receptor assays indicate that myostatin binds both ActRIIa and ActRIIb, although ActRIIb has higher affinity (13). Mice with homozygous and deactivating mutations in ActRIIa have pectoralis and triceps muscles that are 27-40% larger than wild-type mice (14). These muscles are 20-26% larger in mice with mutant ActRIIb receptors suggesting that ActRIIa may play a more important role in regulating myostatin's action, at least in these muscles. The role of activin type II receptors is further supported by transgenic studies in mice overexpressing dominant-negative ActRIIb, whose muscle mass is increased by 125% (13). After myostatin binds ActRIIa/b, the complex recruits the type I

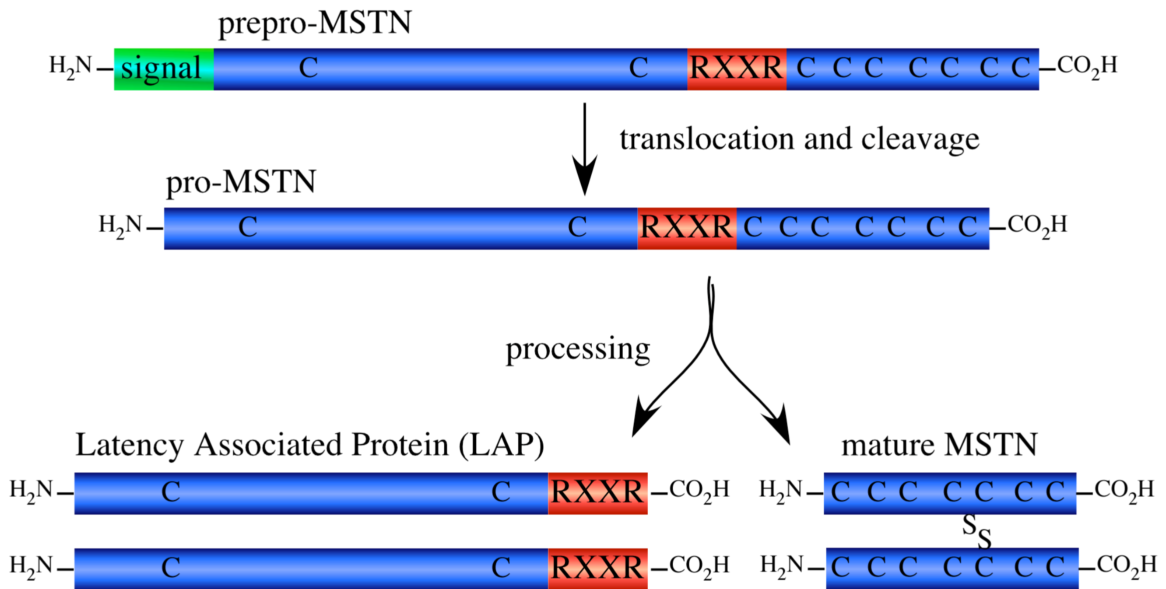

Figure 2. Myostatin structure and processing. Myostatin is synthesized as prepro-MSTN and undergoes two proteolytic events to generate the bioactive form. The first proteolytic event cleaves the signal peptide and the second proteolytic event cleaves at the conserved RXXR site resulting in LAP and mature MSTN. The bioactivity is as a result of the mature MSTN dimer.

receptors activin like kinase (ALK)-4 or 5 (15). The activated kinas domain of the type I receptors the phosphorylated smads 2 and 3. These transcription factors bind smad4 and the complex translocates into nucleus where it regulates gene expression (15, 16). Zhu *et. al*. (16) determined that inhibitory smad7 perturbs the actions of myostatin by binding to smad4 and to the smad2/3 complex. These results suggest that smad2/3 are responsible for the downstream signaling of myostatin.

Myostatin appears to prevent myoblast hyperplasia in mammals by inhibiting cell cycle progression past the G1 and G2 stages (17-19). These actions are mediated in part by reduced Cdk 2 levels and activity, a concomitant increase in p21 Cdk-inhibitor and consequently, the hypophosphorylation of Rb. Myostatin also inhibits myoblast differentiation (20-22), although the teleological significance of this particular effect may appear controversial as conflicting data suggests that myostatin initiates cell cycle withdrawal, which is a necessary prerequisite for differentiation (19, 20, 22, 23). However, studies with primary myosatellite cells from myostatin null mice suggests that myostatin-stimulated cell cycle withdrawal is accompanied by cellular quiescence (23, 24) rather than differentiation. This explains the apparent discrepancy and supports earlier studies indicating that myostatin is a myoblast survival factor (17). A model for myostatin action in mammals therefore suggests that in the absence of other myogenic regulators, myostatin inhibits myoblast hyperplasia by stimulating cell cycle withdrawal and inhibits differentiation by inducing cellular quiescence.

Factors regulating myostatin expression and bioavailability

***Transcriptional regulation*:** There are a number of transcriptional factors that regulate myostatin expression. Analysis of mouse, bovine and human myostatin promoters identified several conserved transcriptional response elements that regulate gene expression (21, 25, 26). These include glucocorticoid response element (GRE), myocyte enhancer factor (MEF)-2, androgen response element (ARE), E-boxes, peroxisome proliferators-activated receptor (PPAR)γ and nuclear factor-kappa B (NF-κB) (25). Deletion of the MEF-2 binding site caused a threefold decrease in the transcriptional activity of myostatin in C2C12 cells. MEF-2 proteins are involved in heterotypic protein-protein interactions with myogenic proteins in the basic helix-loop-helix family (27). The presence of active MEF-2 response elements in myostatin promoters of different mammalian species (26) suggests that these particular elements are well conserved in this vertebrate class (25). Using chromatin immunoprecipitation and luciferase assays, MyoD was also shown to enhance myostatin expression 15-fold by binding to E-Box motifs in the bovine myostatin promoter (26). Recently, it was reported that endoplasmic reticulum (ER) stress, characteristic of sporadic-inclusion body myositis, induces myostatin expression in cultured human muscle fibers (28). Pharmacological inhibitors and electro-mobility shift assays (EMSA) indicated that NF-κB mediated these effects. Each of these factors, MEF-2, MyoD, E-boxes and NF-κB, all contribute to myoblast differentiation, or as with MyoD, are critical to this process. The up-regulation of myostatin expression by these and possibly other myogenic factors is consistent with myostatin's necessary role in the differentiation process; stimulating cell cycle withdrawal and maintaining cellular quiescence.

Myostatin binding proteins: The latent complex consists the two monomeric LAP molecules non-covalently bound to mature myostatin dimer (29). LAP is glycosylated, but the inhibitory action is not effected by glycosylation as LAP expressed in bacteria also blocks myostatin's action (30). The affinity of LAP for mature MSTN is higher than that of other myostatin binding proteins (13). Indeed, transgenic animals overexpressing LAP also increase muscle fiber number and size as well as muscle mass (13, 31). The domain necessary for binding to mature myostatin was partially located to amino acids 42-115 (30). It was estimated that more than 70% of myostatin is bound to LAP in circulation and that LAP plays an important role in regulating myostatin bioavailability (32).

Follistatin, a monomeric glycoprotein expressed in many tissues, binds to MSTN and prevents its binding to the receptors in a manner similar to LAP (13). The affinity of follistatin for myostatin however, was calculated to be 5.84×10^{-10} M (33), which is less than that of LAP (13). Using the yeast two hybrid system, it was shown that the entire follistatin protein is necessary to interact with myostatin and that the interaction may be mediated through several epitopes (33). Transgenic mice overexpressing follistatin under the myosin light chain promoter were double muscled to the same degree (194-327%) as the myostatin knockout mice (13). Furthermore, the systemic delivery of follistatin by subcutaneous injection of Chinese hamster ovary cells expressing follistatin significantly slowed myostatin-induced weight loss in mice (34). Follistatin expression increases in peri-necrotic muscle cells after injection of cardiotoxin (35). This suggests that follistatin may contribute to the regeneration process after muscle damage by inhibiting myostatin's action (35).

Other myostatin-binding proteins include follistatin like-3 (FSTL-3), growth and differentiation factor associated protein (GASP-1), decorin and titin cap. Follistatin like-3 also known as follistatin like related gene (FLRG), is a glycoprotein that is highly similar to follistatin. Circulating myostatin binds FSTL-3 in both mouse and human serum (32), although the affinity of FSTL-3 for myostatin is considerably low compared to that of LAP or follistatin (36). FSTL-3 expression is induced by TGF-β (37) or activin (38) through the activation of smad 3 and 4. Myostatin also signals to the nucleus via smad 3/4 activation. This suggests that myostatin action may be auto-regulated by the induction of FSTL-3 expression. Growth and differentiation factor associated protein (GASP)-1 is a newly described member of the follistatin-domain family that also binds myostatin in mouse and human serum (39). It contains a domain homologous to the 10 cysteine repeats found in follistatin. Unlike follistatin and FSTL-3, however, GASP-1 binds independently to both LAP and MSTN. Furthermore, it inhibits only GDF-11 and MSTN, unlike follistatin and FSTL-3, which inhibit activin as well (13, 32, 40, 41). In addition, LAP, myostatin and GASP-1 have been shown to co-immunoprecipitate, suggesting a possible ternary complex (39). Decorin is a small leucine-rich proteoglycan that plays an important role modulating TGF-β action (42, 43) through interactions with membrane-bound receptors (44, 45). Studies using surface plasmon resonance indicate that decorin binds myostatin, but only in the presence of Zn^{2+} concentrations greater than 10 μM (46) and blocks myostatin's inhibitory actions on mouse C2C12 myoblasts. The concentration of Zn^{2+} in blood ranges from 12-20 μM, which suggests that decorin binds to myostatin in physiological conditions (46). Titin cap (T-cap) is a sarcomeric protein that binds to the N-terminal domain of titin whereby it regulates the cytoskeleton organization of muscle

cells. The association between T-cap and myostatin was initially demonstrated using the yeast two-hybrid assay, but later by co-immunoprecipitation assays (47). T-cap also blocks myostatin action, however, it is an intracellular protein and thus, does not squelch myostatin away from receptors. Rather, T-cap binding to myostatin occurs presumably in the golgi or cytoplasm, wherein it inhibits myostatin secretion (47). The same authors further proposed that myostatin might play a role in limb girdle muscular dystrophy, which results from the defective maintenance of T-cap regulated sarcomere integrity.

Myostatin function in non-skeletal muscle tissues

Myostatin was initially reported to be expressed almost exclusively in developing and fully differentiated skeletal muscle with nearly undetectable levels of expression also occurring in fat (4). McPherron *et. al.* (1997), however, used northern blotting to profile tissue expression and surveyed only a limited number of tissues. Indeed, myostatin is differentially expressed at various stages of development in rat cardiomyocytes, with an increase in expression from day 2 to day 10 postnatally and a decrease in adult hearts (48). Myostatin expression was also detected in 90 day old fetal and adult ovine hearts and was localized to both cardiomyocytes and purkinjee fibers (49). Expression of mRNA and protein increased following infarction and persisted for 30 days in the peri-infarcted region (49). Furthermore, mechanical stretching of rat primary neonatal cardiomyocytes increased myostatin protein levels 4-fold, which were also increased 18-fold in transgenic Akt mice (50). Myostatin also appears to function in cardiac muscle as the addition of exogenous myostatin to fetal (embryonic day 18) and neonatal cardiomyoblasts suppressed proliferation in a time dependent manner by blocking G1 to S phase progression in the cell cycle (48). It also completely abrogated the phenylepinephrine-induced protein synthesis in neonatal cardiomyocytes through Akt and p38 activation (48, 51). Nevertheless, no cardiac phenotype has been described in myostatin null-animals.

Myostatin mRNA was also detected in the mammary gland of lactating pigs, but not in non-lactating pigs, and was primarily concentrated in the tubuloalveolar secretary tubules (52). This suggests that myostatin may regulate mammary gland development during lactation and may indirectly influence galactopoesis (52). The amount of bioactive

myostatin protein was higher in suckling rat pups and decreases with age (53). High levels of myostatin during the early postnatal period may regulate the size of muscle fibers and thereby preferentially divert energy to other organs that take precedent like the brain during development (53). Myostatin was detected in adipose tissue as well, but at lower levels compared to skeletal muscle (4). Decreased adipose tissue as well as leptin levels were observed in myostatin knockout mice (54, 55) and myostatin was shown to inhibit the differentiation of 3T3-L1 preadipocytes (56) and primary bovine preadipocytes (57). The inhibition of adipogenesis with myostatin was primarily due to suppression of adipogenic transcription factors PPAR γ and C/EBP α (57). In a contrasting report, Artaza *et. al*. (2005) showed that myostatin promotes adipogenesis (58), however, the authors used a multipotent mesenchymal cell line (C3H10T1/2) that can differentiate into many cell lineages rather than well characterized and pluripotent relevant 3T3-l1 and primary cell models. Recently, an extensive *in situ* expression map of mouse brain identified myostatin expression in brain (www.brain-map.org) (59). Specifically, it was expressed in cerebrum, cerebellum and brain stem. The function of myostatin, if any, has not been delineated so far in this tissue. However, GDF-11, which is closely related to myostatin and has a nearly identical bioactive domain (95% similar), is thought to play a role in neurogenesis (60). It is secreted by fully differentiated neurons and inhibits the proliferation of neuroprogenitor cells (61, 62) and thus, functions as a negative autoregulator as "chalone". This mirrors myostatin action in mammals, albeit in a different tissue, but together suggests that myostatin may also function in brain.

Myostatin in non-mammalian vertebrates: birds

Myostatin genes and cDNA have been cloned in avian species including the turkey and chicken (4). Chicken myostatin is located on chromosome 7 (63) and its expression is first detected in embryos on day 0. After an initial 5-fold decrease by day 2, myostatin expression subsequently increases 3-fold by day 7 and remains elevated through day 16 (64). However, breed differences in expression was reported (65). Polymorphisms in myostatin gene were associated with body weight at day 7 and day 40 in broiler chickens (66). In situ hybridization of whole mount embryos identified the expression of myostatin in the ventral myotomal region of mature somites (67), suggesting a role during skeletal muscle development similar to mammals. In chicken myosatellite cells derived from red muscle fibers, myostatin mRNA levels increased during cellular fusion as the myotubes form (68). However, myostatin mRNA and protein levels were similar in both broiler and layer chickens during embryonic development as well as in adult muscle tissues, although differences were observed between the poultry lines themselves (69). The levels of MSTN decreased around the hatching period and increased in the post-hatch period, with a similar trend in IGF-I. It was therefore, proposed that the ratio of IGF-I to MSTN may play a role in stabilizing the muscle growth rate during development (70). *In ovo* administration of human IGF-I delayed the increase in myostatin expression in embryonic pectoralis muscle (71). Indeed, the high expression of myostatin during embryonic development suggests that it may play a role similar to mammals. In fact, injection of monoclonal antibodies against myostatin in 3 day- old eggs increased the body weight and muscle mass by 4.2 and 5.5% respectively, compared to controls (72). However, a recent study showed that there are differences in

the proliferative and differentiation responses to myostatin in myosatellite cells derived from chicken pectoralis and biceps femoralis muscles. Cells from the former are more responsive to myostatin's suppressive effects (73) although it is unknown whether this is due to greater tissue sensitivity per se (*ie*. receptor number) or due to differences in tissue-specific bioavailability (*ie*. differential expression of myostatin binding proteins). Chickens fasted for 48 h showed a decreased myostatin expression in pectoralis and sartorius muscle (74). Myostatin has been shown to inhibit proliferation and differentiation of chicken embryonic myoblasts (75), similar to mammalian myoblasts. Administration of polyclonal antibodies against LAP *in ovo* decreased thigh and leg weights of the post hatch chickens, providing evidence that LAP inhibits the biological activity of myostatin in chickens as well as mammals (76).

Myostatin in non-mammalian vertebrates: bony fish

Three attempts were made to reproduce the double muscle phenotype in fish as occurs in myostatin-null mammals. The results, however, are far from conclusive and suggest that myostatin's effects extend beyond skeletal muscle in fish. Injection of antisense morpholinos was reported to enhance somitogenesis and whole embryo size, but did not specifically influence myogenesis (77). The overexpression of a dominant-negative LAP in transgenic zebrafish resulted in a minor increase in muscle cell number, but only in female fish (78). A third study (79) reported to have generated "giant zebrafish" by injecting double-stranded myostatin RNA from a different and unrelated species (tilapia). Each of these studies was performed before the discovery of the second class of fish myostatin genes and as a result, it is difficult to determine if any myostatin transcript was affected. Fish are unique in that hyperplastic muscle growth continues throughout development and in adult fish (80). This is unlike mammals where hyperplastic growth ceases after birth and thereafter, growth occurs through hypertrophy of the muscle fibers (81, 82). Many fish species have economic and agricultural importance. Thus, increasing muscle growth as in myostatin-null mammals will significantly impact the aquaculture community by decreasing the gap between fish supply and demand.

Comparative aspects of myostatin biology need to be defined more completely before they can be exploited in an aquaculture setting, particularly as the function of MSTN in fish has not been determined. Unlike mammals, which have only one copy of the myostatin gene, fish have multiple copies resulting from two genome-wide duplications (83, 84). The first genome duplication occurred before the teleost radiation

early in the evolution of the bony fish (85-87). A second genome duplication event occurred specifically in the salmonids approximately 50-100 million years ago (88, 89), although many paralogs may have since been lost. Phylogenetic analyses of the entire myostatin and GDF-11 gene family identified two distinct myostatin clades in the fishes, MSTN-1 and -2, each of which include two additional subclades in the salmonids. Thus, species of this family should possess four genes: MSTN-1a, -1b, -2a and -2b. These studies also determined that all of the salmonid genes previously characterized were actually MSTN-1 orthologs (83). A new nomenclature for MSTN genes, based on the previous phylogenetic analyses, has since been proposed (83, 84) and will be followed here. Fish MSTN clones were first characterized from zebrafish (6) and have since been sequenced in a number of fish species. To date, however, a number of myostatin orthologs and paralogs have been characterized including both cDNA and genomic clones from different fish species. This includes zebrafish (4, 83, 90), brook trout (91), rainbow trout (92, 93), coho salmon (94), sea bream (95, 96), shi drum (97), Atlantic salmon (98, 99), orange spotted grouper (100), croceine croaker (101), sea bass (102), Channel catfish (103), blue catfish(103), white catfish (103), tilapia (104), white bass (104), stripe bass (105), white perch (105), king mackrel (91), yellow perch (91), little tunny (91), mahi mahi (91), sea perch (106) and fugu (Fernandas, J.M., Kinghorn, J.R, and Johnston, I. A., GenBank accession nos. AY445321 and AY445322).

Similar to mammals, all fish MSTNs have hallmarks of the TGF-β superfamily including a conserved RXXR proteolytic site and several conserved cysteines (83, 91, 93, 104-106). Proteolytic processing also appears to be conserved, in at least two divergent species, and has been demonstrated *in vitro* with tilapia MSTN-1 (104) and *in vivo* with

brook trout (91). Multiple sequence alignments indicate that the LAP region of MSTN is more diverse within and between species, whereas the mature C-terminal domain is highly conserved. In fact, the C-terminal region is 100 % identical in most avian and mammalian species (6) and is 88% identical between mammalian and most fish species (6, 105). This high level of conservation suggests that myostatin function may be equally well conserved among fish as well. However, unlike mammals myostatin is expressed widely throughout development and in a number of adult tissues in fishes.

Embryonic expression. Using *in situ* hybridization, myostatin transcript was detected in the somites of developing mouse embryos (4). Rodgers *et. al*. (2001) first described the developmental expression of a fish homolog using tilapia embryos and larvae (104). The expression of MSTN-1 mRNA was first detected immediately after hatching, which has earliest signs of muscle activity. The MSTN-1 transcript rapidly increased during embryonic and early larval development and decreased during yolk sac absorption. The increased expression of MSTN-1 during early development and with the onset of muscle activity suggests a role in muscle development (104). Initial studies using *in situ* hybridization failed to detect MSTN-1 transcript in zebrafish (78, 107), although later studies used RT-PCR and reported that the expression of MSTN-1 was ubiquitous during development (77, 83) while that of MSTN-2 was regulated (83, 90). By far the most extensive analysis, however, used "real-time" RT-PCR to quantify both transcripts during the development of zebrafish (108). Both were expressed during the blastula stage, which was attributed to maternal transfer, and quickly dropped to undetectable levels during gastrulation. Their expression increased during somitogenesis (11.3-19 hours post fertilization-hpf), although MSTN-2 levels were increased 5-fold before returning to

baseline and those of MSTN-1 steadily rose 30-fold throughout somitogenesis. The expression of MSTN-1 transcript in zebrafish was always greater and the levels steadily rose throughout development unlike MSTN-2, where the expression after somitogenesis did not begin to rise until hatching (108). Using far more limited sampling, the developmental expression of MSTN was also reported in rainbow trout (rtMSTN-1 genes only) (92, 109) and orange spotted grouper (100). The expression of rtMSTN-1a and -1b was similar during embryonic development with low levels during the eyed stage and slightly higher levels in swim-up fry (109). In brook trout, expression of MSTN-1a and -1b was quantified and again both increased gradually during development and through the yolk sac absorption period (110).

Even though myostatin mRNA expression has been reported in many fish species, protein expression has only been reported in brook trout (110). A 42 kDa protein, which corresponds to unprocessed protein, was detected only at 45 days of embryonic development and rises thereafter. However, processed MSTN was not detected in larvae (110). The absence of the 14 kDa mature myostatin in developing embryos suggests either rapid turnover of active peptide or that proteolytic processing of the peptide is regulated. Taken together, these studies suggest that even though small variations exist, the developmental expression pattern is similar in all fish species.

Adult tissue expression. Expression of myostatin in adult fish is very different compared to mammals. In mammals, it is primarily restricted to skeletal muscle (4), although lower levels of the expression were observed in adipose tissue (4, 57), mammary gland (52), cardiac tissue (49) and brain (59). By contrast, myostatin expression in fish is more ubiquitous and occurs in a wide range of tissues, as was first

reported in tilapia (104). Expression of tilapia MSTN-1 was detected in brain, skeletal muscle, gills, eyes, gut, ovary and testis (104). In addition to these tissues mentioned above MSTN-1 expression was reported in skin, heart, spleen, liver and kidney of channel catfish (111) with the highest expression in skeletal muscle. Sea bream, MSTN-1 was similarly expressed in many tissues while MSTN-2 was expressed in far fewer tissues: brain, eye and gonads (95, 96). All of these studies used qualitative RT-PCR to assess tissue expression. It is therefore difficult to compare absolute levels between tissues or genes. Recently, however, the quantitative analysis of both MSTN-1 and -2 expression was reported in zebrafish (108) and both patterns appeared to be quite different. Indeed, MSTN-1 levels were higher in most tissues sampled except eyes and swim bladder. Species variation in the expression of MSTN-1 transcript in red and white muscle is also evident. The expression of MSTN-1 was predominant in red muscle of brook trout, king mackerel and yellow perch, while it was higher in white muscle of little tunny and similar in both red and white muscles of mahi-mahi (96). The difference in the expression in red versus white muscle in different teleost species were attributed to variation in red and white muscle ratio and strategies used for locomotion (91). These studies were performed by northern blotting and therefore, the circumstances could also be explained by cross-hybridization of probe to other MSTN genes.

The differential expression of sea bream MSTN-1 and -2 and of brook trout MSTN-1a and -1b indicate a unique expression pattern for each gene that is also consistent with differential promoter activity. Subsequence analysis of MSTN promoter in zebrafish and brook trout detected several putative myogenic elements (83, 110). However, an androgen response element (ARE) and a glucocorticoid response element

(GRE) were detected only in MSTN-1b promoter of brook trout (110). Similarly, an ARE and GRE were identified only in promoters of zebrafish MSTN-2. The differential presence of these putative elements is also consistent with the differential expression of each gene throughout development and in adult tissues. However, functional studies of each promoter are needed to better understand the underlying mechanisms and to identify conserved or divergent elements.

The expression of only MSTN-1 genes has been described in salmonids. The MSTN-1a and -1b genes differ in the expression levels as well as tissue expressed (91, 92, 98). These studies are far from complete as they were analyzed only in a few tissues. Brook trout MSTN-1b was shown to be abundant in red muscle, but there was little in the brain and none in the ovary. Within the brain, MSTN-1b was reported in the optic lobes, hindbrain, and in the hypothalamus while that of MSTN-1a occurred in red muscle, brain and ovary (91). In fact, the expression of MSTN-1a in the ovary increased with ovulation suggesting a role during spawning.

Even though mRNA expression of MSTN transcripts was reported for various fish species, protein expression was reported in only a few. The most extensive protein analysis was done in sea bream (112). Myostatin immunostaining was identified using antibodies that recognize both MSTN-1 and -2 proteins of sea bream. Immunoreactivity was detected in liver, skeletal muscle and epithelium of gut, kidney and gills. Myostatin immunoreactivity was also detected in the early stages of white muscle development, however, it was not detected in later stages. By contrast, myostatin was strongly detected in red muscles. It was also detected in oocytes and in surrounding granulose cells, but not in testis. The antibodies used unfortunately could not differentiate between the two

different myostatin proteins. The high reactivity of the antibodies in most of the epithelial tissues suggests that myostatin may have a role in the proliferation of epithelial cells where cells are constantly replaced. The wide expression of myostatin protein further suggests that myostatin may have a wider role in fish species. Myostatin protein expression was also described in atlantic salmon by western blotting with custom antibodies (98). However, the mass of the immunoreactive peptides identified was very different from those described in tilapia (104), brook trout (110), mammals (34) or even in another study with atlantic salmon (94).

Factors effecting MSTN expression. In salmonids sexual maturation is accompanied by decreased muscle growth (113). The measurement of rtMSTN-1a and -1b expression of rainbow trout during muscle wasting that accompanies sexual maturation showed that rtMSTN-1a levels were unchanged (92, 109) and rtMSTN-1b expression is significantly decreased in both red and white muscle (92). A similar study in brook trout did not detect any difference in RNA levels, however, a change in the protein level was detected (110). The levels of the 14 kDa bioactive myostatin protein was significantly elevated in red muscle of spermiating males and spawning females. In white muscle, the expression of processed protein is increased in spermiating males, however, an increase in spawning females was not detected (110). This suggests that the mRNA expression may not reflect protein levels. This should be considered when interpreting results based solely on mRNA expression. Stress may also influence the expression of myostatin in fish although not necessarily as it does in mammals where different stressors directly increase myostatin gene expression (114). A short-term fast increased MSTN-1 expression in tilapia larvae while a long term fast reduced it (115). In

the same study, incubating larvae in 1-ppm cortisol for 3 and 6 h decreased the expression of MSTN-1 by 66 and 75%, respectively. Fasting was also shown to elevate larval cortisol levels and thus, the suppression of myostatin expression during fasting was attributed to hypercortisolemia (115). A Similar experiment with adult rainbow trout also reported no difference in the expression of MSTN-1 genes in skeletal muscle of fasted and refed fish for 4, 12, and 34 days in the myotomal muscle (116). In this study, however, the authors did not differentiate between rainbow trout MSTN-1a and -1b genes independently as they measured total MSTN-1 transcript abundance. The authors concluded that myostatin may not mediate muscle recovery during refeeding, although the possibility of increased in expression of one MSTN-1 gene and a proportional decrease in the other cannot be ruled out. Injecting channel catfish with dexamethasone (1.0 mg/kg body weight) decreased the MSTN-1 mRNA levels significantly after 12 h but not after 24 h. A similar study in zebrafish showed delayed growth as well as low skeletal muscle expression of MSTN-1 in response to chronic stress (107). These studies together suggest that changes in myostatin expression in response to stress and glucocorticoids are opposite in fish and mammals. However, a recent study with sea bass reported increased myostatin expression with chronic fasting that was subsequently reduced with refeeding (102). These species-specific differences suggest that the change in MSTN expression during fasting and refeeding may help balance between nutrient demand and growth process only in some fish species. The effect of stocking density stress on adult zebrafish was reported by Helterline *et. al.* (2007), where the expression of both MSTN-1 and -2 increased by 3 orders of magnitude in the spleens of fish stocked at

high density (40 fish/L) (108) suggesting an immunomodulatory effect of myostatin in zebrafish.

In transgenic coho salmon overexpressing growth hormone (GH), the expression of MSTN-1b is significantly higher in red muscle compared to size- and age-matched wild-type fish (94), although differences did not occur in white muscle or brain. Using antibodies raised against recombinant brook trout MSTN-1, 14 kDa protein detected was detected at lower levels in red and white muscle tissues of the transgenic coho salmon compared to wild-type fish. This suggests that low levels of MSTN could be responsible for growth in transgenic coho salmon (94). Injected rainbow trout with recombinant growth hormone increased MSTN-1a expression in white muscle by 56% at day 1 and 3. By contrast, rtMSTN-1b levels decreased by 26% (117). The differential expression in response to GH, cortisol or stress could be due to the different promoter activities that have unique response elements. The same could be explained for differences in myostatin expression between the species.

The effect of myostatin on muscle growth in fish is not well understood as it is in mammals. Most of the information on myostatin has been collected on expression patterns in different fish species. The environment in which different species of fish grow, along their size and growth rate differences, could contribute to some of the differences described. The presence of multiple myostatin genes further complicates things as some of the former studies may have accidentally measured multiple or many transcripts. The results of myostatin expression studies obtained from zebrafish cannot be completely applied to other teleost fish as mosaic hyperplasia is greatly reduced in zebrafish. Indeed, this species does not have indeterminate growth commonly found in

the larger fish cultivars. Thus, future studies with different fish species will likely identify even more differences among the many aspects of myostatin biology in this largest extant vertebrate group.

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CHAPTER ONE

IDENTIFICATION, CHARACTERIZATION AND QUANTITATIVE EXPRESSION ANALYSIS OF RAINBOW TROUT MYOSTATIN-1A AND -1B GENES

*Data reported in this chapter are taken from accepted publication

Abstract

Myostatin is a potent negative regulator of skeletal muscle growth. Although several cDNA clones have been characterized in different vertebrates, the genomic organization and bioactivity of non-mammalian homologues have not. Reported herein is the intron/exon organization and promoter subsequence analysis of two rainbow trout myostatin genes, rtMSTN-1a and -1b (formerly 1 & 2, respectively), as well as a quantitative assessment of their embryonic, larval and adult tissue expression profile. Each gene was similarly organized into 3 exons of 490, 368 and 1600 bp for MSTN-1a and 486, 386 and 1419 bp for MSTN-1b. Comparative mapping of coding regions from several vertebrate myostatin genes revealed a common organization between species including conserved pre-mRNA splice sites; the first among the fishes and the second across all vertebrate species. *In silico* subsequence analysis of the promoter regions identified E-boxes and other putative myogenic response elements. However, the number and diversity of elements were considerably less than those found in mammalian promoters or in the recently characterized zebrafish MSTN-2 gene. A quantitative analysis of the embryonic expression profile for both genes indicates that rtMSTN-1a expression is consistently greater than that of rtMSTN-1b and that neither gene is significantly expressed throughout gastrulation. Expression of both steadily increases 4-fold during somitogenesis and subsides as this period ends. After eyeing, however, rtMSTN-1a mRNA levels ultimately rise 20-fold by day 49 and peak before hatching and yolk sac absorption (YSA). Levels of rtMSTN-1b similarly rise, but do not peak before YSA. An analysis of adult (2 year old fish) tissue expression indicates that both transcripts are present in most tissues although levels are highest in brain, testes, eyes,

muscle and surprisingly spleen. These studies suggest that strong selective pressures have preserved the genomic organization of myostatin genes throughout evolution. However, the different expression profiles and putative promoter elements in fishes versus mammals suggests that limitations in myostatin function may have recently evolved.

Introduction

Muscle growth results from the proliferation of myoblasts and their subsequent differentiation into muscle fibers. This process is regulated *in vivo* through mechanisms that involve cell-to-cell interactions, cell-to-matrix interactions and extra-cellular secreted factors including myostatin (also known as growth/differentiating factor (GDF)-8) (1). This member of the transforming growth factor (TGF) β superfamily is a potent negative regulator of skeletal muscle growth. Indeed, a myostatin-null phenotype in domestic mammals is characterized by extreme gains in muscle mass, commonly referred to as "double muscling" (2, 3). In addition, a 5' splice site mutation in the first intron of the human myostatin gene has recently been reported in a child with extraordinary musculature (4). Increased muscle growth in all of these models results from both muscle cell hyperplasia and hypertrophy as myostatin directly influences myosatellite cells (5-10). These results together suggest that the biological functions of myostatin are conserved in all mammals, although they have yet to be described in other vertebrates.

A recent phylogenetic analysis of the entire myostatin/GDF-11 subfamily (11) indicates that bony fish possess multiple myostatin genes and that a gene duplication event during early fish radiation (12, 13) produced two distinct myostatin clades: MSTN -1 and -2. A second duplication event within salmonids, likely resulting from tetraploidization, produced two subsequent divisions, one in each clade. This suggests that most if not all salmonids possess four distinct myostatin genes: two within the first clade (1a & 1b) and two in the second (2a & 2b). Both of the previously identified rainbow trout cDNA clones, formerly named *Tmyostatin*-1 and -2, are actually MSTN-1

orthologues. They were therefore renamed rtMSTN-1a and -1b, respectively, which reflects their true evolutionary relationship to other myostatin genes (11).

Myostatin genes have been characterized in mice (14), humans (15), cattle (16) and pigs (17). Although cDNA clones have been characterized in many diverse fish species (11, 18-24), very few genes have been fully characterized. This is particularly disconcerting as bony fish, especially teleosts, represent the largest group of extant vertebrates and many of these species are commercially important. Therefore, a better understanding of the genomic sequence and organization of different fish myostatin genes as well as species-specific expression patterns will significantly interest comparative and agricultural biologists alike. This information will be particularly important in the identification and cloning of MSTN-2 genes from different salmonids and will help in distinguishing paralogues from orthologues.

Reported herein is the isolation and characterization of the rtMSTN-1a and -1b genes including their respective promoter regions. We additionally report the quantitative assessment of each gene's expression pattern using detailed RNA panels generated from multiple stages of embryonic/larval development and from different adult tissues. These studies indicate strong sequence conservation among all vertebrate myostatin genes. However, the expression patterns and putative promoter elements suggest that the function of myostatin in fish may not be as limited as it is in mammals.

Materials and Methods

Isolating genomic myostatin clones. Genomic DNA was extracted from rainbow trout (*Oncorhynchus mykiss*) fin clips. Briefly, 3 ml of lysis buffer (30mM Tris, 8M Urea, 4% w/v Chaps, pH 8.0) was added to 50 mg of tissue and incubated overnight with proteinase K (20mg/ml) at 60 °C. Three consecutive phenol:chloroform:isoamyl alcohol extractions were then performed and DNA quality was verified on a 1% agarose gel. Promoter regions were cloned using the Universal Genome walker kit (BD Biosciences, www.bdbiosciences.com) and the manufacturer's protocol. Briefly, genomic DNA was digested with the blunt end restriction enzymes Dra I, EcoR V, Pvu II, and Stu I and subsequently ligated to the provided adaptor linkers. Nested PCR with 94ºC initial denaturing was then performed using gene specific primers homologous to the known 5' coding region of each gene (Table 1) and adaptor primers with the Advantage 2 PCR kit (BD Biosciences). Cycle parameters were as follows and were used as default unless otherwise specified: an initial denaturation at 94°C for 1 min, 7 cycles of 94°C for 30 sec and 72°C for 3 min followed by 30 cycles of 94°C for 30 sec and 67°C for 3 min and a final extension for 4 min at 67°C. The PCR products were sub-cloned into the Topo TA vector (Invitrogen, www.invitrogen.com) and sequenced in the university's genomic core facility. Putative regulatory elements were identified by subsequence analysis using Matinspector software (Genomatrix Inc, www.genomatix.de), which searches consensus sequences of known *cis* regulatory sequences. Intron sequences were obtained by nested PCR using gene specific primers for rtMSTN-1a or -1b coding regions and adaptor primers (Table 1) as follows: initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 3 min and a final extension at 72°C for

3 min. The resulting amplicons were then cloned as described and sequenced. Flanking primers specific to each myostatin gene were then used to amplify and clone the complete genes using Pfu polymerase (Stratagene, www.stratagene.com) and the pCR4-blunt Topo vector (Invitrogen). Intron/exon boundaries were determined by aligning the cDNA sequences to their respective gDNA sequences using ALIGN X (VectorNTI, www.Invitrogen.com).

The 3' untranslated regions were isolated using a 3' RACE (rapid amplification of cDNA ends) kit (Invitrogen, www.invitrogen.com). Total RNA from juvenile skeletal muscle was extracted using Trizol and reverse transcribed using Superscript reverse transcriptase, both according to the manufacturers' protocols (Invitrogen). cDNA was then amplified by PCR using gene specific forward primers (3'UTR1a/1 & 3'UTR1b/1, Table 1) and universal adaptor primers (UAP) provided in the kit. The PCR conditions were as follows: an initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 2 min and a final extension at 72 °C for 2 min. Nested PCR was subsequently performed with the 3'UTR2 forward primer and the abridged universal adaptor primer (AUAP) provided in the kit. The resulting amplicons were sub-cloned and sequenced as described.

Embryonic and tissue collections. Rainbow trout were reared at the National Center for Cool and Cold Water Aquaculture, Kearneysville, WV, according to the guidelines approved by the institutional animal care and use committee. An RNA panel was generated from 5000 pooled eggs from multiple females (Trout lodge, October 2004) that were fertilized by milt from two males. Following fertilization, eggs were incubated at approximately 13°C throughout embryonic development. In addition to the unfertilized

Table 1: Primer sequences and annealing temperatures (°C) for rtMSTN-1a and -1b

Primer name	Sequence (5' - 3')	An. temp.
MSTN-1a F	CTT CAC ATA TGC CAA TAC ATA TTA	60
MSTN-1a R	GCA ACC ATG AAA CTG AGA TAA A	60
MSTN-1b F	TTC ACG CAA ATA CGT ATT CAC	60
MSTN-1b R	GAT AAA TTA GAA CCT GCA TCA GAT TC	60
18s F	TGC GGC TTA ATT TGA CTC AAC A	60
18s R	CAA CTA AGA ACG GCC ATG CA	60
3'UTR 1b/1	AAC TCT GTA GTC CGC CTT CAC GCA	65
3'UTR 1a/1	AAC TCT GTA GTC CGC CTT CAC ATA	65
3'UTR 2	CAC CTG CAG AAG TAC CCC CAC ACC	65
Adaptor primer 1	GTA CTA CGA CTC ACT ATA GGG C	67
Adaptor primer 2	ACT ATA GGG CAC GCG TGG T	67

eggs (Day 0), developing embryos were collected as whole egg samples daily for the first 14 days, every other day until hatch (Day 24) and every third day thereafter. Each sample contained 18 eggs or embryos or 9 post-hatched larvae that were pooled and several samples were collected at each time point. Tissues were also removed from 2 year-old adult fish weighing approximately 2 kg. All samples were flash frozen in liquid nitrogen and stored at -80°C until RNA isolation. Samples were first powdered using a liquid nitrogen cooled Bessman Tissue Pulverizer (Spectrum Laboratories, www.spectropor.com) and total RNA was extracted using TRI-Reagent with the high salt solution modification to remove excess glycosylated proteins. The RNA was reconstituted in 20-50 μL of nuclease free water and treated with DNAse (DNAse RQ-1, Promega, www.promega.com) to remove contaminating genomic DNA. Samples were then re-extracted with TRI-Reagent and RNA quality was assessed by agarose gel electrophoresis.

RNA Quantification using "real time" RT-PCR. Total RNA (2μg) was reverse transcribed with 1 μg of random primers (Promega) and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT, Promega) in 40 µl. Subsequent real time RT-PCR assays were conducted using the ABI Prism 7900HT Sequence Detection System (www.appliedbiosystems.com) and gene specific primers (MSTN-1a F, MSTN-1a R, MSTN-1b F & MSTN-1b R, Table 1). For each sample, 1 μl of cDNA was combined with 7.5 μl of 2x SYBR® Green PCR master mix (Applied Biosystems). For each reaction, 6 μl of this mixture was added to 9 μl of the primer mix containing 500 nM of each primer. The reactions were carried out as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles consisting of 95°C for 15 sec and 60°C for 1 min. The cycling

reaction was followed by a dissociation curve to verify amplification of a single product and amplicons were also verified by DNA sequencing.

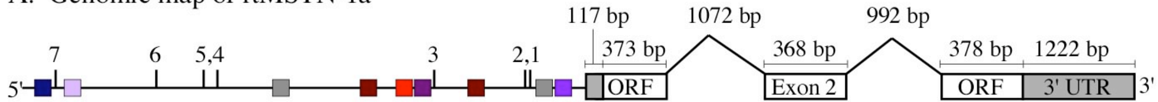
The relative standard curve method was employed to quantify gene expression. For each primer set, a serial dilution of a mixed tissue cDNA was used to construct a standard curve for each assay plate. The standard curve was constructed by plotting the threshold cycle (C_T) versus the natural log of input RNA (ng). This curve was then used to calculate the relative abundance of each transcript in each sample. Myostatin values were then normalized to those of 18s to control for differences in RNA and cDNA loading. Each sample was run in triplicate on a single plate and each plate was run in duplicate. Assays were repeated with different samples and all data are presented as normalized gene expression.

Results

Genomic organization and comparative mapping of rtMSTN-1a & -1b genes. Complete genomic clones for both rtMSTN-1a and -1b genes were isolated and sequenced (Figs. 3 & 4). This includes approximately 2 kb upstream of each initiator. The annotated gene and promoter sequences were then deposited into GenBank and assigned the accession numbers DQ136028 and DQ138300, respectively. Each gene is organized into three exons of similar size with appropriate intron/exon splice sites, a pattern that is conserved with other fish species (11, 25, 26) and with mammals (14-17). The three rtMSTN-1a exons are 490, 368 and 1600 bp in size, respectively, and are separated by 1072 and 992 bp introns (Fig. 3A). The rtMSTN-1b gene is similarly organized with 3 exons of 486, 386 and 1419 bp with two 564 and 778 bp introns intervening (Fig. 4A). The 3' UTRs were also cloned by 3' RACE using RNA from adult skeletal muscle and were determined to be 1.2 kb in the rtMSTN-1a transcript and 1.1 kb in rtMSTN-1b. This is in contrast to the significantly shorter 3' UTRs previously reported (21). In addition, two polyadenylation signal sequences (AATAAA) were detected in each 3'UTR at -17 to -11 bp and -83 to -76 bp from the poly A site in rtMSTN-1a and -19 to -13 bp and -86 to -80 bp in rtMSTN-1b (Figs. 3D & 3D).

The proteolytic processing sites and the entire bioactive domains for both myostatin proteins are entirely encoded within the third exons, which is also true for all previously characterized vertebrate genes (Fig. 5). Comparative mapping of coding regions revealed a common organization between species including conserved pre-mRNA splice sites. Codons flanking the first splice sites (3' end of exon 1 & 5' end of exon 2) are highly conserved among the fishes while the second site (3' of exon 2 & 5' of

A. Genomic map of rtMSTN-1a



B. rtMSTN-1a promoter

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-2284                      CTTT CCCCTACGGT TGATTTCAAT AAAGAAGTGT
-2250 CATACATCCA CACGGATTGT GAACCGAAAT ATGTGTGCCG CCTCTCCTTT
-2200 CTATTCAATC TCGTTATCGC CTTCAAACCG CTGCATATGC GTCCAATGAT
-2150 TTGAACTTGA AGCTTGGAAG GCAGCGATAG CATTGGTCGG TTTCTCTCTC
-2100 TGCACGAAAT GTGCACTACG TTACCAAACT GGGCACCATA CCACATTGTG
-2050 CAGTAATTTA ACTAAATGCT AAATCAATTT AAGGTCTTAA AGCCATGAAT
-2000 AGTCATACCT GGATGACTAC ACCTTCTTGC CATCATTCTA CTCGAGATTG
-1950 TCCATAGGTC ATCGTTGAAA TTGTTGCCTG TTTGACTACA CTTTCTAATG
-1900 GCGGCTGTAG CTTGTAGGAC GTAATTATAT TTTTAAAACA CTCTCAACAG
-1850 CAATTAGTAG CTGTGTCGAC TATGTCGTGG AGGTAAATTG TGCGCATACA
-1800 ATCAGGATAC TGATACTTGT ACAATATCAC CCTCTTGTGG CATGCTTGAC
-1750 TTCTCAGTGA TTTATTACAA TGTTTAAAGT AAGGAATATA TTCAGTTGTT
-1700 GCTATAAACT AATGCCATAG ATTATAATCC TGTCTCCCAG GCCTTTGCTG
-1650 GAAGTGTACC CCCGTGCAGC TTCCCTGTGT CTAAATTTTA GGCTACTACT
-1600 GAGTCTGCCA AGGGGTCTGG ACCATTATGG CACAGGAGGT ACGGTAGTGT
-1550 GCTTTCGCTT AAACCGCAGA GGTTTTATGA GTATTGATAG CAAGCCATAT
-1500 GTGCTCGGAT ATATTTCAAA CCAATTGTTG TCAGCTAAAA AAAAAATATA
-1450 TATATGACGA GTTCGTTCTT GACGAATGCG TAAATCTGTA AAACTCATGG
-1400 AGGAGTGTGC GTAAAACTGT ATAGTCCATA AGTGTAAATT AGTTAAAGTC
-1350 GTTTAGTATG TTTTCTTTCA CATAAGGGAT ATAGTCTCAA ATTCCATAAT
-1300 GATAATAGGC TATAAACCAT TGACATGACT CGTGACCAAG TCATTGTGTC
-1250 ATGCCATCCA TATTGCTGCG TAAAATGTAC ATAATTTAGA CGATTTGATT
-1200 GACTTTCATT AGAACCTATA TGTTACTTTT CATCTGTAAA TGAAAATAAT
-1150 ATTTCATAAC CTGTGTGTAA TGTTGCAACT GTAGTTGTCG ATAAGCCGCA
-1100 CGATGGCACT AAACGTCTAG ACAAAAAAGC TTTCGCAAAT CAGACACCCC
-1050 AAGGCAACAA TAACCAGTAG CCTATAGTTT AGTACAGTAT AGTAGGCCTA
-1000 CATTACAGGC CATTTCGCTA TATATTTGGA ATGGTATCTA TCCTATAGCC
-950  TATTGATTAA TACATTTTGC CTTTGGAGAG TAGGTTTGGA GACATGTAGG
-900  TCTACTTCAT GTAGCCTATA GGTCTACATA GACTAAATAT TGGTTCCTGT
-850  CATATTTGTA AATAATTGAC CTTACATTTT CAAGAGAGAT TCAGAAAGAT
-800  AGGCAAGCTG ATCATATAAC TAACTGATTG TAAAAGAAGT GGTCATAACT
-750  CAGATTAATT TGATCCCTGT AGCTTTGATG GGATGCAGGG GCGGACTGGC
-700  CATCTGGCAT TCCCGACAAA TGTCTGAAGG GTTGGTAAAT TCTTTGCCTA
-650  GAGGGCCTGT CTAACTTTTT TTATTTATTT TTTTTTGCGC AAAATGATAA
-600  TTATCTGGCT AATAATGGTG GCCTCAATTA ATAAAATGGG CTGGTGTGGG
-550  GGCCTTGATG AAACATTTTT AGTTGTGGGT GCCTCAAGGG GGAAAATGTG
-500  CCATTGTGTT ATAAATGCCA GAACCGATTT CTGGTTCCAG TCCACCCCTG
-450  GTGTGATGGG ATAGGCTGAA TAAAACAGGT TTCTCAGTGG CATTCAGATG
-400  TTGACAGGCT GTGGCTGACT TAAGTTCATG CAATAAATCA ATAACCATAT
-350  CCGAAATGTT TATTTGACCA TTTGCAGTAC TGTCTTGTCT GATTGATAGT
-300  GTAGTTGTCT CAAAACTATT CCAAGCCTTG CCATTGCGTA CCTCTTTAAG
-250  GTTTGACAGA AAAATACAGT TCATGCCTGT CTGTTAAATT CATTGTTGCC
-200  AGCCCAACCA ATCATAGATT TTGACGACAC AAAAGAGAGG CCAAAGTTGC
-150  AATATAAAAA GGGCTGCCGA ATTAAAGTAT GACACCTATC AGTGTAGAGC
-100  CTGATTTCAA ACACAGGCAA CTCTGTAGTC CGCCTTCACA TATGCCAATA
-50   CATATTACAT TTGGGATTCA ATTTTTATAG CAAACTCCGC ACCTTAGATA
```

C. Promoter elements of rtMSTN-1a

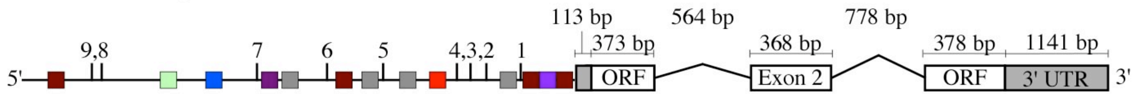
Name	Location
Comp1	-236 to -256,-1190 to -1210
HAND2/E12	-694 to -708
MEF 2	-477 to -499,-772 to -794
MusIn	-2161 to -2184
SRF	-2257 to -2281
TATA box	-143 to -147
TEF-1	-683 to -699

D. 3' UTR of rtMSTN-1a

```
TGAGCGAGAG CTCTGCCGGT GAGGGGGAGG GGCTCAGCCA GGGTCTCCC
CCCTGGACTT TGGGACAGAT CCATCCACCA CTACCAGTGC TTTCTGCAG
AACACGGTGC AATAGAGCCA GAATAGCGGC TAAAGAAATG CCCGTTCAT
TCGCTGAGCA GCGCTTCAAC CACGGACATG TCTCGTTCAT TTTTTTTCT
TCATTTTTGT TTTCTTCCCT CCTCCAATCA CATGTTCGCA GCCACAATG
GCCTAAAACC ACATGGATGT AGGAACACAA CGCCTGTTCG ACTTGGGAG
TGGACGTAGT AGCCTAAAGT ACGCCACATG TTCCCACAAA ACTGTTAAA
TGTCACACGC TCCGTCCAGT TATTCAAACA TACAGACATG GATGGACAC
GCATACACCA GACCTGGGTT CAAATAGTAT TTGTTTTTCT TTCAAATAC
TTTGACTGTT TGATGGAGAC AGGTGGGGTT TGCACTTTGG GGAATAATC
CATTGATTCC ATTGCACCAG GCAAGCACAG CTTAAGTACT GGAAAGAAA
ACAAATCATA TTTCAACCCA GGTCAGAAAC ATAGGTGCAC ACACACACA
CACACAGACT AATTTCCCCC TTAAGATTTT ACAGCTACGC TTACCTATC
GGTGTGATAA TCATATAAGC AATATTTGAG AGTGAAACCG GGAATCCTC
AACGACTTTT GAAAGGGCTT GGAAAACTAT AGCTGAGGTC TCAGTCTGA
ACTGGCCTTC ATGCATAATG CCATAAACAC ACACAGCAAC AGTGCATTC
TTATTCAACT TTTAATCATA GCTTTACCAC CATTCAACTG CCTGAACTG
CCCTACTCAC TTGAATTATT CTTATTGTAA ATCAACATTA CTGGACAGG
AGGACTTGAA CCGGAGGCAC AATGAATGCA GTCTACACAT TGAGATGTG
TTTAAGACAG ATAAATATAT TTTGAAAAGT ATGAATGTTA AAACCACGT
TTAAACTCTG TCTTACAAAT AACACAGTTT GCACTATGGC AAACCAATA
GAAAGAATTG GTTGCTACAA AAGTTGTAAA AACTGATTTT GATACGTTT
GCTAATTTGT ATTGTATACA TATGCCATTG TTTCCATTAG CAGTTGCCT
TTCTACACCA CTGTTAATAA ATGTATAAGA CCACAAACTA GCAAGAAAA
CAGTACAAAT GCGACTCTCT ACCTGTTAAT CAAATAAAGG TGCTTCCTT
AAAAAAAAAA AAAAAA
```

Figure 3. Genomic structure & organization of the rtMSTN-1a gene. (A) Map of rtMSTN-1a gene and putative myogenic *cis* regulatory elements within the promoter region. Exons are boxed with the open reading frame (ORF) in white and untranslated regions (UTR) in gray. Each individual *cis* element is placed relative to its position within the promoter region and is color-coded as indicated in C. Putative E-boxes are numbered. (B) Sequence of the promoter region with color-coded *cis* elements. Boxed are consensus sequences (CAN(T/A)TG) for E-boxes. Nucleotide position corresponds to the initiator. (C) Key to the color-coded promoter elements in A and B and their corresponding positions. (D) Sequence of the 3’ untranslated region. The newly identified sequence is underlined and two potential polyadenylation signal sequences are boxed.

A. Genomic map of rtMSTN-1b



B. rtMSTN-1b promoter

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-2425                                  ATCAT GGGAAGCGAC CCTTGAAACC
-2400  CCCTCCCCAC CTTTAACTAT AACCCACCTT TATTGAACTA ATAAAGGCAT
-2350  ATGTGGAAGA ATGTCACTAT TCAAATGTGA ACATAAAAGG TCCAGGAGAA
-2300  ATACTAGCCC GGATGGAGGA AGGGCGAAAT AAATGGTTGT CCGCGTTGTA
-2250  AATAGGCTTC TAAGAATTCT TACATGAGCG TATTTCTGTT CAATATGTTT
-2200  AGAGGTAGCG TCTAGTCCAT TTCATACACA GTTACCAGAA AAATAGGTAC
-2150  ATGGAATATT GCTGAAGCGT GGGATAGGCA GCCTACCTTC AAAAAGACTC
-2100  TTGGAAAAGT AGGACTGGAT GGGTTTCCCT CCATGAATAT CAAATTCATC
-2050  CCGTAGGAAA ACGCATCGTT TATCTTGAAC GACACGTTTC AATAAAGAAG
-2000  TCTCATACAT CCACACGGTT TGTGAACCGA AATCACCAAA CGTGCCGCCT
-1950  CTCGGACTGT GTCTACTCCG TCTCCTTATC AAACCGCCGA ATATGCGTCC
-1900  AAGGCTTGAA CATGAAACTT GGAAGGCAGC GACCACATTG GTCAGGTTTC
-1850  TCTCTCAGCA CGAAATTTCC ACCAAGGTAC CAAACTGGAC ATTCAATGTT
-1800  CCACCATACC ACATTGTGCA GTAATCCCAC TAAATACAAC ATCGATTTAA
-1750  TTAAACTCTC ACATCCTGGA ATAGTCGGAC ATGGAGGACT ATACCTTCTT
-1700  CTTGCCATCA TTCTACTGGA GATTCTACAT AAATCATCGT TGAAAGTGTT
-1650  GCCTGTTTGA CTCAACGTTC TGAAGGCAGT TAGTAAACTG CAGAACGGGT
-1600  TGTGGAGATA AATTGTGCGC GTTCACACGG GTTACTGTTA CTTGTAGAAT
-1550  ATCACCCTCT TGTGGCGCAT GGTGGCTCCA TGCTTGACTC CCCAGTGACT
-1500  ATTGAAATGT TTAAAGTAAG GAATACAGTC AGTTGTTTCT ATAAGCTATC
-1450  TACCTATAGA TTACATTCCT GTCTCTTGCC TTTGATGGAC GTGTACCCAC
-1400  GATTAGCTTC CTTGTATCAA AGTGTTCTAA GGCTACTCAT GCGCCTGTCG
-1350  AGAGGTCTGG ATCATTATGG CACGAGGTAT ATTAGCGAGC TTTCCCAATA
-1300  GCCACTGCAG TCGCACTGTG TCAGATTTTC AGTTATGAGT TATCGGATAT
-1250  GTTCAAAACC AATTGGTGTC ATTTAAAAAA ATATATATGA CGCATTCATA
-1200  AAACAGTAAA ACTCATTGGA CGAGTGTGCG TAAAACGGTA TAATGTACAT
-1150  TAGTTAAATT CGTTTATGTT TTTGTAAATA CAGTATGGTA TATTTTCTTT
-1100  CACATAAGGG TTCCAGTCTC AAATTCGATA ACGACAATAG GCTATAATTA
-1050  AACCATTCAT TGACCTAACA TAGTGCCATG CCATTCATAT TGCTGCGTAA
-1000  AATGTGCATA AAGTGGGCAA TTTGATTGAC TCTTGTGAGC CATACACATT
-950   TTCTAAATGC CGGTTGCGTT GATCTAAATT ATAATGCATA TGTTACTGTT
-900   TATTTGTGAA GGAAAATAAT ATCTCATAAC CTGTGCGTAA TTTTGCAACT
-850   GTTGTTGATT AACCGCATGA TGGCACTAAA CGTCTGGACT AAAAAGCTTT
-800   CGTAAATAGT AAGCGCAAAC ACCTCAAGAC AACAATATCC AGTAGCCTAT
-750   AGATTAATAC AGTATCGTAG GCCTACAGAA GAGCCCATTT CGCCATAAAT
-700   GTTCAATGCA ATAGTATCTA TCCTATAGCC TATTGACAAA AGTATTTAGC
-650   ATTTGGAGAC AAGTTTAGAA AGAAGTAGGT CTACTCCATG TAGCCTATAT
-600   CAATATAGAC TAAAGAGTGG TTCCTGTCAT ATTTGTCAAT TAATATACCT
-550   TGCATTTTCG AGAGAAACAG ATTTAGAGAG TTAGGCAAAC TGGTCATATA
-500   AACTAACTGA TCACAGGGGA AGGGAAGTCA TAACACCGAT TCATTTGAAC
-450   CCTGTTGCGT TGATGGGATG AGCGGAAAAA ACGCCTTTTC TCAGTGGCGT
-400   TCCTATGGTG ACAGGCTTGC GGATTTAAGT TTATGCAATT AATCAATATA
-350   ACCGCATCCG TAATTTGATT TGACCATTTG CAGAACTGTC TTGTCTTTGA
-300   TTGGTAGTGT AGTTGTCTCA AAACTATTCC GAACCTTGCC ATACCTCTTT
-250   TAAGGGTTGA CAGAAAAATA CAGTTGATGT CTGTCCGTTA AATTCATTGT
-200   TGCCAGCCCA ACCAATCGTC GATTTTGACG ACACAAAAGA GAGGCCAAAG
-150   TTGCAGTATA AAAAGGGCTG ACGAATTAAA GTATGCCACC TATCAGTGTA
-100   GAGCCTGATT TTAAACACAG GCAACTCTGT AGTCCGCCTT CACGCAAATA
-50    CGTATTCACT TTTGGATTTT TTTTTTATAG CAAACTCCGC ACCTTAGATA
```

C. Promoter elements of rtMSTN-1b

Element	Position
Comp1	-232 to -252,-830 to -850 -963 to -983, -1403 to -1423
Hand2/E12	-694 to -708
MEF 2	-77 to -99,-128 to -150 -1044 to -1066, -2373 to -2395
MEF 3	-1850 to -1862
Muscle TATA	-1660 to -1676
TATA Box	-139 to -144
TEF-1	-1427 to -1443

D. 3' UTR of rtMSTN-1b

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TGAGCGAGAG TTCTGCTGGG GAAGGGGAGG GGCTTAGCCA GGGTCTCCAC
CCTGGACTTT GGGACAGATC CATCCATCAC TACCAGTGCT TTCTGCAGAA
CACAGTGCAA TAGAGCCACA ATAGCGGCTA AAGAAACACC TGTCCATTCG
CTGAGCCGGC TTCAACCACG GACATGTCTC GTTCGTTTTT TTCATTTTCA
TTTTCTTCTC TCCTCCAATC ACATGTTCGC CCCACAATGG CCTAAAGTCA
CAAGGATGTA GGAACACAAT GCCTGTTGGA CTTGACATGG ACACACACCA
GACCTAGGTT CAAATAGTAT TTGTTTTTTT TCTTCAAATA CTTTAATGGA
GAAGGGTGGG GTTTGCACTT TGGGGAATAA TCCATTGGTT CCATTGAACC
AGGCAAGCTC AATCAAGTGC AGCTTAAGTA TTGGAAATAT AACAAATCCT
TTTTAAACCC AGGTCTGACA CACATAGACT AATCCCCCAA CCCCCAAGCT
TTTACAGCGT ACGTTTAACT ATCGGTGTGA TAATCATATA AGCAATTTTT
GAGAGTGAAA CCGGGAATCC TCAAGGACTT TTTAAAGGGC TTGGAAAACT
ATAGCTGAGG ACTCAGTCTG AACTGGCCTT CATGCACAAT GCCATAAACA
CCATATACTC ACACAGCAAC AGTGCATTAT TATTCAACTT TTAATCATAG
CTTTACCACC ATTCAACTGC CTGAACTGCC CTACTCACTT GAATTATTCT
TATTGTAAAT CACCATTACT GGACAGGAGG ACTTGAACCG GAGGCACAAT
GAAAGCAGTC TACACATTGA GATGTGTTTA AGACAGATAA ATATATTTTG
AAAAGTATGA ATGTTAAAAC CACGTTTAAA CTCTGTCTTA CAAATAACAC
AGTTTGCACT ATGGCAAACC AATTGAAAGA ATTGGTTGCT ACAAAAGTTG
TAAAAACTGA TTTTGATACG TTTGCTAATT TGTATTGTAT ACATATGCCA
TGGTTTCCTT TAGCAGTTGC CTTTTTACAC CACAGTTAAT AAATGTATAA
GGCCACAAAC CAGCAAGAAA ACAGTACAAA TGCGGCTATA TAACCTGTTA
ATCAAATAAA GGTGCTTGCT TATAAAAAAA AAAAAAAAAA AAAA
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Figure 4. Genomic structure & organization of the rtMSTN-1b gene. (A) Map of rtMSTN-1b gene and putative myogenic *cis* regulatory elements within the promoter region. Exons are boxed with the open reading frame (ORF) in white and untranslated regions (UTR) in gray. Each individual *cis* element is placed relative to its position within the promoter region and is color-coded as indicated in C. Putative E-boxes are numbered. (B) Sequence of the promoter region with color-coded *cis* elements. Boxed are consensus sequences (CAN(T/A)TG) for E-boxes. Nucleotide position corresponds to the initiator. (C) Key to the color-coded promoter elements in A and B and their corresponding positions. (D) Sequence of the 3' untranslated region. The newly identified sequence is underlined and two potential polyadenylation signal sequences are boxed.

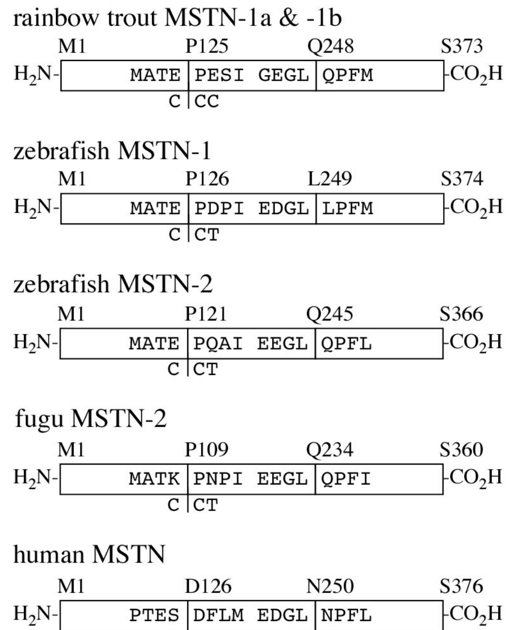

Figure 5. Comparative mapping of exon boundaries in different myostatin genes. All vertebrate myostatin genes cloned to date are organized into three exons. The three adjoining boxes for each protein represent the coding regions for each exon. Amino acid sequences coded by exon boundaries are shown inside the boxes. The first amino acid coded by each exon is shown above as is the last residue of the third exons. In all fish genes, the codon of the proline residue located at the first exonic boundary is partially coded by the first and second exon as shown. Locations of the nucleotide splice sites for rtMSTN-1a and -1b are shown in figures 1 and 2 and the sequences fit the known consensus.

exon 3) is conserved in fish and mammals (Fig. 5). Amino acid consensus sequences were therefore identified by comparative sequence analysis of intron/exon coding junctions. These include MAT(E/K) | PXXI for the first junction and (G/E)(E/D)GL | XPFΦ for the second (X = any amino acid, Φ = hydrophobic, likely L, I or M). Indeed, multiple sequence alignments with myostatin proteins from different vertebrates, both previously published (18) and repeated with newly discovered clones (data not shown), indicate that these motifs are highly conserved, the first in fishes and the second in all vertebrates.

In silico analysis of rtMSTN-1a & -1b promoters. Subsequence analysis of the 2 kb promoter regions upstream of each gene using *MatInspector* software identified several putative muscle specific transcription factor binding sites or *cis* regulatory elements. These include Comp1 (cooperates with myogenic proteins 1), HAND2 (heart, autonomic nervous system, neural crest derivative 2), MEF 2 (myocyte enhancer factor 2), MusIn (muscle initiator), SRF (serum response factor) and TEF-1 (transcriptional enhancer factor 1) binding sites in the rtMSTN-1a promoter (Fig. 3). The rtMSTN-1b promoter contained all of these putative elements except for the MusIn site and additionally contained a MEF 3 site (Fig. 4). Each promoter also contained an appropriately placed TATA box and several putative E-boxes while a muscle TATA box was also found in the rtMSTN-1b promoter. A comparative analysis of all cloned myostatin gene promoters from fish revealed features common to all or most promoters and some features unique to a particular promoter as well (Fig. 6). Every promoter contained several E-box motifs and all but the brook trout (bt) MSTN-1b promoter contained multiple Comp 1 sites and TATA boxes in close proximity to the transcription

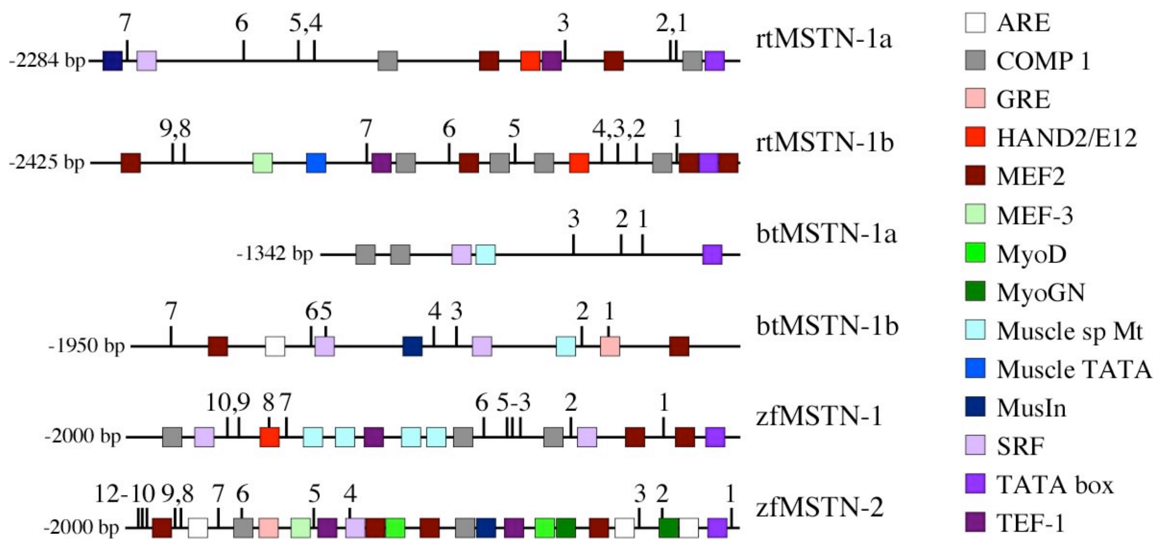

Figure 6. Comparative subsequence analysis of fish myostatin gene promoters. Promoter maps of rainbow trout myostatin (rtMSTN)-1a and -1b, brook trout (btMSTN)-1a and -1b and zebrafish (zfMSTN)-1 and -2 are shown with putative *cis* regulatory elements boxed. Putative E-boxes are also indicated and are numbered in ascending order starting from the transcription start site on the right. Each element is placed relative to its position within each promoter and is color-coded according to the key.

start sites. Studies with mammalian promoters indicate that MEF2, GRE (glucocorticoid response element) and MyoD (myogenic differentiation factor) binding sites all regulate myostatin promoter transactivation (27-29). These sites were also identified in the fish promoters. All but the btMSTN-1b promoter contained multiple MEF2 sites and many were located within the first 500 bp. A GRE was only identified in the btMSTN-1b and zebrafish (zf) MSTN-2 promoters while the latter additionally contained the only MyoD binding site as well as two myogenin binding sites. Roberts and Goetz (2003) previously identified a MyoD binding site in the btMSTN-1b promoter, although this site was not identified in our analysis using the same yet updated software package.

Embryonic expression. A quantitative analysis indicated that both rtMSTN-1a and -1b were similarly expressed at low levels during the early stages of development. However, levels of both transcripts rose substantially after eyeing with rtMSTN-1a mRNA levels always greater than those of rtMSTN-1b (Fig. 7A). Expression of both peaked and dropped immediately before hatching and then continued to rise thereafter. A similar peak in rtMSTN-1a expression also occurred just before yolk sac absorption, although this was not observed with rtMSTN-1b (Fig. 7C). A closer analysis of early developmental stages indicated that neither gene was significantly expressed during gastrulation, although expression of both steadily rose during somitogenesis peaking and then subsiding at its end (Fig. 7B).

Adult tissue expression. Expression of both rtMSTN -1a and -1b was detected in every tissue sampled including brain, pituitary gland, heart, ovary, testis, kidney, stomach, pyloric ceca, intestine, liver, pancreas, peripheral blood leukocytes, erythrocytes, spleen, gills, brachial arches, fins, skin, eyes, white and red skeletal muscle

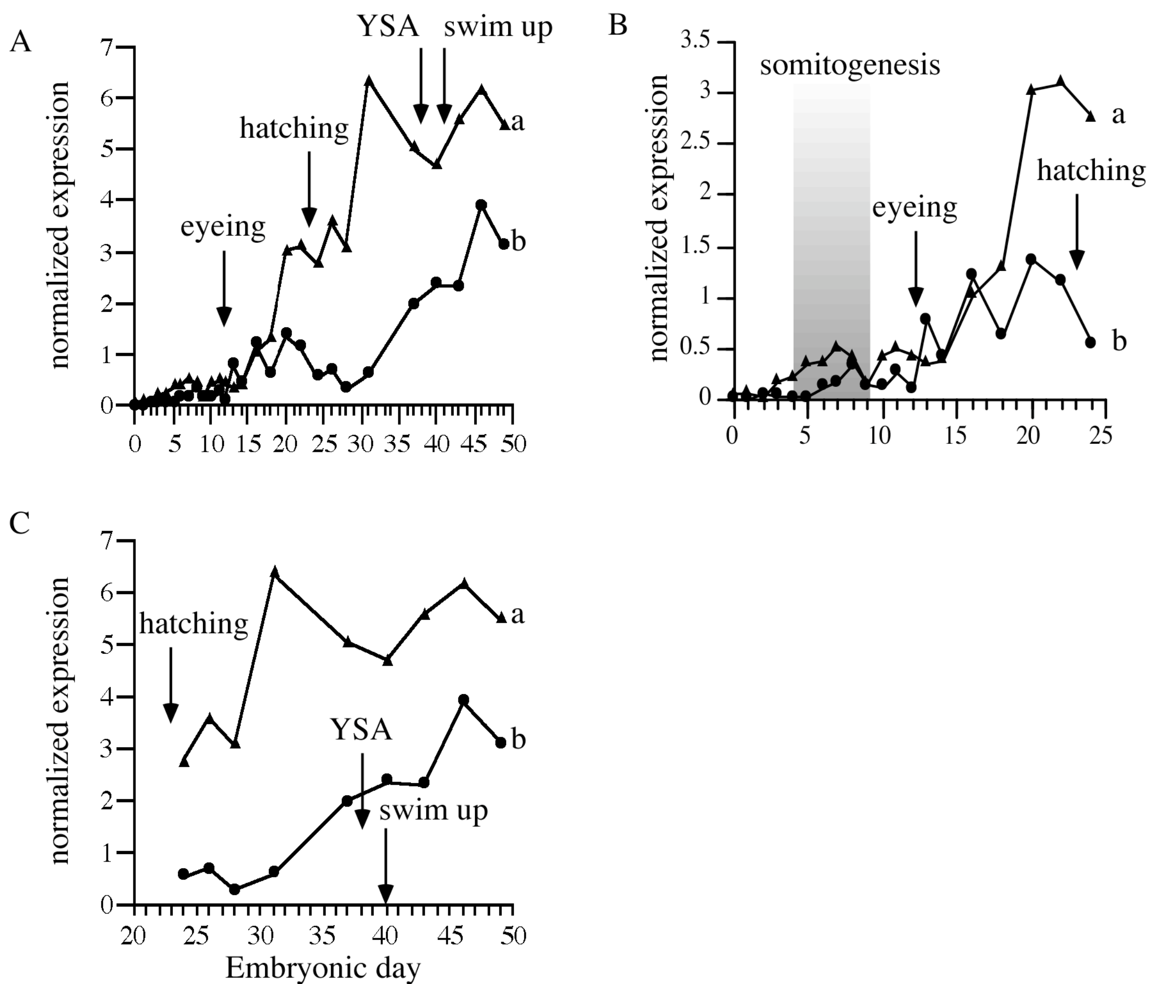

Figure 7. Developmental expression of rtMSTN-1a and -1b. A RNA panel was constructed from 5000 fertilized eggs and developing embryos sampled at the indicated days. Levels of rtMSTN-1a and -1b mRNA were quantified using gene-specific “real-time” RT-PCR assays. Expression levels throughout the entire period from day 0 (unfertilized) to day 49 are shown in panel A, whereas more detailed assessments of early (days 0 to 24) and late (days 24 to 49) stages are shown in panels B and C, respectively. Key developmental events are indicated by the labeled arrows and the gray box (YSA, yolk sac absorption). Each value represents a mean of 3 replicate measurements of a single pooled sample (n = 18 embryos or 9 post-hatched larvae) at each time point. Assay variance was controlled as described in Materials and Methods.

and fat (Fig. 8). This includes some tissues not known to express either myostatin (pituitary, stomach, pyloric ceca, pancreas, leukocytes, erythrocytes, spleen, brachial arches, fins, skin, eyes & fat) and several others previously thought not to express rtMSTN-1b in particular, which has only been identified in brain and skeletal muscle (21). Expression of both genes was highest (note log scale) in brain, testes, eyes, muscle and, surprisingly, spleen. Individual tissue levels of both transcripts were similar in many tissues, but not all. Those of rtMSTN-1a were approximately 50-fold higher than rtMSTN-1b levels in fins and expression of the latter was almost 100-fold greater in leukocytes and gills and 1000-fold greater in heart.

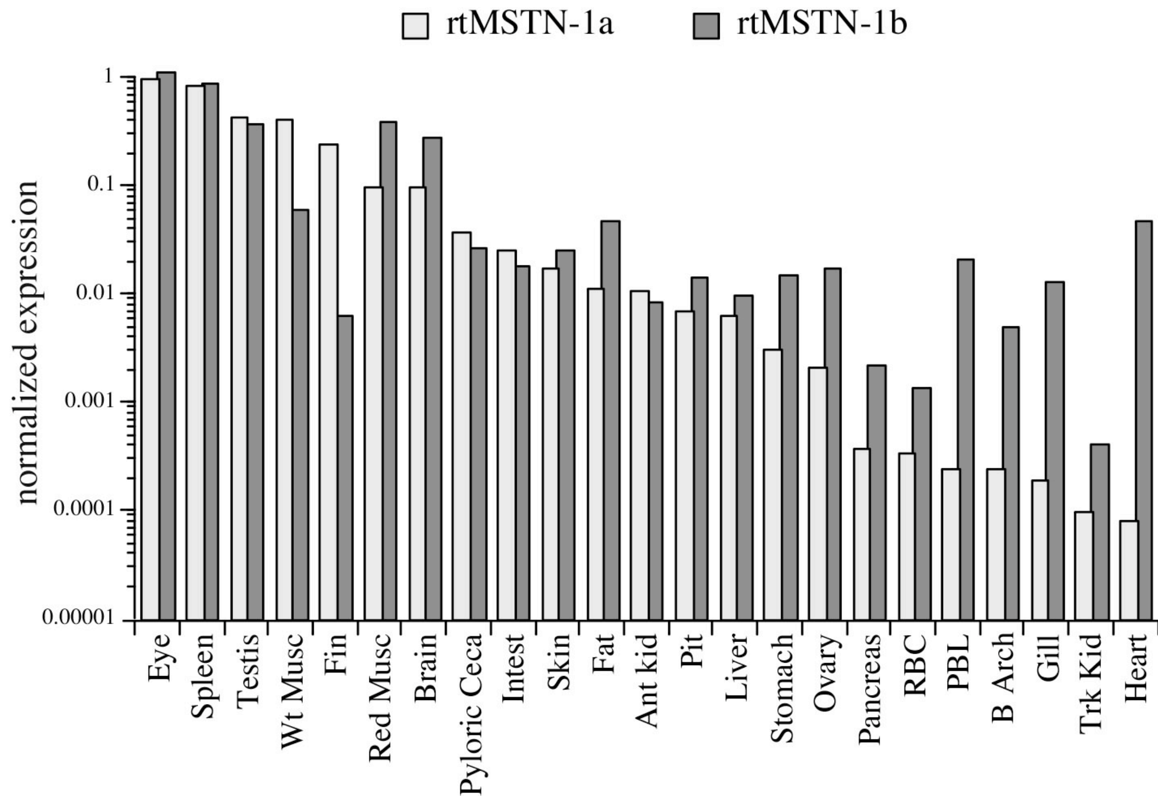

Figure 8. Adult tissue expression of rtMSTN-1a and -1b. Levels of mRNA for both genes were quantified using gene-specific "real time" RT-PCR assays and total RNA removed from the indicated tissues. Assays were performed on pooled tissue samples, but were run in triplicate and were repeated twice. Mean values are shown. (Pit, pituitary gland; Ant kid, anterior/head kidney; Trk, trunk; Intest, whole intestine; PBL, peripheral blood lymphocytes; RBC, red blood cells; B Arch, branchial arch w\o gill fillaments; Wt Musc, white muscle).

Discussion

The genomic organization of both rainbow trout MSTN-1 genes (Figs. 3 & 4) is highly similar to that of other homologues previously characterized in mammals and nearly identical to those in other fish. Indeed, exon boundaries and pre-mRNA splice sites are even conserved (Fig. 5), especially the second, which separates the coding region of the latency-associated peptide (LAP) from the bioactive domain of mature myostatin. The amino acid identity of the mature bioactive domains of most fish and mammalian species is 88% (18) indicating that both primary sequence and gene organization are highly conserved among vertebrates. Although a more comprehensive analysis of genes from divergent fish species and from other vertebrate classes is needed to determine the degree of conservation across taxa, these data suggest that strong selective pressures are likely responsible and were particularly important in preserving fidelity of the third exon. Teleosts commonly possess multiple copies of individual genes. This is a result of an early genome duplication event prior to the teleost radiation, but after the divergence of ray- and lobe-finned fishes (12, 13). A second duplication event specifically within the salmonids (30) gave rise to additional ("a" & "b") myostatin paralogues within each MSTN-1 and -2 sister clade (11), although none of these genes has been identified to date. Nevertheless, the high degree of genomic and sequence conservation shared among all myostatin genes and among fish homologues (Fig. 5) should aid in their isolation and characterization.

Subsequence analysis of the rtMSTN-1a and -1b promoter regions identified several putative *cis* regulatory elements that could contribute to the myogenic process. Some of these elements were also identified in the comparable promoters of brook trout

(*Salvelinus fontinalis*) and zebrafish myostatin genes including multiple MEF2 sites in each (Fig. 6). A putative MyoD site was also identified in the brook trout MSTN-1b promoter by (31), although this particular site was not identified in our analysis using the same, yet updated software. Among these fish genes, however, MyoD sites were identified in the zebrafish MSTN-2 promoter, which also contained far more putative myogenic elements than its counterpart (11). Mammalian myostatin promoters contain E-boxes and other elements critical to the differentiation and maturation of skeletal muscle including both MyoD and MEF2 binding sites. Indeed, both of these sites have been implicated in the regulation of myostatin gene expression in different animal and cellular systems (28, 32, 33). Expression of both rtMSTN-1a and -1b genes increases as somitogenesis progresses and rapidly decreases as it ends (Fig. 7B). This is consistent with increased transactivational activity of these and other myogenic regulatory factors and with myostatin's developmental expression profile in mouse embryos (14). A functional assessment of promoter activity is needed to definitively determine whether these transcription factors regulate either rtMSTN-1a or -1b gene expression in developing skeletal muscle. The ubiquitous nature of MSTN-1 expression in fish, however, suggests that additional elements unrelated to myogenesis altogether may be active as well.

Former attempts to define the developmental and tissue-specific expression profiles of fish MSTN-1 genes revealed a far more diverse expression pattern than which occurs in mammals (20-22, 24, 25, 31, 34-36). These studies were still somewhat limited and mostly qualitative assessments. Rescan *et al.* reported that rtMSTN-1a mRNA levels were substantially higher than those of rtMSTN-1b in most adult tissues and at three

stages of development (eyeing, hatching & free-swimming larvae). The one exception was adult brain where expression appeared equal for both genes. This study also indicated a very limited distribution of rtMSTN-1b expression, which was restricted to brain and skeletal muscle. By contrast, Ostbye *et al.* reported a much wider tissue distribution and apparently higher levels, in some tissues, of Atlantic salmon MSTN-1b expression. Both of these studies used qualitative RT-PCR assays that do not account for primer efficiency and other aspects of non-quantitative PCR amplification and could have easily underestimated rtMSTN-1b expression. By contrast, our use of comprehensive RNA panels and a quantitative "real-time" assay suggest that both rtMSTN-1a and -1b genes are expressed much earlier embryologically, specifically during somitogenesis, and in more adult tissues. Expression of both genes was detected in all tissues sampled and was surprisingly high in spleen and eyes, which possibly indicates novel functional roles for myostatin in the growth and/or differentiation of immune cells and of proliferative cells of the eye (37). Johansen and Overturf also analyzed developmental expression of rtMSTN-1a and -1b using a quantitative RT-PCR assay. Although only a few developmental stages were sampled (eyed, hatched/sac present & swim-up fry), their results also indicate that the expression of both genes rises substantially after eyeing and that rtMSTN-1b mRNA levels are significantly higher than previously reported. Myostatin expression in mammals is first detected within the developing myotome (2, 3), although former attempts to localize myostatin message in fish somites have produced mixed results (11, 26, 38). Nevertheless, our results are the first to identify a temporal expression pattern in fish that is consistent with a functional role during the early stages

of muscle development as levels of both rtMSTN-1a and -1b rise substantially throughout somitogenesis and begin to subside just before this developmental period ends.

The expression patterns described and the subsequence analysis of the different promoters further support a role for both MSTN-1 genes during fish myogenesis, although the ubiquitous expression pattern in different adult tissues suggests that the cytokine's functional role is far more diverse than that in mammals. The presence of multiple fish genes that are differentially expressed throughout development and in adult tissues also suggests that the precise role of a particular gene may vary between tissues. A better understanding of physiological factors that influence the expression of each gene and of the transcriptional machinery involved will therefore help distinguish the potential divergent actions of myostatin in fish and mammals.

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CHAPTER THREE

CHARACTERIZATION OF RAINBOW TROUT MYOSTATIN-2 GENES (rtMSTN-2A & -2B): GENOMIC ORGANIZATION, DIFFERENTIAL EXPRESSION AND PSEUDOGENIZATION

*Data reported in this chapter are taken from accepted publication

Abstract

Myostatin is an extremely potent negative regulator of vertebrate skeletal muscle development. A phylogenetic analysis suggests that salmonids should possess four distinct genes, although only MSTN-1 orthologs have been characterized. Described herein are the rainbow trout (rt) MSTN-2a and -2b genes, subsequence analysis of their promoters and their quantitative expression profiles. Both genes are similarly organized, contain several putative myogenic response elements and are legitimate MSTN-2 orthologs based on Bayesian analyses. However, rtMSTN-2b contains two in-frame stop codons within the first exon and unspliced variants of both transcripts were expressed in a tissue specific manner. Complete splicing of rtMSTN-2a only occurred in brain, where expression is highest, while rtMSTN-2b transcripts were mostly present in unspliced forms. The presence of stop codons in the rtMSTN-2b open reading frame and the expression of mostly unspliced transcripts indicates that this particular homolog is a pseudogene. These results confirm our previous phylogenetic analysis and suggest that all salmonids likely possess four distinct myostatin genes. The tissue-specific expression and differential processing of both rtMSTN-2 transcripts as well the pseudogenization of rtMSTN-2b may reflect compensatory and adaptive responses to tetraploidization and may help limit rtMSTN-2a's influences primarily to neural tissue.

Introduction

Myostatin is a member of the TGF-β superfamily and a potent negative regulator of skeletal muscle growth in mammals (1). Myostatin null phenotypes in rodents and cattle are characterized by extreme gains in muscle mass commonly referred to as "double muscling" (2-4). Indeed, extraordinary musculature has also been reported in a child with a 5' splice site mutation in the myostatin gene's first intron (5), indicating that myostatin's function is widely conserved among mammals. Unlike mammals where myostatin expression is restricted primarily to skeletal muscle (4, 6) and to a much lesser extent in heart (7) and mammary glands (8), it is widely expressed in many different fish tissues (6, 9-13). Nevertheless, tissue-specific functions of myostatin have yet to be described and the existence of multiple myostatin genes (9, 12, 14-16) further complicates our understanding of myostatin biology in fish. Many salmonid species are highly prized for their commercial importance and rainbow trout in particular are an emerging and alternative animal model for basic and biomedical research. Thus, improving muscle growth by controlling myostatin expression or bioactivity in these species could significantly impact aquaculture and academic communities alike.

An early genome duplication in the bony fish, before the teleost radiation, resulted in multiple copies of many genes (17, 18). A second duplication event occurred in the salmonids approximately 50 to 100 million years ago (19), although many paralogs may have been lost. Some of these genes have been characterized in rainbow trout and include homologs for Wilms' tumor gene (19), glutamate synthetase (20), the ID (Inhibitors of DNA binding/differentiation) genes (21) and major histocompatibility genes (22). Our previous phylogenetic analysis of the myostatin gene family (9) identified two distinct

fish clades, MSTN-1 and MSTN-2, and determined that all of the salmonid myostatin genes previously identified were actually MSTN-1 orthologs. These genes were therefore reclassified as either MSTN-1a or MSTN-1b. This study also predicted the existence of two additional myostatin paralogs, namely MSTN-2a and -2b, in most if not all salmonid species, although none of these genes have been identified to date.

Reported herein is the discovery of MSTN-2a and -2b genes in the rainbow trout (rt), the genomic organization of both alleles, including their respective promoter regions, and the quantitative assessment of rtMSTN-2a gene expression using comprehensive RNA panels derived from multiple developmental stages and various adult tissues. These results are the first to confirm the existence of four myostatin genes in any salmonid species and reveal a strong conservation of genomic organization among all vertebrate homologs. In addition, we identified rtMSTN-2b as a pseudogene as well as unspliced transcripts of both rtMTSN-2a and -2b, which has never been described in any vertebrate.

Materials and Methods

Animals. Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Washington State University hatchery, a Center of Reproductive Biology core facility. Fish were reared and used according to protocols pre-approved by the Institutional Animal Care and Use Committee (IACUC). The RNA panels were generated from embryos and adult tissues of rainbow trout obtained from the National Center for Cool and Cold Water Aquaculture according to the guidelines approved by a separate IACUC.

Cloning and characterizing rtMSTN-2a and -2b genes. Genomic DNA (gDNA) was isolated from fin clips (~50 mg) after incubating in 3 ml of lysis buffer (30mM Tris, 8M Urea, 4% w/v Chaps, pH 8.0) containing 20 mg/ml proteinase K at 60°C. Three consecutive phenol:chloroform:isoamyl alcohol extractions were then performed and gDNA quality was verified on a 1% agarose gel.

Degenerate primers (DegP-1 and DegP-2, Table 2) were designed to recognize conserved regions within the C-terminal domain of different fish MSTN-2 genes (shi drum, seabream, fugu & zebrafish) and were used to amplify custom rainbow trout genomic libraries. Briefly, gDNA was digested with the blunt end restriction enzymes Dra I, EcoR V, Pvu II, and Stu I and subsequently ligated to adaptor linkers according to the kit manufacturer's protocol (BD GenomeWalker™ Universal kit, BD Biosciences, www.bdbiosciences.com). Nested PCR was performed using degenerate primers and the adaptor primers (Table 2) with the Advantage 2 PCR kit (BD Biosciences). Cycling parameters were as follows and were used as default unless otherwise specified: an initial denaturation at 94°C for 1 min, 32 cycles of 94°C for 30 s, 65°C for 3 min and a final extension period of 4 min at 67°C. The PCR products were sub-cloned into the Topo TA

Table 2: Primer sequences and annealing temperatures for rtMSTN-2a and -2b

Primer name	Sequence (5'- 3')	An. Temp. (°C)
DegP-1	TCN CCN GAC CAC TAR TTG GGC	65
DegP-2	CGY TTG GGV GCR ATK ATC CAG TCC CA	65
β-actin F	TCT GGC CGT ACC ACC GGT AT	60
β-actin R	CGT GTT GGC GTA CAG GTC CTT	60
PS-I F/PS-II F	TTG TCA CTG TGT TAT ATA GGC CTG GAA	60
PS-I R	GCG GGA GAT TTG CAA GAA GAC AGT TG	60
PS-II R	TAT GCG CGA GTT CCC TCA GCC	60
PS-III F	AAC CTG CGG CCG GCT GAG GGA A	60
PS-III R	GGT TAT CTT CCC ATA GAT GAT CTG C	60
Adaptor primer 1	GTA CTA CGA CTC ACT ATA GGG C	65
Adaptor primer 2	ACT ATA GGG CAC GCG TGG T	65
QMSTN-2a F	AAT CTC CCC GCA TAA AAG CAA CCA C	66
QMSTN-2a R	CAC CAG AAG CCA CAT CGA TCT T	66
18s F	TGC GGC TTA ATT TGA CTC AAC A	60
18s R	CAA CTA AGA ACG GCC ATG CA	60
3'UTR2a	CTT AGT GAG GAC ATG AAA CTG CCC ATA	60
MSTN-1a F	CTT CAC ATA TGC CAA TAC ATA TTA	60
MSTN-1a R	GCA ACC ATG AAA CTG AGA TAA A	60
MSTN-1b F	TTC ACG CAA ATA CGT ATT CAC	60
MSTN-1b R	GAT AAA TTA GAA CCT GCA TCA GAT TC	60

N= A/T/G/C; R=A/G; Y=C/T; V=A/C/T & K=G/T

vector (Invitrogen, www.invitrogen.com) and sequenced in the university's genomic core facility. This resulted in partial sequences of two new myostatin genes. Complete genomic clones were isolated using the gene-specific primers based on the partial sequences previously cloned. Whole gene sequences were then isolated by PCR using a high fidelity polymerase (Pfu) (Stratagene, www.stategene.com) and resequenced.

The 3' untranslated region of rtMSTN-2a was isolated using a 3' RACE (rapid amplification of cDNA ends) kit (Invitrogen). Total RNA from juvenile skeletal muscle was extracted using Trizol and reverse transcribed using superscript reverse transcriptase, both according to the manufacturer's protocols (Invitrogen). cDNA was then amplified by PCR using a gene specific forward primer (PS-I F, Table 2) and the universal adaptor primers (UAP) provided in the kit. The PCR conditions were as follows: an initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 2 min and a final extension at 72°C for 3 min. Nested PCR was subsequently performed with the 3'UTR2a primer and the abridged universal adaptor primer (AUAP) provided in the kit. The resulting PCR product was sub-cloned and sequenced as described.

Computational analysis. Putative cDNA sequences for each novel myostatin gene were constructed in silico from the predicted open reading frames using Genescan (www.genes.mit.edu/GENESCAN.html) and were confirmed by sequencing RT-PCR amplicons. The 2.4 and 1.5 kb regions upstream of the rtMSTN-2a and rtMSTN-2b coding regions, respectively, were also cloned and sequenced using similar protocols. Putative regulatory elements in the promoters were identified by subsequence analysis using Matinspector software (Genomatrix Inc, www.genomatix.de), which searches for

known consensus sequences of cis regulatory elements. Alignments of the putative rtMSTN proteins were performed using Vector NTI Align X (Invitrogen).

Phylogenetic analyses was performed using Bayesian inference as implemented in MrBayes v.3.1 (23) and the newly discovered myostatin genes, rtMSTN-2a and -2b, along with previously characterized myostatin genes from the following species: human (GenBank accession #AF019627), chicken (AY448007), seabream (AF258448 & AY046314), shi drum (AF316881 & AY059386), fugu (AY445322 & AY445321), zebrafish (AY258034 & AY687474), striped bass (AF290910), white perch (AF290910), white bass (AF197194), tilapia (AF197193), king mackerel (AF317667), little tunny (AF317666), Atlantic salmon (AJ344158 & AJ297267), brook trout (AF313912 & AF247650), rainbow trout (AF273035 & AF273036), coho salmon (AY434465 & AF394687), blue catfish (AY540992) and channel catfish (AF396747). Amino acid alignments were created using Clustal X 1.83 (24) using default parameters. Bayesian analyses used a mixed amino acid model prior, 10,000,000 generations performed with four chains (Markov Chain Monte Carlo) sampling every 1000 generations, and resulted in a distribution of 10,000 trees. In order to test for the occurrence of stationarity, convergence and mixing within the 10,000,000 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 (25). Trees sampled during the “burn-in” of the chain, the first 2,000,000 generations, were discarded to assure that only trees sampled after stationarity was established were

included and the remaining trees were loaded into PAUP*4.0 (26), Consensus trees were then created to display branches with posterior probabilities greater than 50%.

Qualitative analysis of rtMSTN-2a and -2b expression in adult tissues. Brain, skin, gill filaments, gill arches, heart, kidney, spleen, intestine, stomach, liver, eyes and red and white skeletal muscle were collected from 2 year old male and female rainbow trout, snap frozen in liquid nitrogen and stored at -80°C. Samples were powdered and total RNA was extracted using TRI-Reagent (Invitrogen, www.invitrogen.com) according to the manufacture's protocol and treated with DNase (DNase RQ-1, Promega, www.promega.com) to remove contaminating gDNA. Samples were then re-extracted with TRI-Reagent and RNA quality was assessed by agarose gel electrophoresis. Assays were also performed using the RNA panel described below. Three different primer sets (PS), each spanning an intron, were used to amplify specifically rtMSTN-2a and -2b transcripts (Fig. 13A). The PS-I R primer (Table 2) is specific for rtMSTN-2a and primers PS-II R and PS-III F are specific for rtMSTN-2b gene transcripts. However, the other primers cross-hybridize to both rtMSTN-2 transcripts. Nevertheless, primer specificity was confirmed by sequencing amplicons. The PCR conditions were as follows: an initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 90 s and a final extension at 72°C for 3 min. After the initial 30 cycles, an additional 30 cycles of PCR was performed using 5 μL of the PCR product as template. To confirm that the unspliced amplicons were not from gDNA contamination, equal quantities of RNA from gills were treated with or without RNase A following the manufacturer's protocol (Fermentas, www.fermentas.com) and re-extracted with TRI-Reagent. cDNA was then synthesized and amplified with primers specific to

rtMSTN-2a (PS-I), rtMSTN-2b (PS-II/PS-III) or both rtMSTN-2 genes (PS-I F and PS-III R). The PCR protocol included an initial denaturation at 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min and a final extension at 72°C for 3 min. An aliquot of 5 μL was then re-amplified using the same protocol. Qualitative analysis of rtMSTN-1a, -1b and -2a was performed in different sections of the brain with gene specific primers (Table 2). The PCR protocol included an initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 90 s and a final extension at 72°C for 2 min.

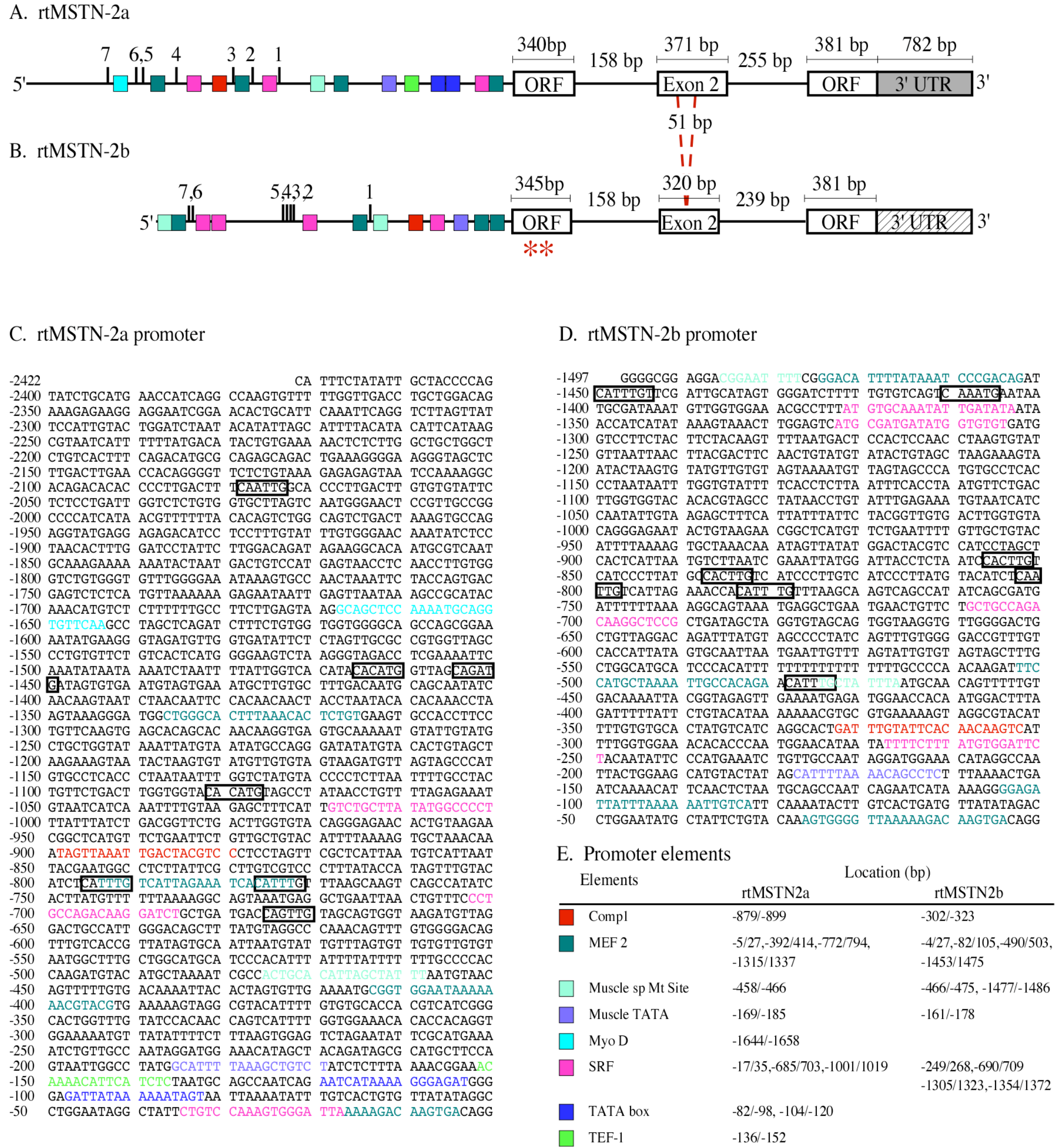
Quantitative analysis of gene expression in developing embryos and in adult tissues. An RNA panel was generated from 5000 pooled eggs from multiple females (Trout Lodge, October 2004) that were fertilized by milt from two males. Following fertilization, eggs were incubated at 13°C throughout embryonic development. Unfertilized eggs (Day 0) and developing embryos were collected as whole egg samples daily for the first 14 days, every other day until hatch (Day 24) and every third day thereafter. Each sample pool contained 18 eggs/embryos or 9 post-hatched larvae that were pooled and several samples were collected at each time point. Frozen samples were first powdered using a liquid nitrogen cooled Bessman Tissue Pulverizer (Spectrum Laboratories, www.spectropor.com) and total RNA was extracted using TRI-Reagent with the high salt solution modification to remove the excess glycosylated proteins and treated with DNAse as described. Samples were then re-extracted with TRI-Reagent and RNA quality was assessed by agarose gel electrophoresis. The adult tissues were collected and mRNA was extracted as previously described.

Total RNA (2 μg) was reverse transcribed with 1 μg of oligo-dT primers (Promega) and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT, Promega). Subsequent real time RT-PCR assays were conducted using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, www.appliedbiosystems.com) and gene specific primers (QMSTN-2a F and QMSTN-2b R, Table 1). For each sample, 1 μl of cDNA was combined with 5 μl of 2x SYBR® Green PCR master mix (Applied Biosystems). For each reaction, 6 μl of this mixture was added to 9 μl of the primer mix containing 500 μM of each primer. The reactions were performed as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycling reaction was followed by a dissociation curve to verify amplification of a single product and amplicons were also verified by DNA sequencing. The relative standard curve method was employed to quantify gene expression. A serial dilution of a mixed tissue cDNA was used to construct a standard curve for each assay plate. The standard curve was constructed by plotting the threshold cycle (CT) versus the natural log of input RNA (ng). This curve was then used to calculate the abundance of each transcript in each sample. Myostatin values were then normalized to those of 18s to control for differences in RNA and cDNA loading. Each sample was run in triplicate on a single plate, each plate was run in duplicate and data was presented as normalized gene expression values.

Results

Genomic organization of rtMSTN -2a and -2b gene. Complete genomic clones for both rtMSTN-2a and -2b genes were isolated and sequenced (Figs. 9A & 9B). This includes approximately 2.4 kb upstream from the translational start site for rtMSTN-2a and 1.5 kb for rtMSTN-2b. The annotated gene and promoter sequences of rtMSTN-2a and -2b were deposited into GenBank and assigned the accession numbers DQ138301 and DQ177320, respectively. The genomic organization of both genes was similar to all myostatin genes characterized to date (4, 9, 13, 27-30). The three exons of rtMSTN-2a are 340, 371 and 1063 bp in size, respectively, and separated by 158 and 255 bp introns (Fig. 9A). Similarly, the rtMSTN-2b is also organized into three exons with 346, 320 and 381 bp (partial) respectively, and are separated by 158 and 239 bp introns (Fig. 9A). The 3' UTR of rtMSTN-2a was also cloned by 3' RACE using RNA and was determined to be 782 bp, which is considerably shorter than the 3'UTRs of the rtMSTN-1 genes (13). A partial 3'UTR of rtMSTN-2b was also cloned and found to be indistinguishable from that of rtMSTN-2a. Therefore, the exact size of rtMSTN-2b's 3'UTR could not be determined.

In silico analysis of rtMSTN-2a and -2b promoters. Subsequence analysis of the upstream 2.4 and 1.5 kb sequence for rtMSTN-2a and -2b, respectively, identified several putative muscle specific transcriptional factor binding sites or cis regulatory elements. These regulatory elements include COMP1 (cooperates with myogenic proteins), MEF2 (myocyte enhancer factor), SRF (serum response factor), TEF-1 (transcriptional enhancer factor 1), MyoD (myogenic differentiation) and Muscle sp Mt site (Fig. 9). Each promoter also contained a muscle specific TATA box and several putative E-boxes. Most



Elements	Location (bp) rtMSTN2a	Location (bp) rtMSTN2b
Comp1	-879/-899	-302/-323
MEF 2	-5/27,-392/414,-772/794, -1315/1337	-4/27,-82/105,-490/503, -1453/1475
Muscle sp Mt Site	-458/-466	-466/-475, -1477/-1486
Muscle TATA	-169/-185	-161/-178
Myo D	-1644/-1658	
SRF	-17/35,-685/703,-1001/1019	-249/268,-690/709 -1305/1323,-1354/1372
TATA box	-82/-98, -104/-120	
TEF-1	-136/-152	

Figure 9. Genomic structure and organization of the rtMSTN-2a and -2b genes. (A & B) Maps of rtMSTN-2a and -2b genes and putative myogenic *cis* regulatory elements within the promoter regions starting from the initiator. Exons are boxed with the open reading frame (ORF) in white, the untranslated region (UTR) of rtMSTN-2a in gray and the putative 3'UTR of rtMSTN-2b hatched. Each individual *cis* element is placed relative to its position within the promoter and is color-coded as indicated in E. Putative E-boxes are numbered. The 51 bp cassette missing from the second exon of rtMSTN-2b is indicated by red lines. The asterisks below the first exon in B indicate in-frame stop codons. (C & D) Sequence of the promoter regions for rtMSTN-2a and -2b, respectively, with color-coded *cis* elements. Boxed are consensus sequences (CAN(T/A)TG) for E-boxes. Nucleotide positions correspond to the start codon. (E) Key to the color-coded promoter elements in A-D and their corresponding nucleotide positions

importantly, a MyoD binding site and several MEF2 sites were identified, although the MyoD site was only found in the rtMSTN-2a promoter (–1658 and –1644). The presence of these putative sites is particularly intriguing as both regulate myostatin expression in different mammalian systems (31-33).

Sequence analysis. The coding sequences of both genes are similar (Fig. 10 & 11), although rtMSTN-2b's is smaller and lacks 51 bp from the second exon (Fig. 1), which corresponds to 17 amino acids present in the other rtMSTN proteins (Fig. 3). The putative rtMSTN-2 proteins possess all of the elements conserved in other myostatin proteins (Fig. 10). However, two in-frame stop codons were detected within the rtMSTN-2b ORF (Fig. 10B), which were coded by the first exon (Fig. 9B). A multiple sequence alignment of the four rtMSTN amino acid sequences, including the stop codons in rtMSTN-2b, revealed several unique regions including a 9 residue epitope that is missing from the rtMSTN-2 sequences (Fig. 11). Individual comparisons indicate that rtMSTN-2a and -2b are 81% identical overall and 94% in the conserved C-terminal domain while rtMSTN-2a is 66% and 91%, respectively, to the rtMSTN-1 proteins. However, the C-terminal domains of the rtMSTN-2 sequences contained two notable differences: a methionine substitution for lysine 354 (rtMSTN-1a) and a histidine substitution for arginine 369 (Fig. 11). This particular arginine is replaced by either histidine (Seabream, Shi drum and Fugu) or leucine (Zebrafish) in other MSTN-2 proteins.

Phylogenetic analyses and comparative exon mapping. The current Bayesian inference analysis of different fish myostatin sequences produced only minor differences from the previous maximum likelihood analysis of the entire myostatin gene family (9). Multiple independent analyses converged on the same posterior probability values for

A rtMSTN-2a

```
     M  Q  F  M  L  Y  L  T  L  L  G  V  L  S  T  T  M  G  M  N
1    ATGCAATTTATGCTTTACCTGACACTCTTAGGTGTACTTAGCACCACCATGGGGATGAAT
     K  T  T  R  R  Q  A  N  V  T  E  E  G  E  V  Q  Q  C  S  N
61   AAAACCACGAGGCGCCAAGCCAACGTAACAGAAGAGGGAGAAGTACAGCAATGCTCAAAC
     C  E  F  R  E  Q  S  R  L  M  R  L  H  N  I  R  S  Q  I  L
121  TGCGAGTTCAGAGAACAGAGCAGACTAATGAGACTCCACAACATCAGATCCCAAATTCTC
     S  I  L  R  L  E  Q  A  P  N  I  S  R  E  M  I  R  Q  L  L
181  AGCATTCTGAGGCTGGAGCAAGCTCCAAACATCAGCCGAGAAATGATCAGGCAGCTCTTA
     P  K  A  P  P  L  T  Q  L  I  D  Q  Y  E  H  R  V  E  D  E
241  CCGAAAGCGCCTCCCTTGACACAACTTATAGACCAGTATGAGCATCGTGTGGAGGATGAG
     E  R  A  T  T  E  T  I  I  T  M  A  K  P  G  P  M  S  Q  Q
301  GAGCGTGCCACCACTGAAACGATCATAACCATGGCAAAACCTGGACCAATGTCACAACAA
     D  G  I  P  S  C  C  F  F  N  L  S  P  K  I  R  P  N  N  I
361  GACGGAATACCGTCTTGTTGTTTCTTCAATCTCAGTCCGAAGATTCGACCTAACAACATT
     L  H  A  Q  L  W  V  H  L  R  P  A  D  T  V  T  T  V  F  L
421  TTACATGCACAACTTTGGGTGCACCTGCGACCAGCTGACACAGTCACAACTGTCTTCTTG
     Q  I  S  R  I  K  A  T  T  E  G  N  S  R  I  R  I  L  S  L
481  CAAATCTCCCGCATAAAAGCAACCACTGAGGGAAACTCACGCATACGGATCCTCTCCCTG
     K  I  D  V  A  S  G  A  S  S  W  Q  S  V  D  I  N  Q  L  L
541  AAGATCGATGTGGCTTCTGGTGCAAGCTCTTGGCAAAGTGTAGACATCAATCAATTACTC
     K  T  W  L  R  Q  P  E  T  H  Y  G  L  E  I  K  A  Y  D  S
601  AAAACCTGGCTTCGTCAGCCAGAAACTCATTATGGTCTCGAGATCAAAGCTTATGATTCG
     K  G  Q  D  L  A  V  T  V  A  E  L  G  E  E  G  L  Q  P  F
661  AAAGGGCAGGACTTGGCTGTCACGGTAGCTGAGCTGGGAGAAGAGGGACTGCAACCCTTC
     M  E  V  K  I  L  E  S  L  K [R  S  R  R] A  S  G  L  D (C)
721  ATGGAGGTGAAGATATTGGAGAGCCTAAAACGTTCCCGGAGGGCCTCAGGCCTGGACTGT
     D  E  E  S  S  E  T  L (C)(C) R  Y  P  L  T  V  D  F  E  A
781  GATGAGGAGTCTTCAGAGACGCTGTGTTGCCGCTACCCCCTCACCGTTGACTTTGAGGCC
     F  G  W  D  W  I  I  A  P  K  R  Y  K  A  N  Y (C) S  G  E
841  TTTGGGTGGGACTGGATCATTGCCCCCAAGCGCTACAAGGCCAACTACTGTTCTGGAGAG
    (C) E  Y  M  H  L  Q  K  Y  P  H  T  H  L  V  N  K  A  N  P
901  TGTGAGTACATGCACCTCCAGAAGTACCCCCACACTCACCTGGTGAACAAGGCTAACCCC
     R  G  T  T  G  S (C)(C) T  P  T  K  M  S  P  I  N  M  L  Y
961  CGGGGTACCACAGGGTCCTGTTGTACTCCGACTAAGATGTCCCCCATCAACATGCTCTAC
     F  N  R  M  E  Q  I  I  Y  G  K  I  P  S  M  V  V  D  H (C)
1021 TTCAACCGCATGGAGCAGATCATTTATGGGAAGATACCATCTATGGTGGTGGACCACTGT
     G (C) G  *
1081 GGCTGCTCCTGA
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B rtMSTN-2b

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     M  Q  C  M  L  Y  L  K  L  I  G  A  L  G  T  T  M  G  V  N
1    ATGCAATGTATGCTTTACCTTAAACTCATAGGTGCACTTGGCACCACCATGGGGGTGAAT
     E  T  T  R  H  Q  T  N  V  T  T  V  D  G  E  V  Q  Q  C  S
61   GAAACCACAAGGCACCAAACCAACGTAACAACAGTGGATGGAGAAGTACAGCAATGCTCA
     T  C  E  F  R  E  Q  N  R  L  M  R  L  A  T  S  D  P  K  F
121  ACCTGCGAGTTCAGAGAACAGAACAGACTAATGAGACTCGCAACATCAGATCCCAAATTC
     S  A  F [*] G  S  S  R  L  Q  T  N  I  S  R [*] M  I  R  Q
181  TCAGCATTCTGAGGCTCGAGCAGGCTCCAAACAAACATCAGCAGATAAATGATCAGGCAG
     L  L  P  K  A  P  I  L  S  Q  F  T  D  Q  Y  E  H  R  V  E
241  CTCTTACCAAAAGCTCCCATCCTTTCACAATTTACAGACCAGTATGAGCATCGTGTGGAG
     G  E  E  R  A  P  T  E  T  I  I  S  M  A  T  P  G  P  M  S
301  GGTGAGGAGCGTGCCCCAACTGAAACGATCATATCCATGGCAACACCTGGACCAATGTCA
     Q  Q  D  G  I  P  S  C  C  F  F  N  L  S  P  K  I  R  P  N
361  CAACAAGACGGAATACCATCTTGTTGTTTCTTCAATCTCAGTCCGAAGATTCGACCTAAC
     N  I  L  H  A  Q  L  W  V  N  L  R  P  A  E  G  N  S  R  I
421  AACATTTTACATGCACAACTTTGGGTGAACCTGCGGCCGGCTGAGGGAAACTCGCGCATA
     R  I  L  S  L  K  I  D  V  A  S  G  A  S  S  W  Q  S  V  D
481  CGGATCCTCTCCCTGAAAATCGATGTGGCTTCTGGTGCAAGCTCTTGGCAAAGTGTAGAC
     I  N  Q  L  L  K  T  W  L  H  Q  P  E  T  H  Y  G  L  E  I
541  ATAAATCAATTACTCAAAACCTGGCTTCATCAGCCCGAAACTCATTATGGTCTCGAGATC
     K  A  Y  D  S  K  R  Q  D  L  A  V  T  V  A  E  L  G  E  E
601  AAAGCTTATGATTCGAAAAGGCAGGATTTAGCTGTCACAGTAGCAGAGTTGGGAGAAGAG
      G  L  Q  P  F  M  E  V  K  I  S  E  N  L  K [R  S  R  R] D
661  GGACTGCAACCCTTCATGGAGGTGAAGATATCGGAGAACCTAAAGCGTTCCCGGAGGGAC
     S  A  L  D (C) D  E  E  S  S  E  T  L (C)(C) R  Y  P  L  T
721  TCAGCCCTGGACTGTGATGAGGAGTCCTCAGAGACGCTGTGTTGCCGCTACCCCCTCACC
     V  D  F  E  A  F  G  W  D  W  I  I  A  P  K  R  Y  K  A  N
781  GTCGACTTTGAGGCCTTTGGCTGGGACTGGATCATTGCCCCCAAACGCTACAAGGCCAAC
     Y (C) S  G  E (C) E  Y  M  H  L  Q  K  Y  P  H  T  H  L  V
841  TACTGTTCTGGAGAGTGTGAGTACATGCACCTCCAGAAGTACCCCCACACTCACCTGGTG
     N  K  A  S  A  R  G  T  T  G  P (C)(C) T  P  T  K  M  S  S
901  AACAAGGCTAGCGCCCGGGGTACCACAGGGCCCTGTTGTACTCCAACTAAGATGTCCTCC
     I  N  M  L  Y  F  N  R  M  E  Q  I  I  Y  G  K  I  P  S  M
961  ATCAACATGCTCTACTTCAACCGCATGGAGCAGATCATCTATGGGAAGATACCATCTATG
     V  V  D  H (C) G (C) S  *
1021 GTGGTGGACCACTGTGGCTGCTCCTGA
```

Figure 10. Annotated cDNA sequences of rtMSTN-2a and -2b. Nucleotide positions are numbered, conserved cysteine residues in the C-terminal regions are circled and the proteolytic processing sites are boxed. The in-frame stop codons in rtMSTN-2b are highlighted by gray boxes.

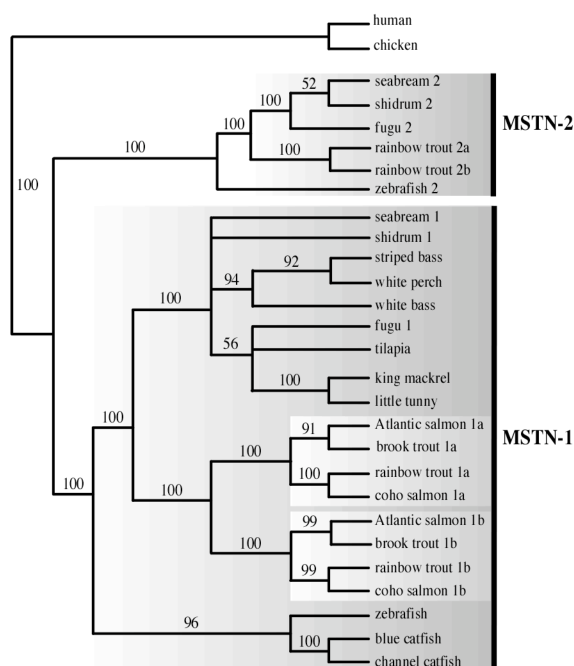
```
MSTN-1a 1     MHLTQVLIYL SFMVAFGPVG LGDQTAHHQP PATD...DGE QCSTCEVRQQ IKNMRLHAIK SQILSK.LRL
MSTN-1b 1     MNLMQVLIYL SFMVAFGPMG LGDQTAHHQS PATD...DGE QCSTCEVRQQ IKNMRLHAIK SQILSK.LRL
MSTN-2a 1     M...QFMLYL TLLGVLSTTM GMNKTTRRQA NVTE.EGEVQ QCSNCEFREQ SRLMRLHNIR SQILSI.LRL
MSTN-2b 1     M...QCMLYL KLIGALGTTM GVNETTRHQT NVTTVDGEVQ QCSTCEFREQ NRLMRLATSD PKFSAF*GSS
              M   Q   YL                 T   Q     T          QCS CE R Q     MRL

MSTN-1a 67    KQAPNISRDV VKQLLPKAPP LQQLLDQYDV LGDDNKDGLM EEDDEHAITE TIMTMATEPE SIVQVDRKPK
MSTN-1b 67    KHAPNISRDV VKQLLPKAPP LQKLLDQYDV LGDDNKDGLM EEDDEHAITE TIMTMATEPE SIVQVDGKPK
MSTN-2a 66    EQAPNISREM IRQLLPKAPP LTQLIDQYEH RVED...... ...EERATTE TIITMAK.PG PMSQQDGIPS
MSTN-2b 68    RLQTNISR*M IRQLLPKAPI LSQFTDQYEH RVEG...... ...EERAPTE TIISMAT.PG PMSQQDGIPS
                  NISR      QLLPKAP  L     DQY                     E A TE TI   MA  P      Q D  P

MSTN-1a 137   CCLFSFSSKI QVNRIVHAQL WVHLLPADEV TTVFLQISRL MPVTDGGRHI GIRSLKIDVN AGVSSWQSID
MSTN-1b 137   CCFFSFNSKI QANRIVRAQL WVHLQPPDEV TTVFLQISRL IPVTDGGRNI QIRSLKIDVN AGVSSWQSID
MSTN-2a 126   CCFFNLSPKI RPNNILHAQL WVHLRPADTV TTVFLQISRI KATTEGNSRI RILSLKIDVA SGASSWQSVD
MSTN-2b 128   CCFFNLSPKI RPNNILHAQL WVNLRPAE.. .......... .....GNSRI RILSLKIDVA SGASSWQSVD
              CC F    KI   N I  AQL WV L P                      G   I    SLKIDV     SSWQS D

MSTN-1a 207   VKQVLSVWLR QPETNWGIEI NAFDSKGNDL AVTSAEAG.E GLQPFMEVTI SEGPKRFRRD SGLDCDENSP
MSTN-1b 207   VKQVLSVWLR QPDTNWGIEI NALDSKGNDL AVTSAEAG.E GLQPFMEVKI SEGPKRSRRD SGLDCDENSP
MSTN-2a 196   INQLLKTWLR QPETHYGLEI KAYDSKGQDL AVTVAELGEE GLQPFMEVKI LESLKRSRRA SGLDCDEESS
MSTN-2b 181   INQLLKTWLH QPETHYGLEI KAYDSKRQDL AVTVAELGEE GLQPFMEVKI SENLKRSRRD SALDCDEESS
                Q    WL  QP T  G EI    DSK  DL AVT AE G E GLQPFMEV I  E  KR RR    LDCDE S

MSTN-1a 276   ESRCCRYPLT VDFEDFGWDW IIAPKRYKAN YCSGECEYMH LQKYPHTHLV NKANPRGTAG PCCTPTKMSP
MSTN-1b 276   ESRCCRYPLT VDFEDFGWDW IIAPKRYKAN YCSGECEYMH LQKYPHTHLV NKANPRGTAG PCCTPTKMSP
MSTN-2a 266   ETLCCRYPLT VDFEAFGWDW IIAPKRYKAN YCSGECEYMH LQKYPHTHLV NKANPRGTTG SCCTPTKMSP
MSTN-2b 251   ETLCCRYPLT VDFEAFGWDW IIAPKRYKAN YCSGECEYMH LQKYPHTHLV NKASARGTTG PCCTPTKMSS
              E  CCRYPLT VDFE FGWDW IIAPKRYKAN YCSGECEYMH LQKYPHTHLV NKAN RGT G  CCTPTKMSP

MSTN-1a 346   INMLYFNRKE QIIYGKIPSM VVDRCGCS*
MSTN-1b 346   INMLYFNRKE QIIYGKIPSM VVDRCGCS*
MSTN-2a 336   INMLYFNRME QIIYGKIPSM VVDHCGCS*
MSTN-2b 321   INMLYFNRME QIIYGKIPSM VVDHCGCS*
              INMLYFNR E QIIYGKIPSM VVD CGCS
```

Figure 11. Alignment of rtMSTN-1a, -1b, -2a and -2b amino acid sequences. Alignments were performed using VECTOR NTI Align X. Identities between proteins are shown in the consensus while gaps (.) and stop codons (*) are indicated in the individual sequences. The in-frame stop codons in rtMSTN-2b are also circled. The conserved proteolytic RXXR site is boxed and the residues highlighted in gray indicate notable differences in the conserved carboxy-terminal bioactive domain.

branches, suggesting that convergence and mixing are occurring. Most of the major internal branches are well supported, and the consensus tree clearly supports the placement of the newly discovered proteins within the MSTN-2 clade. This not only confirms the identity of rtMSTN-2a and -2b as legitimate MSTN-2 orthologs, but also validates our previous phylogenetic analysis and suggests that other salmonid MSTN-2 genes likely exist as well. Comparative mapping of coding regions of different myostatin genes reveals a similar organization in all species. Codons flanking the first and the second exon are highly conserved among the fishes despite minor differences in rtMSTN-2a and -2b, while the codons flanking the second and third exon are highly conserved among all vertebrate species (Fig. 12B). The previously identified consensus sequence (13) for the codons flanking the first and second exon, MAT(E/K) | PXXI (X= any amino acid), was slightly different in the rtMSTN-2 proteins [MAT(E/K) vs. MA(T/K)] whereas the consensus between the second and third exons, (G/E)(E/D)GL | XPFϕ (ϕ + hydrophobic amino acid), was found in both rtMSTN-2 coding frames. This further indicates a high level of conservation of myostatin genes as reflected in both gene sequences and organization.

Qualitative analysis of rtMSTN-2a and -2b expression. Amplification of rtMSTN-2 transcripts in different adult tissues was performed using three primer sets. A schematic of hybridization sites and amplicons sizes are shown in Figure 5A. In most tissues, both transcripts were expressed at low or undetectable levels except for brain where rtMSTN-2a was readily detected (Fig. 13B & 13C). However, amplicons corresponding to both spliced and unspliced transcripts were detected for both rtMSTN-2a and -2b and were confirmed by sequencing. Primer set (PS)-I, which is specific for rtMSTN-2a, amplified

A Majority rule consensus tree



B Exon map of myostatin proteins

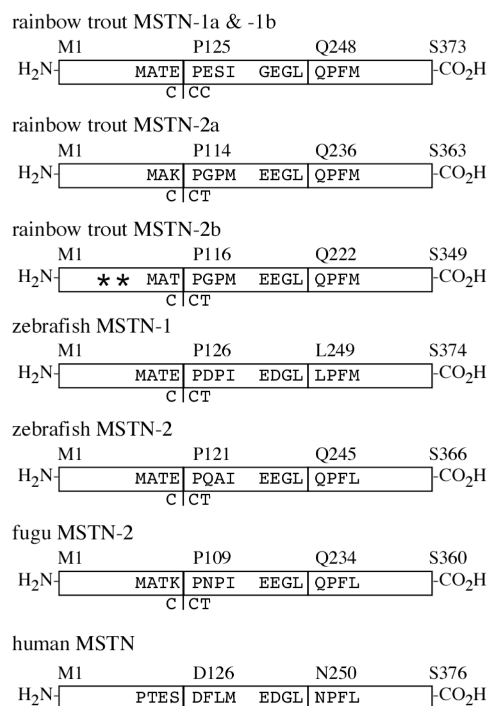


Figure 12. Phylogenetic analysis and comparative exon mapping of the myostatin proteins. (A) Bayesian inference analysis was performed on a single matrix composed of homologous myostatin sequences from the indicated species. Two independent analyses were performed and produced identical results. Bayesian posterior probability values are shown above each branch when greater than 50%. The individual clades are shaded and labeled. (B) Comparative exon mapping of the myostatin proteins. All the vertebrate myostatin genes characterized to-date are organized into three exons. The first amino acid coded by each exon and the last amino acid of each protein (sequential white boxes) are shown above. Amino acids coded by adjacent regions to each exonic boundary are shown inside the boxes. In all the fish genes, the codon of the proline residue located at the first exonic boundary is partially coded by nucleotides in the first and second exons as shown. The in-frame stop codons in rtMSTN-2b are indicated with asterisks.

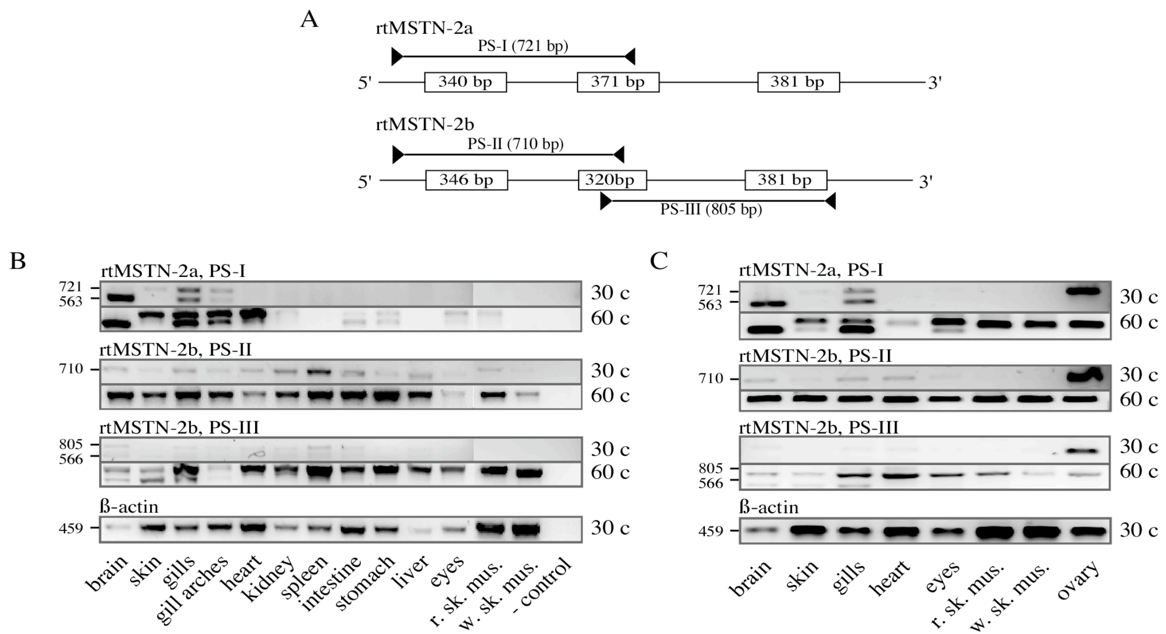


Figure 13. Expression of rtMSTN-2 transcripts. (A) Primer annealing locations (triangles) on rtMSTN-2 gene maps used for expression analysis. Exons are boxed and amplicon sizes (lines connecting primers) resulting from the amplification of gDNA or unprocessed transcripts are indicated. (PS, primer set; bp, base pairs). (B) Expression analysis of rtMSTN-2a, -2b and β-actin in different male rainbow trout tissues using the primer sets shown above. Initial amplification for 30 cycles was performed and 5 μL of the product was then used for another 30 cycles of PCR. Exact amplicon sizes are indicated and were verified by sequencing. (C) Gene expression in different female tissues.

both spliced and unspliced transcripts in most tissues, although the amplified variant appeared to be more abundant. However, only the spliced variant was detected in brain. By contrast, only unspliced rtMSTN-2b transcripts were detected regardless of the tissue. This was confirmed using two gene-specific primer sets that spanned either the first (PS-II) or second (PS-III) intron. To rule-out the possibility of gDNA contamination of our cDNA, we amplified β-actin using gDNA or gill cDNA as template and intron spanning primers (Fig. 14A). However, the larger amplicons produced with gDNA template were not produced with cDNA even after 60 cycles. Amplifying gill cDNA with primers that recognize both rtMSTN-2 transcripts produced unspliced amplicons for both genes and spliced for rtMSTN-2a (verified by sequencing). To further illustrate that these amplicons originated from RNA, samples were treated with or without RNase before cDNA synthesis and were eventually amplified with primers specific to rtMSTN-2a, -2b or both transcripts (Fig. 14B). Regardless of primers, all of the amplicons produced in the control samples either disappeared or were significantly reduced with RNase treatment. Taken together, these results indicate that the detection of unspliced transcripts for both rtMSTN-2 genes in different tissues is not due to gDNA contamination. The expression pattern shown represents a single male fish, however, it was additionally confirmed in a female fish and by using the RNA panel described herein. Only unspliced transcripts were present in ovaries (Fig. 13C) and testes (data not shown) and no evidence of sexually dimorphic mRNA processing was detected in any tissue. Nevertheless, this is the first report of myostatin splice variants for any species and possibly reflects a novel mechanism for limiting rtMSTN-2a influences to the brain and for further silencing rtMSTN-2b.

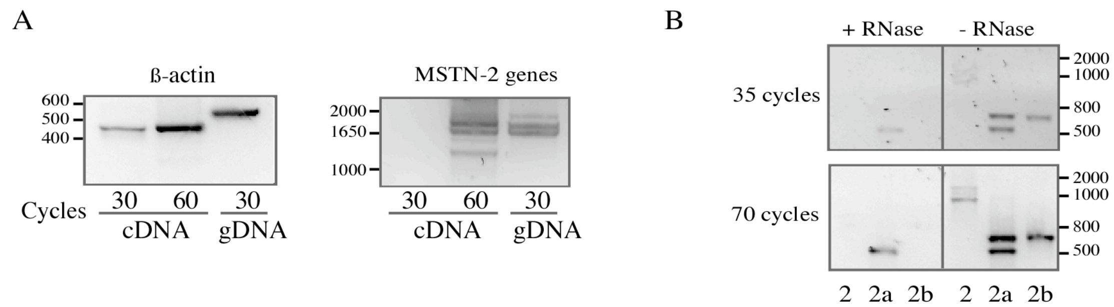


Figure 14. Pre-mRNA processing of rtMSTN-2 transcripts. (A) Amplification of β-actin and rtMSTN-2 genes from gill cDNA and gDNA. Total RNA was treated twice with DNase, re-extracted and used to synthesize cDNA. Primers for β-actin and those that recognize both rtMSTN-2 genes (forward primer of PS-I and reverse primer of PS-III) were then used for 30 and 60 cycles of PCR. The same primers were also used to amplify gDNA. (B) Expression of MSTN-2 transcripts at 35 and 70 cycles using cDNA synthesized from RNase-treated and non-treated gill RNA. Separate, but equal RNA pools, were treated with RNase and nuclease free water, re-extracted and used for cDNA synthesis before PCR (2, "all" MSTN-2 primers; 2a & 2b, gene specific primers).

Embryonic and adult tissue expression. Embryonic expression of rtMSTN-2a remained very low throughout development and did not differ significantly between sample times (data not shown). In adults, expression of rtMSTN-2a was detected in all tissues sampled and was highest in brain, which was 10-fold higher than in testis, spleen, and red and white skeletal muscle and at least 100-fold higher than all other tissues (Fig. 15A). Qualitative analysis of rtMSTN-1a, -1b and -2a expression in different brain regions revealed gene specific expression patterns (Fig. 15B). Although rtMSTN-1b was similarly expressed in all regions (myelencephalon, cerebellum, proencephalon and optic lobe), rtMSTN-1a and -2a expression was barely detected in the proencephalon and appeared highest in the cerebellum, albeit using a qualitative assay. Comparing the normalized expression of all the three genes in selected tissues revealed that rtMSTN-1b expression was consistently highest (Fig. 15C). In the brain, it was 12-fold higher than rtMSTN-2a expression, which in turn was 8-fold higher than that of rtMSTN-1a. In red and white muscle, however, rtMSTN-2a expression was 6-10 fold lower than rtMSTN-1a expression and over 100-fold lower in eye.

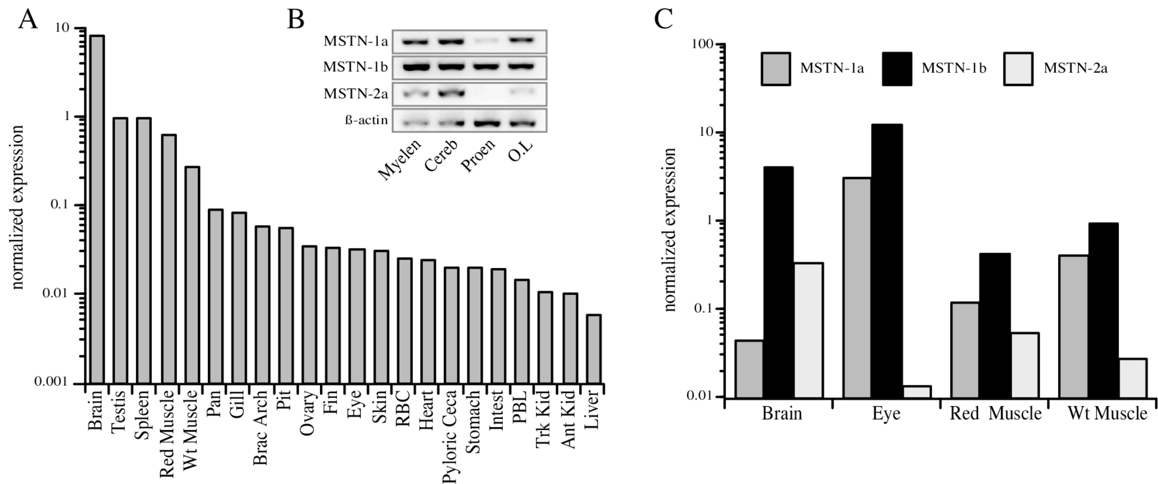


Figure 15. Comparative expression analysis of different rainbow trout myostatin genes in different adult tissues. (A) Real-time RT-PCR was used to quantify rtMSTN-2a expression in different adult tissues. Assays were repeated twice and run in triplicate from pooled tissue samples. (Pit, pituitary; Ant kid, anterior/head kidney; Trk, trunk; Intest, whole intestine; PBL, peripheral blood lymphocytes; RBS, red blood cells; red Muscle, red skeletal muscle; Wt, white). (B) Qualitative analysis of rtMSTN-1a, 1b and -2a expression in different brain sections. (Myelen, myelencephalon; Cereb, cerebellum; Proen, proencephalon; O.L., optic lobe). (C) Real time RT-PCR was used to quantify and compare normalized expression of rtMSTN-1a, -1b and -2a in brain, eye and red and white skeletal muscle using the pooled tissue samples in A.

Discussion

An early genome duplication event prior to the teleost radiation, but after the divergence of ray- and lobe-finned fishes, resulted in multiple copies of many genes (17, 18). A second event specifically in the salmonids produced additional copies of some genes (19) in this family. To date, two myostatin genes have been identified in zebrafish (9, 27), brook trout (34), rainbow trout (15), Atlantic salmon (12), coho salmon (35), seabream (16, 36), shi drum (37) and fugu (unpublished). However, all the salmonid genes are actually MSTN-1 paralogs as no MSTN-2 genes have been identified in these species until now. Indeed, a phylogenetic analysis (Fig. 12A) and sequence alignment (Fig. 11) clearly indicate that rtMSTN-2a and -2b are legitimate MSTN-2 paralogs. Organization of the newly discovered rtMSTN-2 genes (Fig. 9A & 9B) is highly conserved with three exons, identical to rtMSTN-1 genes and other vertebrate genes characterized to date. The second exon/intron boundary, which separates the coding regions for the latency-associated peptide from that of the bioactive domain of both rtMSTN-2 genes, is also highly conserved (Fig. 12B). However, comparable introns in both rtMSTN-2 genes have similar sequences, but are significantly smaller than their rtMSTN-1 counterparts (13), which is further indicative of their orthologous relationship. These date validate our previous phylogenetic analysis (9) and strongly suggests the presence of additional myostatin genes in other salmonid species. Whether or not all genes are functionally active, similarly expressed or lost/silenced to pseudogenization in all salmonids remains to be determined. These studies are dependent upon the characterization of genes from additional salmonid species and possibly from other basal

fish groups. Thus, comparative mapping of different myostatin genes (Fig. 12B) and the identification of consensus sequences for exon boundaries should prove invaluable.

Subsequence analysis of rtMSTN-2a and -2b promoter regions identified several putative *cis* regulatory elements that could contribute to the myogenic process. Some of these promoter elements were also found in other fish myostatin genes including brook trout (14) and zebrafish (9). However, putative MyoD binding site was only found in promoters for zebrafish MSTN-2 and rtMSTN-2a. In mammals, MyoD and MEF2 are critical to the differentiation of skeletal muscle and induce myostatin expression (31-33). However, rtMSTN-2a expression was relatively low in skeletal muscle when compared to brain expression (Fig. 15), which is consistent with tissue expression levels of other MSTN-2 genes (16, 38). MyoD is also expressed in brain and helps in the early differentiation of neurons (39). Nevertheless, MyoD binding sites alone cannot explain the possible preferential expression of MSTN-2 genes in brain as MSTN-1 gene expression also occurs in the tissue. Indeed, MSTN-1a and -1b are both differentially expressed in brook trout (34) and in rainbow trout brains (Fig. 15B). A recent computational analysis of the structural changes that occurred throughout the evolution of the myostatin gene family failed to identify markers of positive selection in any taxonomic group (40). Thus, diversity in muscle mass among vertebrates may have evolved from changes in the *cis* regulation of myostatin gene expression rather than from changes in the protein structure *per se* (40).

The rtMSTN-2 genes are highly similar to each other and their coding sequences are shorter than that of the rtMSTN-1 genes, which is not necessarily true in other species (9, 16, 37). Like other myostatin genes, the bioactive domains of both are entirely coded

by the third exons and each coding sequence contains the modular architecture common to other TGF-β and myostatin gene family members. A prominent difference between the two genes is that rtMSTN-2b lacks a 51 bp cassette found in rtMSTN-2a (corresponding to 17 amino acids) and contains two in-frame stop codons within the first exon. Qualitative expression analysis of rtMSTN-2b indicates that neither intron is spliced from the primary transcript regardless of tissue (Fig. 13). Both introns contain in-frame stop codons, which prohibits the production of functional protein even in the presence of alternative translation start sites. The presence of stop codons in the coding sequence and in the unspliced intronic regions indicate that rtMSTN-2b gene is a pseudogene. Both spliced and unspliced variants of rtMSTN-2a were also detected in all tissues surveyed except brain, which only expressed the correctly spliced transcript. The pre-mRNA splice sites for both rtMSTN-2 transcripts are identical, thus the presence of correctly spliced rtMSTN-2a in some tissues is likely due to higher levels of rtMSTN-2a expression rather than differential processing. Whether or not MSTN-2b orthologs from other salmonid species are also pseudogenes remain to be determined. Furthermore, the exact timing of rtMSTN-2b pseudogenization or of any other salmonid MSTN, cannot be estimated until other MSTN-2 genes are isolated from these species.

The bioactive C-terminal domain of myostatin is highly conserved in all species. In fact, the amino acid identity shared between most fish and mammalian species is 88% (6). These comparisons were made with MSTN-1 orthologs and although the bioactive domains of MSTN-2 proteins are also well conserved, notable differences exist. These include methionine for lysine and histidine for arginine substitutions in the C-terminus (Fig. 11). The latter conserved substitution is also seen in other fish MSTN-2 proteins

(fugu, seabream and shi drum) and may be unique to many MSTN-2 orthologs. The former however, occurs in a region hypothesized to have contributed to enhanced musculature in domesticated and wild bovids (4, 41, 42). Myostatin's actions are mediated by binding to the activin receptor ActIIRb (43, 44) as in fact any perturbation in ActIIRb signaling increases muscle mass (44-46). The carboxy terminal of activin is nearly identical to that of myostatin and is critical to ActIIRb signaling (47). Any change in myostatin's structure could potentially impact the activation of ActIIRb. Future functional studies are therefore needed to determine whether MSTN-1 and -2 proteins bind ActIIRb equally or to other receptors.

Quantitative analysis of rtMSTN-2a expression in different stages of embryonic development revealed no significant differences during any stage (data not shown) suggesting that rtMSTN-2a may not play a significant role throughout development. This is consistent with the expression of MSTN-2 orthologs in other fish species including seabream (16) and zebrafish (9, 38), although MSTN-2 expression did appear to rise slightly during early somitogenesis in zebrafish. Nevertheless, this is in stark contrast to the developmental expression patterns of other fish MSTN-1 orthologs (13, 14, 38, 48), particularly rtMSTN-1a and -1b, which increased significantly during somitogenesis and after hatching and yolk sac absorption (13). In adult tissues, expression of rtMSTN-2a was comparable to that of MSTN-1a and -1b, however, it was at least 10-fold higher in brain than in any other tissue. In addition, brain was the sole tissue that only expressed the correctly spliced transcript. Teleost fish exhibit an enormous capacity to replace damaged neurons in the central nervous system, a process that involves a subdivision of the cerebellum (49, 50). Each myostatin gene was differentially expressed in different

brain sections although rtMSTN-2a levels were highest in the cerebellum (Fig. 15B). Qualitative analysis of MSTN-2 expression in sea bream tissues indicates that it occurs primarily in brain (16), which is consistent with its expression in zebrafish, although more sensitive and quantitative assays detected low levels of MSTN-2 expression in other zebrafish tissues as well (51). These data together suggest that the biological actions of rtMSTN-2a, and possibly other MSTN-2 orthologs, may be limited primarily to the brain and may help regulate neurogenesis.

The rainbow trout is a unique model organism and representative of the salmonidae family. A better understanding of myostatin genes in this fish will add significantly to our understanding of the gene family's evolution. Although similar to other myostatin genes in many ways, both subtle and obvious differences distinguish the trout genes. Whether or not pseudogenization or alternative splicing also occurs among MSTN-2 genes of other salmonids or if their expression occurs primarily in neural tissue remains to be determined. These studies will nevertheless be aided by the characterization of MSTN-2 genes from other species and by additional computational and functional analysis.

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CHAPTER FOUR

MYOSTATIN REGULATES CARDIOMYOBLAST GROWTH AND DEVELOPMENT

Abstract

Myostatin is a highly conserved and potent negative regulator of skeletal muscle growth. It is expressed primarily in skeletal muscle, although expression has also been detected in heart, fat and mammary glands. Its non-muscle actions, however, are not completely known. Myostatin was recently shown to inhibit protein synthesis in primary cardiomyocytes suggesting that its actions may be similar to those in skeletal muscle. To test this hypothesis, we determined the effect of myostatin on basal and stimulated H9C2 cell proliferation and on differentiation. Myostatin suppressed basal proliferation in a dose-dependent manner, using 0, 2.7, 11 and 22 nM recombinant peptide, after 48 h. By contrast, insulin-like growth factor (IGF)-I stimulated proliferation in a dose-dependent manner after 24 and 48 h. Co-incubating cells with myostatin (2.7 & 11 nM) and 2.7 nM IGF-I resulted in the significant and dose-dependent inhibition of IGF-stimulated proliferation. Inhibition occurred whether IGF-I or the long R3 IGF-I analog (LR3), which does not bind the IGF binding proteins, was used. In fact, LR3 was slightly more effective in stimulating proliferation, but was equally susceptible to myostatin inhibition. Thus, IGFBPs may not significantly contribute to myostatin's inhibitory action in these cells. Myostatin also suppressed differentiation as measured by the quantitative expression of equilibrative nucleoside transporter (ENT)-1. Proliferating and differentiating H9C2 cells as well as L6 skeletal muscle cells were also surveyed for the expression of receptors and other genes that negatively regulate myostatin action or bioavailability. Expression of both activin type II receptors (ActRIIa & ActRIIb) was unaffected by differentiation and levels of ActRIIa were higher than those of ActRIIb. Both type I receptors, activin like kinase (ALK)-4 and 5, were similarly unaffected by

differentiation and ALK5 expression was much higher than that of ALK4. Furthermore, the expression of follistatin increased with differentiation while that of follistatin-like 3 (FSTL-3) decreased. Growth/differentiation factor-associated serum protein (GASP)-1 expression was not observed in these cells. These studies indicate that H9C2 cells express the necessary receptors required for myostatin signaling and that myostatin inhibits their proliferation and differentiation in a manner similar to skeletal muscle cells. Myostatin is believed to act primarily through ActRIIb in skeletal muscle cells. However, the differential expression of the type I and II receptors suggests that the functional role of ActRIIa and ALK5 in both cardiac and skeletal muscle may be more significant than previously believed.

Introduction

Myostatin is a potent negative regulator of skeletal muscle growth and a member of the transforming growth factor (TGF)-β superfamily (1). Null-mutations in the gene produced extreme gains in skeletal muscle mass among several different mammalian species (2-5). Although primarily expressed and initially characterized in mammalian skeletal muscle, myostatin expression has been reported in a number of fish tissues (6-8) and has also been detected in mammalian heart (9), mammary gland (10), adipose tissue (11) and brain (Allen brain atlas, www.brain-map.org) (12). The functional significance of myostatin expression and bioactivity in tissues other than skeletal muscle, however, has not been determined.

Myostatin is expressed in both fetal and adult ovine hearts, specifically in the cardiomyocytes and Purkinje fibers (9, 13). This suggests that myostatin may influence the growth and development of cardiac muscle as well. Myostatin is differentially expressed in cardiomyocytes during cardiac development and high levels of expression are correlated with a low proliferation index (14). Expression increases following myocardial infarction, particularly in cardiomyocytes of the peri-infarcted region, and persists for at least 30 days (9). In addition, levels of processed myostatin peptide were elevated in the myocardium following volume-overload heart failure (15, 16). These changes in expression are similar to those occurring in damaged skeletal muscle (17, 18) and suggest that myostatin may influence cardiac muscle repair. Indeed, recent studies with Akt transgenic mice (19), *in vitro* models of cyclic stretch and IGF-I stimulated cardiomyocytes (13) all suggest that myostatin not only regulates some cardiac muscle growth processes, but that it may function as a cardiac chalone as it does in skeletal

muscle (20). Indeed, myostatin expression is elevated in all of these models and may therefore provide a negative feedback mechanism to limit cardiac muscle growth and thus, hypertrophy. However, heart size of myostatin-null animals including the "mighty mouse", is normal (2) as are the size of individual cardiomyocytes (21). The low level of myostatin expression in hearts of normal animals together with the sharp increase following myocardial infarction (9) suggests that myostatin may influence cardiac muscle growth process during pathophysiological conditions of the heart. A better understanding of myostatin's functional role in regulating cardiac muscle growth could help developing novel therapies for treating cardiac disorders and to determine why myostatin null-animals do no have a cardiac phenotype.

Reported herein is the validation of the H9C2 cell model for defining basic cardiac muscle growth process. Our studies model demonstrated that myostatin suppresses basal as well as IGF-I stimulated proliferation of cardiomyoblasts in a dose dependent manner. Myostatin also partially suppressed cardiomyoblast differentiation. These findings indicate that myostatin can potentially limit cardiomyoblast proliferation and differentiation in a manner similar to that in skeletal muscle cells. The lack of cardiac phenotype, however, in myostatin-null animals may be due to the continual expression of high affinity binding proteins, including follistatin and FSTL-3, that are not similarly expressed in skeletal muscle cells.

Materials and methods

Animals. Rat and mouse tissue samples were obtained from animals bred at the experimental animal laboratory building, Washington State University. Samples were obtained according to protocols pre-approved by the Institutional Animal Care and Use Committee (IACUC).

Cell culture. H9C2 cardiomyoblasts, a clonal line derived from embryonic rat heart, were cultured at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Sigma, www.sigmaldrich.com) supplemented with 10% fetal bovine serum (FBS), 0.2 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 250 ng/ml amphothericin B. Proliferation assays were performed on cells originally plated at 50% confluency and in serum-free DMEM supplemented with 2.7, 11 or 55 nM long R3 (LR3) IGF-I (Diagnostic systems laboratory, www.dslabs.com) for 24 and 48 h. Cells were also cultured with 2.7 nM LR3 with 0, 2.7 nM or 11 nM human myostatin (Metamorphix, www.metamorphixinc.com) for 48 h of incubation. At the end of the incubation period, total cell number was measured using the Cell Titer 96 proliferation assay (Promega, www.promega.com. To differentiate, cells were grown to 70% confluency in DMEM supplemented with 1% FBS and stimulated with 10 nM retinoic acid (Sigma) daily as described (22). Briefly, retinoic acid was diluted in the dark with DMSO, aliquoted and stored as 1 mM stock solutions and used at a final concentration of 10 nM. To determine the effect of myostatin on H9C2 differentiation, cells were cultured in differentiation medium for 3 to 6 days with or without addition of 11 nM myostatin. Myostatin was added at the beginning of the experiment and additionally on day 3 in the

6-day group. Quantitative RT-PCR was performed to measure the expression of the differentiation maker equilibrative nucleoside transporter (ENT)-1.

Expression analysis. Total RNA was extracted from cells, adult rat heart and skeletal muscle tissue using Trizol reagent (Invitrogen, www.invitrogen.com) and treated with DNase (DNase RQ-1, Promega) according to the manufacturer's protocol. The RNA integrity was assessed by agarose gel electrophoresis and 5 μg total RNA was used for cDNA synthesis using the First stand cDNA synthesis kit (Invitrogen). Semi-quantitative RT-PCR was performed for activin type II receptors (ActRIIa and ActRIIb), activin-like kinase (ALK)-4, ALK-5, follistatin (FS), follistatin-like (FSTL) -3, growth/differentiation factor associated serum protein (GASP)-1, ENT-1, BMP and activin membrane-bound inhibitor (BAMBI) and β-actin in adult rat heart, skeletal muscle and in proliferating and differentiating H9C2 and L6 cells. Primers and the annealing temperatures are included in Table 3. The PCR protocol includes an initial denaturation of 94 °C for 1 min, followed by 30, 35 or 40 cycles of 94 °C for 30 s, a gene-specific annealing temperature (Table. 3) for 30 s and 72 °C for 90 s.

Real time RT-PCR assays were conducted using iCycler iQ Real-time PCR Detection System (Bio-Rad, www.biorad.com) and gene specific primers for ENT-1 and elongation factor (EF)-2 (Table. 3). The samples were amplified for 95 °C for 10 s, followed by 57 °C (ENT-1) or 62 °C (EF-2) for 30 s and 72 °C for 30 s using primer concentrations that were empirically determined (ENT-1 FP = 400 nM, ENT-1 RP = 200 nM; EF-2 FP = 200 nM, RP = 200 nM). Melt curve analysis was performed with each primer set to assure specificity. In addition, amplicons were isolated on a 1% agarose gel to check for any genomic DNA contamination or non-specific amplification. Master mix

Table 3. Primer sequences and annealing temperatures

Primer name	Sequence (5'- 3')	An. Temp. (°C)
ActRIIa FP	TGG CGT TCG CCG TCT TTC TTA TC	60
ActRIIa RP	AGT GTG GAC AGA TGG TGC AAC	60
ActRIIb FP	CAG GTT GGC ACC AGA CGG TAC	55
ActRIIb RP	TCG ATG GTC ACG CAG AGC TGG	55
ALK-4 FP	TGC CCA GTG GAC ACC TCA AGG	60
ALK-4 RP	TGG ACT CCT CCA GAA TTG CAC CTC	60
ALK-5 FP	CCG AGA CAG GCC ATT TGT TTG T	60
ALK-5 RP	TCG TGG ATT CCG CCA ATG GAA C	60
FSTN FP	GCG GGC TCT GCC TCC TGC TG	70
FSTN RP	TTC CAC CAC CGC ACT GGA TGT C	70
FLRG FP	TCT GCT GGT ACT TTG GGG CCA C	65
FLRG RP	AGC CTG GTC CAA CTT CAC CCA	65
GASP-1 FP	GCT CCC CTT CGA GGC CTA GC	63
GASP-1 RP	GTC CTC ACT GTC TGG GGG CTT CAG	63
MSTN FP	GCT GAT TGC TGC TGG CCC AGT GG	60
MSTN RP	GAG CAC CCA CAG CGG TCT ACT ACC	60
β-actin FP	CGC TGC GCT GGT CGT CGA CAA CG	60
β-actin RP	ATC GTA CTC CTG CTT GCT GAT CCA C	60
ENT-1 FP	TCA TGC GAA AGC ACC GAG	57
ENT-1 RP	GGC ACA GAT CAT GGC AAC	57
Myf-5 FP	TGT ATC CCC TCA CCA GAG GAT	55
Myf-5 RP	GGC TGT AAT AGT TCT CCA CCT GTT	55
EF-2 FP	GAC ATC ACC AAG GGT GTG CAG	62
EF-2 RP	GCA GTC AGC ACA CTG GCA TA	62
BAMBI FP	TGT CCT CTC TCC TCC CAA GA	60
BAMBI RP	TGT CCA TGG AAG CTG TAG TG	60
flBAMBI FP	GTA GGC GTT GCT CTC TGT G	62
flBAMBI RP	GCGAGT TAG TTG TCT CCA GAT AA	62

FP, forward primer; RP, reverse primer

containing iQ Super mix (Bio-rad) was aliquoted before addition of cDNA. These samples were subsequently divided in half and primers were added. Samples were run in duplicate and each analysis was also duplicated. Relative gene expression of ENT-1 was, therefore, normalized to that of the EF-2 using the Q-Gene method (23).

Production of stable cell line. Mouse cDNA encoding BAMBI was amplified from heart by RT-PCR using primers flBAMBI-FP and fl-BAMBI-RP (Table. 3) that amplify the entire coding region. The PCR product was sub-cloned into the expression vector pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen) and verified by sequencing at the university's genomic core facility. H9C2 cells were transfected with 5 μg of vector with Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Stably transfected cells were derived in 750 μg/ml G418 to obtain a polyclonal cell line and were used to study the effect of myostatin on the proliferation.

Statistical analysis. At least three independent experiments were performed for the proliferation assays and two for differentiation experiment analysis. An ANOVA (1 and 2 way) was performed and mean differences were determined using Fisher's PLSD test. Significance was accepted at $P \leq 0.05$.

Results

Expression of myostatin receptors and related genes in H9C2 cells and heart. Semi-quantitative expression analysis was performed in adult skeletal muscle and heart tissue of rat, proliferating H9C2 and L6 cells and in cells differentiating for 3 or 6 days (Fig. 16). Expression of ENT-1 was used as a marker for cardiac-specific differentiation and as expected increased. The expression of ActRIIa did not change in differentiating H9C2 cells. In contrast, it increased in L6 cells with differentiation. ActRIIa expression was detected 5 cycles earlier than that of ActRIIb, suggesting that is expressed at higher levels. Changes in ActRIIb expression were similar to those with ActRIIa. The expression of both type I receptors ALK-4 and -5, increased as both cell types differentiated, although absolute levels of ALK-5 expression were higher than those of ALK-4. The pseudoreceptor, BAMBI, mRNA was expressed similarly in all samples.

Myostatin binding proteins are expressed in proliferating cardiomyoblasts, throughout differentiation and in adult cardiac muscle. Follistatin like-3 gene expression was seen only in heart muscle and decreased in differentiating H9C2 cells. In contrast, follistatin expression increased with differentiation, occurred in both cell types and was similar in adult cardiac and skeletal muscle. Myostatin expression decreased during differentiation in H9C2 cells, however, its expression in L6 cells was not observed even after 40 cycles.

Myostatin inhibits cardiomyoblast proliferation. H9C2 cells express all the important receptors necessary for myostatin signaling. Therefore, we determined if myostatin inhibits proliferating cardiomyoblasts. Exogenous addition of myostatin significantly ($p \leq 0.05$) inhibited basal proliferation of H9C2 cells in a dose-dependent

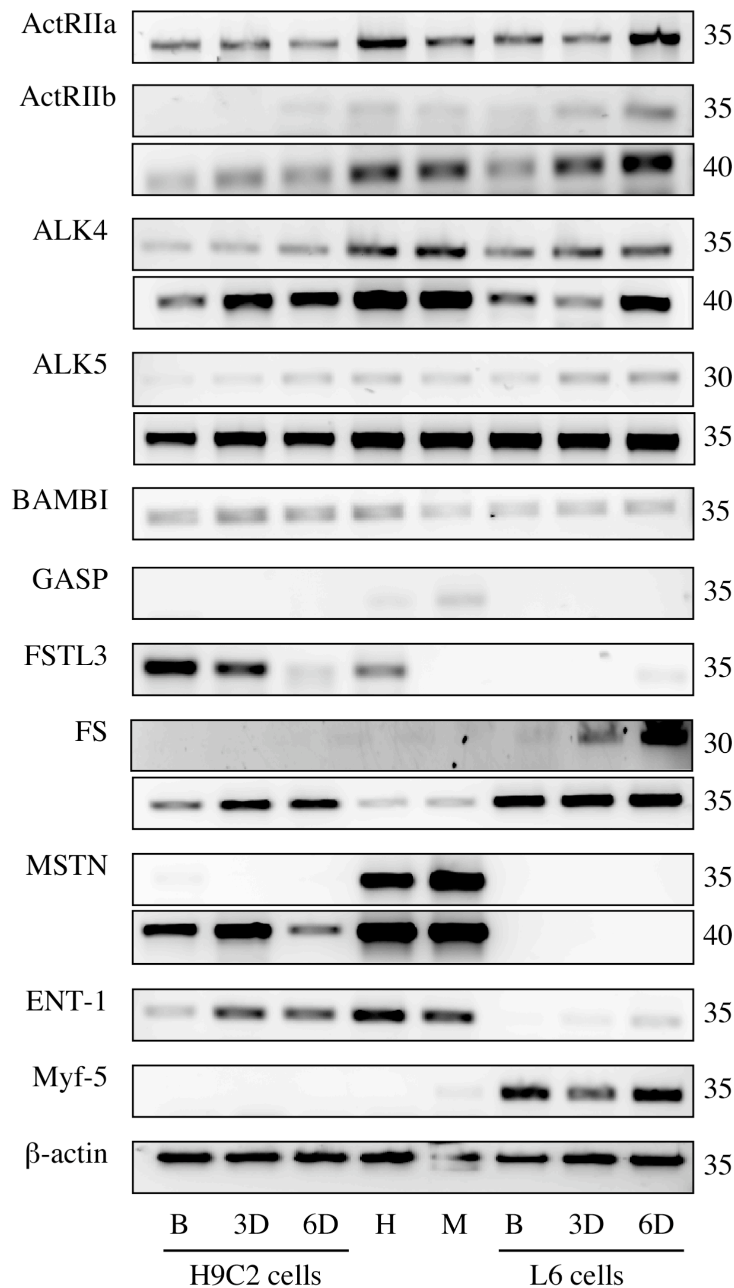


Figure 16. Semi-quantitative expression analysis of myostatin receptors and related genes during differentiation. Transcripts for activin type II receptors (ActRIIa & ActRIIb), activin like kinase (ALK4 & ALK5), BMP & activin membrane-bound inhibitor (BAMBI), follistatin like-3 (FSTL-3), follistatin (FS), growth and differentiation factor associated protein (GASP) and myostatin (MSTN) were amplified using semi-quantitative RT-PCR. Equilibrative nucleoside transporter (ENT)-1 was used as marker for differentiation and β-actin was used as a loading control. (B, myoblasts; 3D & 6D, days in differentiation medium; H, adult rat heart; M, adult skeletal muscle; H9C2, cardiomyoblasts; L6, skeletal myoblasts; cycle number indicated on the right).

manner when cells were cultured in serum free medium for 48 h (Fig. 17). Cell growth was inhibited by 8% and 21% with 11 and 22 nM MSTN, respectively, although the smaller dose of myostatin, 2.7 nM, did not have a significant effect.

Effect of IGF-I on H9C2 proliferation. IGF-I is a potent regulator of skeletal muscle growth and a determinant of animal size. It also stimulates proliferation of skeletal muscle cells (24). Addition of IGF-I stimulated the proliferation of H9C2 cells at 24 h and 48 h (Fig. 18). This proliferative effect was only observed in serum free medium, but was dose-dependent with a minimum dose of 2.7 nM significantly increasing total cell number.

Effect of myostatin on IGF-I stimulated proliferation of H9C2 cells. IGF-I is a potent stimulator of cell proliferation and myostatin is a negative regulator skeletal muscle proliferation. It has been reported that IGF binding proteins mediate the actions of myostatin in porcine myogenic cells (25, 26). Therefore, we examined whether myostatin inhibits the actions of IGF-I or LR3, an IGF-I analog that does not bind IGF binding proteins (IGFBP), on cardiomyoblast proliferation. The proliferative effect of 2.7 nM LR3 on H9C2 cells is more pronounced than 2.7 nM IGF-I. However, myostatin significantly ($p \leq 0.05$) suppressed the stimulatory effect of both and in a similar manner (Fig. 19). In addition, the suppressive effects were dose-dependent as IGF-I stimulated growth was inhibited by 14 and 23 % with 2.7 and 11nM myostatin, respectively. Myostatin had similar effects on LR3-stimulated proliferation (13 & 20%) suggesting that IGFBP sequestration of IGF-I is not involved.

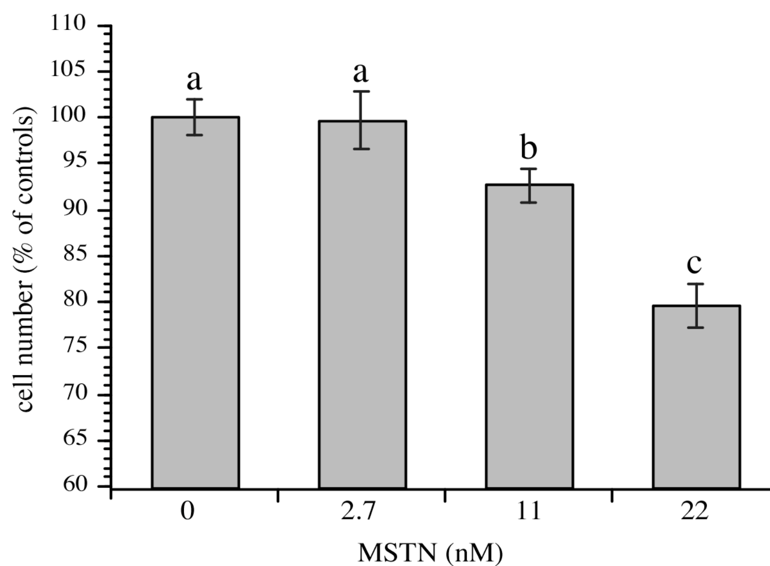
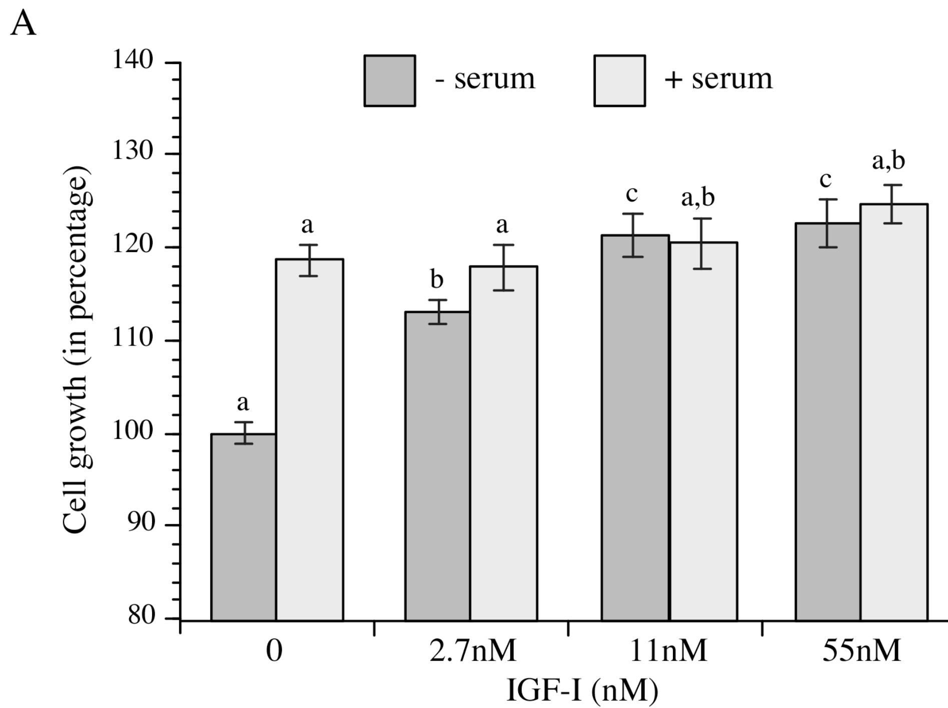


Figure 17. Myostatin inhibits basal cardiomyoblast proliferation. H9C2 cells were cultured in the presence or absence of myostatin, in serum-free conditions and with the different doses indicated. Cells were grown for 48 h before the total cell number was measured. Results from multiple experiments were pooled and data are expressed as % of controls. Significant differences ($p \leq 0.05$) are indicated by different letters whereas same letters denote no differences.



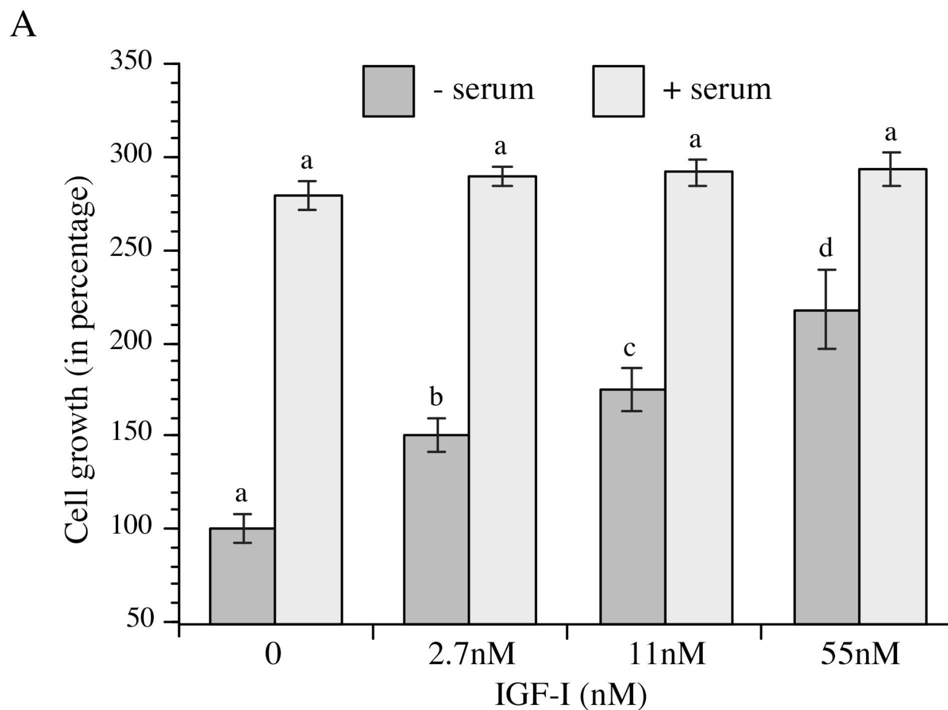


Figure 18. IGF-I stimulates cardiomyoblast proliferation. H9C2 cells were cultured with IGF-I for 24 h (A) and 48 h (B). Results from multiple experiments were pooled by expressing data as % of controls for each time point. Significant differences ($p \leq 0.05$) are indicated by different letters (comparisons within -/+ serum groups, not between groups) whereas the same letters denote no differences.

Myostatin suppress H9C2 differentiation. Myostatin inhibits the differentiation of skeletal muscle cells by suppressing MyoD activity (27). Therefore, we examined myostatin's effect on the differentiation of cardiomyoblasts using quantitative expression of ENT-1 as a marker. The levels of ENT-1 mRNA increased more than 3-fold after 3 days in differentiation medium and remained constant thereafter (Fig. 20). The addition of 11 nM MSTN, however, partially suppressed ($p \leq 0.05$) the rise in ENT expression. This suppressive effect was not observed after 6 days in differentiation medium, nevertheless, myostatin appears to inhibit the differentiation of cardiac as well as skeletal muscle cells.

Overexpression of BAMBI. Blocking myostatin's activity by overexpressing follistatin resulted in enhanced muscle growth comparable to myostatin null-animals (28). We determined whether the similar overexpression of an activin pseudoreceptor, BAMBI, would block myostatin's effect on H9C2 cells. Stable H9C2 cells were generated by transfecting a BAMBI cDNA construct driven by the cytomegaloviral promoter. Semi-quantitative analysis for BAMBI confirmed that the pseudoreceptor mRNA was overexpressed, but only 2-3 fold (Fig. 21). The effect of myostatin on the basal and LR3 stimulated proliferation of these cells was then determined. However, myostatin's suppressive action on proliferating H9C2 was not blocked by BAMBI overexpression. Addition of 11 nM myostatin suppressed 13% of the basal proliferation and 19% of IGF-I stimulated growth.

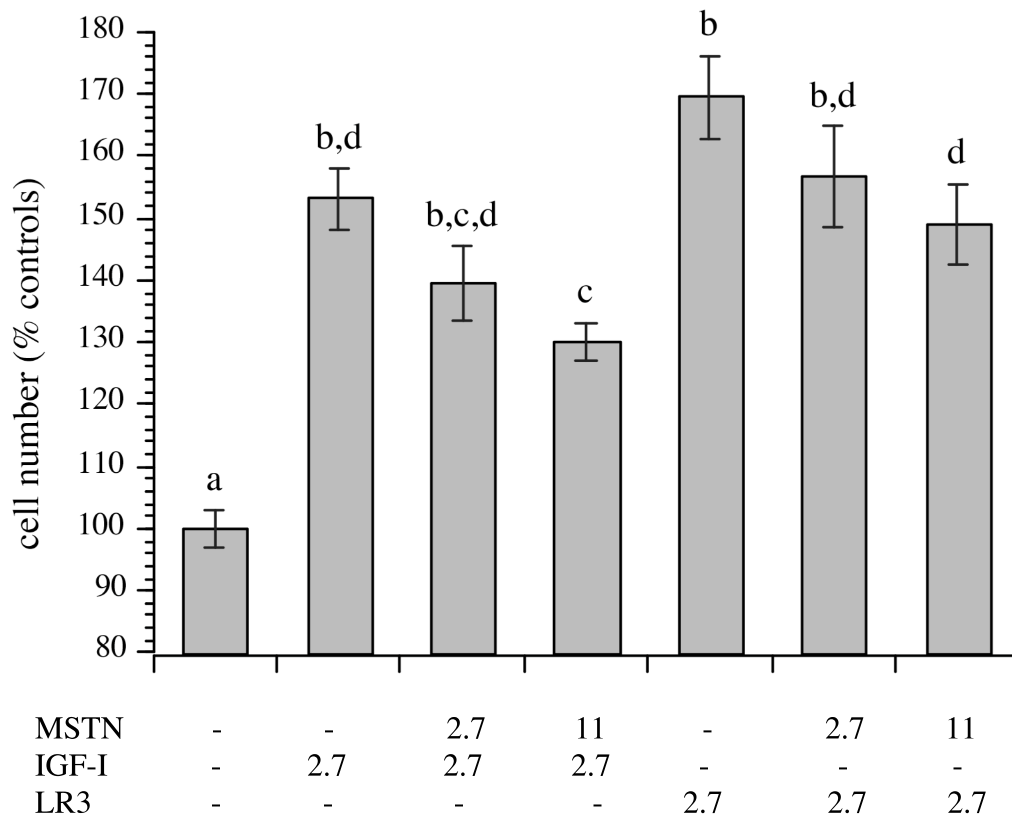


Figure 19. Myostatin inhibits IGF-I and LR3 stimulated cardiomyoblast proliferation. H9C2 cells were cultured in the presence of IGF-I or LR3, an IGF-I analog that does not bind IGFBP, with myostatin doses indicated in serum free conditions. Cells were grown for 48 h before total cell number was measured. Results from multiple experiments were pooled and data are expressed as % of controls. Significant differences ($p \leq 0.05$) are indicated by different letters whereas same letters denote no differences.

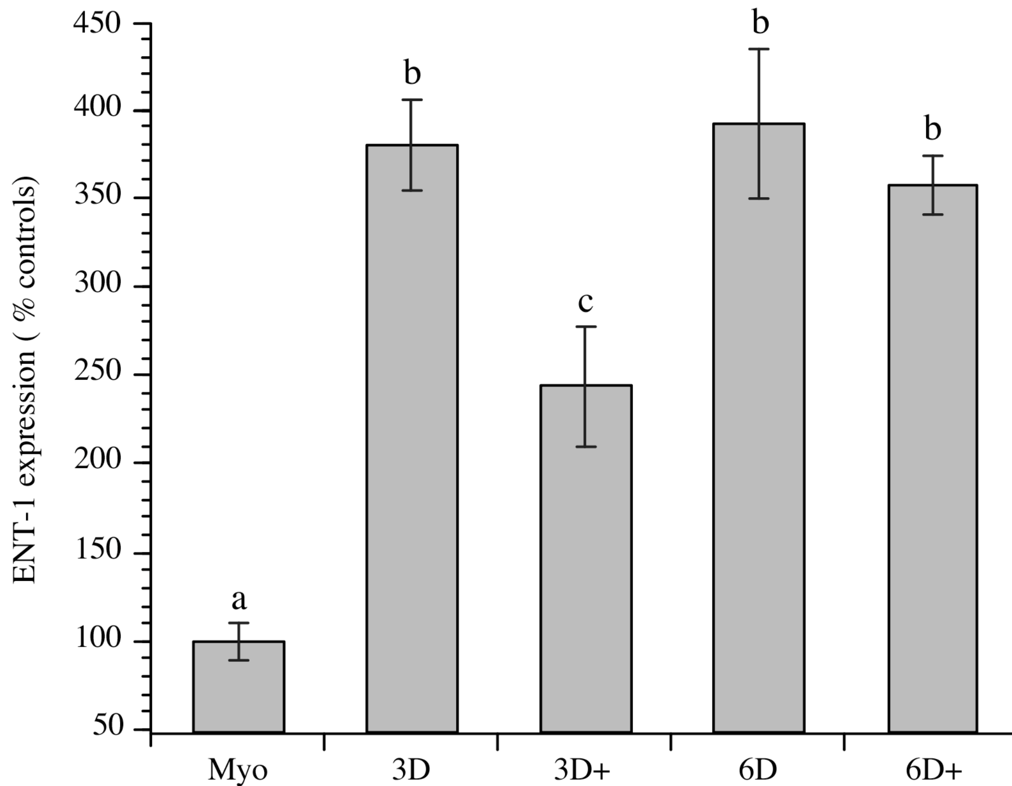


Figure 20. Myostatin suppress cardiomyoblast differentiation. H9C2 cells were cultured in the presence or absence of 11 nM MSTN and stimulated to differentiate in 1% FBS and 10 nM retinoic acid (added daily). The culture medium was changed after 3 days and myostatin was once gain added. Equilibrative nucleoside transporter (ENT)-1 was used as a differentiation marker and was quantified using real-time RT-PCR. (Myo: proliferating H9C2 cells; 3D: H9C2 cells grown for 3 days in differentiation medium; 3D+: H9C2 cells grown for 3 days in differentiation medium with 11 nM myostatin; 6D: H9C2 cells grown for 6 days in differentiation medium; 6D+: H9C2 cells grown for 6 days in differentiation medium with 11 nM myostatin). Significant differences ($p \leq 0.05$) are indicated by different letters whereas same letters denote no differences.

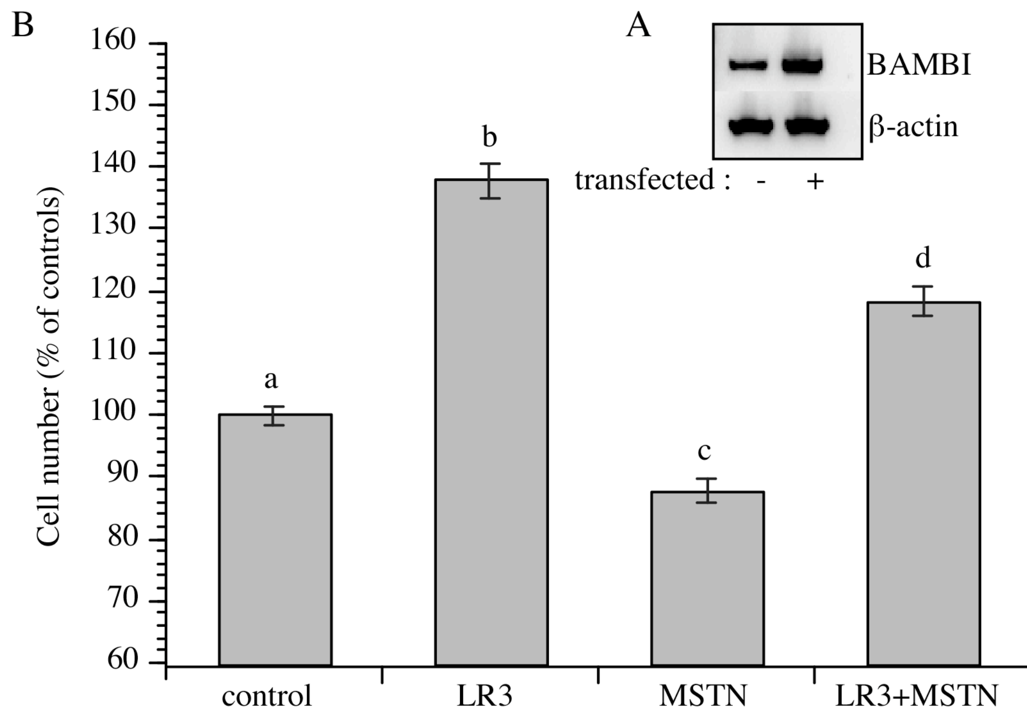


Figure 21. BAMBI overexpression does not block myostatin's effect on H9C2 cell proliferation. H9C2 cells were stably transfected with BAMBI. BAMBI expression in the polyclonal cell line was verified by semi-quantitative RT-PCR (A). Cells were then cultured in the presence of 2.7 nM LR3, 11 nM myostatin or a combination of both, to determine if BAMBI overexpression blocks myostatin's effect (B). Significant differences ($p \leq 0.05$) are indicated by different letters whereas same letters denote no differences.

Discussion

Myostatin's role as a negative regulator of skeletal muscle growth is well established from myostatin null-animals (2, 4, 29, 30) and from *in vitro* studies (27, 31). Myostatin null animals did not show any cardiac phenotype (2) and there is no differences in cardiomyocyte size or heart/body weight ratio (21) compared to wild-type animals. Myostatin is primarily expressed in skeletal muscle (2) in mammals, although lower levels of expression in other tissues (10-12) including heart (9) and cardiomyocytes (13, 14, 32) occurs. A sharp increase in the expression of myostatin levels was reported in heart after cardio-infarction (9), volume-overload hypertrophy (15), in transgenic mice with chronic Akt (19), and in cardiomyocytes during mechanical stretching (13). The presence of myostatin but an absence of a clear role in heart physiology led us to determine myostatin's actions in these cells. It was reported that myostatin acts through activin type II receptors, which recruit ALK-4/5. These type I receptors then phosphorylate smad 2/3 (33). We determined that cardiac muscle and H9C2 express the necessary receptors for myostatin signaling (Fig. 16). We observed that ActRIIa mRNA expression was several times higher than that of ActRIIa in all the samples. A similar observation was also observed in different skeletal muscle tissues of mice (34). This is interesting because myostatin has a slightly higher affinity for ActRIIb compared to ActRIIa, as determined by receptor binding assays (28, 33), and is thought to signal primarily through ActRIIb. This differential expression suggests that ActRIIa may play a more important role than was previously believed. In fact, our hypothesis is supported by Lee *et. al*. (34) who generated transgenic mice overexpressing a double mutant ActRIIa.

These animals have higher skeletal muscle weights compared to transgenic mice with mutant ActRIIb.

Myostatin is highly expressed in the developing fetal heart and ultimately decreases in the adult heart (9). This pattern of expression was also seen in differentiating H9C2 cells, which further validates the model. In another study, myostatin mRNA and protein levels increased in cardiomyocytes from embryonic day 18 to 10 days after birth and then decreased greatly in adult heart tissue (14). The authors observed that the expression of myostatin increased 6-fold in cardiomyocytes of postnatal animals compared to embryonic period. Proliferation of cardiomyoblasts mostly during the early postnatal period (14, 35). Thus, an increase in myostatin expression during this period suggests a potential role for myostatin in regulating heart development. We determined the effect of myostatin on the basal and IGF-stimulated cardiomyoblast proliferation and found that myostatin suppresses both in a dose dependent manner (Fig. 17). Similar results were recently shown using primary fetal and neonatal cardiomyocytes (14), although IGF-stimulated cells were not used. The authors also reported the inhibition of proliferation by blocking the G1 to S phase cell transition through the increase in Cdk2, decrease in p21 levels and phosphorylation of smad proteins (14), suggesting that the inhibitory effect of myostatin is similar to that in skeletal muscle cells (18, 36-38). IGF-I is a potent stimulator of cell proliferation in skeletal muscle cells (39). IGF-I stimulated H9C2 cell proliferation in a dose dependent manner (Fig. 18) and these effects were suppressed by myostatin whether IGF-I or LR3 was used to stimulate the cells (Fig. 19). Myostatin's inhibitory effects in porcine myogenic cells are mediated in part by IGFBP-3 and -5 (25, 26). However, thus does not appear to happen in H9C2 cells (Fig. 19).

A role of myostatin in suppressing phenylephrine induced protein synthesis (14, 32) and IGF-I induced protein synthesis (13) in cardiomyocytes is well documented. The inhibition of protein synthesis occurs through the suppression of Akt phosphorylation/activation (32). Therefore, we examined if myostatin inhibits cardiomyoblast differentiation. Our study showed that myostatin suppresses the differentiation of H9C2 cardiomyoblasts (Fig. 20). Addition of 11 nM myostatin significantly suppressed the expression of ENT-1 after 3 days in cells stimulated to differentiate, although these effects were lost at 6-days. This can be explained by the fact that myostatin is added once in 3 days and the cells were stimulated daily with retinoic acid, so the suppressive effect on differentiation was likely nullified by daily stimulation with retinoic acid. Nevertheless, ENT-1 expression and cardiomyoblast differentiation were clearly inhibited by myostatin after 3 days.

Our study shows that myostatin inhibits proliferation and differentiation of cardiomyoblasts. Others have confirmed myostatin's anti-proliferative effects in primary cells and that myostatin also inhibits protein synthesis (14, 32). However, there is absence of a cardiac phenotype in myostatin null animals (21). This could be explained by the differential expression of both FSTL-3 and FS in proliferating and differentiating cells (Fig. 16). Both are secreted proteins that bind myostatin and inhibit its activity (40). FSTL-3 is highly expressed in proliferating H9C2 cells, while FS expression increases with differentiation (Fig. 16). Thus, H9C2 cells are always exposed to these myostatin binding proteins. We hypothesize that FSTL-3 and FS perturbation of myostatin's actions in developing cardiac muscle, but not in skeletal muscle may explain the lack of cardiac

phenotype in myostatin-null animals. Further studies by blocking FS and FSTL-3 to observe myostatin's actions need to be performed to support our hypothesis.

BAMBI is an activin type I pseudoreceptor that lacks an intracellular kinase domain (41). This could be a good candidate gene for blocking MSTN's activity intracellularly as it acts like a naturally occurring dominant negative. To test this hypothesis, we produced polyclonal stable H9C2 cells and determined the effect of myostatin on basal and IGF-stimulated growth. We found that myostatin's activity was not inhibited in stable cells overexpressing BAMBI (Fig. 21). These results could be because the polyclonal stable cell lines produced higher levels of BAMBI, but not high enough to squelch ALK4/5 signaling (Fig. 21). Other approaches such as using monoclonal stable cell line or viral delivery mechanisms to overexpress BAMBI could also be utilized greatly enhance BAMBI expression and to block myostatin's activity.

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CHAPTER FIVE

SUMMARY AND FUTURE DIRECTIONS

Summary

Since the discovery in 1997, myostatin has been reported to be involved as a negative regulator of skeletal muscle proliferation and differentiation, and its function appear to be well conserved in mammals. This attracted attention from researchers interested in manipulating myostatin's activity for agricultural, therapeutic and biomedical reasons. Indeed, blocking myostatin action improves muscle conditions in animal models of Duchenne muscular dystrophy (1-4). The effect of manipulating myostatin increases domestic animal meat production and feed efficiency and thereby decreases the cost of production (5, 6). However, the knowledge and functional significance of myostatin in aquaculture species is not complete.

The evolution of fish is quite unique compared to mammals. Genome duplication occurred during the evolution of bony fish and resulted in multiple myostatin genes. A second genome duplication occurred specifically in salmonids around 50-100 million years ago and resulted in additional myostatin gene copies. As a result, salmonids possess four myostatin genes: rtMSTN-1a, -1b, -2a and -2b (7). Past researchers identified two myostatin genes in rainbow trout (8), although phylogenetic analyses (9) suggest that those were actually MSTN-1 orthologs. We discovered two new MSTN genes in rainbow trout that are orthologous to the MSTN-2 clade and were named rtMSTN-2a and -2b. The genomic and upstream 2 kb promoter regions were sequenced for all four genes. The genomic structure of each was identical and appears to be well conserved in all vertebrates. This suggests that there is a strong selective pressure for maintaining the genomic structure of the myostatin gene. Analysis of the promoter region for each gene identified several putative myogenic elements. However, the putative MyoD element,

which regulates myostatin expression in mammals (10), was identified only in the promoter of rtMSTN-2a. Of the newly discovered genes, rtMSTN-2b is a pseudogene and like rtMSTN-2a, is either not correctly spliced or is alternatively spliced, limiting mature transcripts (7). In fact, correctly spliced rtMSTN-2a transcripts mostly occur in brain.

The developmental expression of rtMSTN-1a and -1b is consistent with a role during somitogenesis and early muscle development, possibly including the periods of hatching and yolk sac absorption. This suggests that rtMSTN-1 genes could be playing a role in hatching and yolk sac absorption. The expression of rtMSTN-2a is far more limited, is very low and does not change during embryonic development. Adult tissue expression of rtMSTN-1 genes was ubiquitous, suggesting a wider role. In contrast, the expression of rtMSTN-2a is specific to brain and could be playing a role during neurogenesis. These results show that myostatin biology in fish is different from mammals and needs to be completely studied before manipulation of myostatin genes is utilized in aquaculture.

We studied the affect of myostatin on cardiomyoblast proliferation and differentiation. A semi-quantitative expression analysis of receptor and genes that regulate of myostatin action showed that H9C2 cardiomyoblasts express a complete complement of factors also found in heart. The expression of myostatin binding proteins (FS and FSTL-3) may explain why myostatin-null animals do not show a cardiac phenotype. Nevertheless, myostatin action in cardiomyoblasts appears to be similar to that in skeletal muscle cells. Myostatin suppressed both basal as well as IGF-stimulated proliferation and in addition suppressed cardiomyoblast differentiation. Blocking myostatin's actions in cardiac tissue could be potentially beneficial, especially during

pathological conditions of heart where its expression is sharply increased (11). Our attempts to disrupt myostatin signaling by overexpressing BAMBI in a stable cell line failed, but were hampered by low expression levels. Use of alternative techniques such as viral based vectors to produce high levels of BAMBI expression is therefore required.

Future directions

Studies in fish. Future studies in fish should concentrate on characterizing myostatin genes in salmonids and using this information to explore mechanisms of functional divergence. Additional myostatin genes belonging to the myostatin-2 clade could be isolated and characterized in more diverse salmonid species by constructing degenerate primers from available MSTN-2 genes. It would be interesting to see if any of these genes are also pseudogenes or if differential processing occurs. Together with expression analysis and computational analysis, the myostatin gene family could serve as a powerful model for understanding the impacts of different selection pressures and gene functions.

The study of promoter activity of the rtMSTN genes would complement these studies as changes in promoter structure and function also contribute to divergence. Recombinant plasmids can be constructed with fluorescing reporter genes (YGP, GFP and RFP) driven by rtMSTN-1a or -1b or -2a promoters. The constructed plasmids can then be injected during 1-2 cell stage embryos of rainbow trout. Such studies would determine the promoter activity of individual myostatin genes during development. Identifying the promoter activity and determining where and when the myostatin genes are active will help determine the specific role of each and how such differences arose. These studies would also contribute important information about conserved or divergent response elements. DNase I foot printing and electro-mobility shift assays could be performed on all the rtMSTN promoters with nuclear protein from different tissues. This could identify potential promoter binding elements *in vitro*. However, functional studies should also be performed. These include luciferase reporter assays in primary

myosatellite cells, which are easily cultured (12, 13) or even in whole embryos. The use of deletion mutants of promoters with mutated response elements would further confirm the functional studies and would identify specific and conserved promoter elements.

Gene knockdown technology could also be used to study the function of rtMSTN genes. Antisense morpholinos could be used for this purpose. Myostatin C-terminal domain of rtMSTN genes is highly similar and so could compensate for the knockdown gene. To address this, multiple myostatin gene knockdown studies should also be performed. These studies are still in infancy (14) and difficult to perform in rainbow trout because of slow developmental rate and may not be relevant in zebrafish, which have determinant growth. A better approach would be to overexpress individual rtLAPs, although this requires functional studies on LAP:MSTN interactions using rainbow trout peptides.

Transgenic follistatin mice have a similar muscle phenotype as the myostatin knockout mice (15). These studies could be duplicated in fish. Recombinant plasmid under EF2α could be constructed which will be expressed ubiquitously throughout the development and adult life of fish. The recombinant plasmid could then be used to generate recombinant retrovirus that could be used to infect the embryonic stages of the fish. Retrovirus was shown to have higher embryonic infection rate compared to injecting plasmids, at least in zebrafish (16, 17). These studies would determine if inhibitors of myostatin, such as follistatin or FSTL-3 function in fish as they do in mammals.

Studies in cardiomyoblasts. Future studies should be directed to determine if FSTL-3 is responsible for the lack of cardiac phenotype in myostatin-null animals. Cardiomyoblasts could be transfected with anti-sense oligonucleotides to determine if

myostatin has enhanced effect in suppressing proliferation and differentiation of cardiomyoblasts. Another approach is to use FSTL-3 antibodies to immunoneutralize it in conditional medium of cardiomyoblasts. These studies could potentially explain why the myostatin knockout animals do not have cardiac hypertrophy.

Cardiomyoblasts express all the necessary receptor genes for myostatin signaling and myostatin expression increases in pathological conditions of the heart, especially myocardio-infarction (11). To study the significance in such pathological conditions, an *in vitro* system could be utilized. Cardiomyocytes could be cultured in the presence or absence of ischemic buffer, which simulates an infarct *in vitro*. Myostatin or FSTL-3 could be added and level of apoptosis and protein synthesis quantified. Similar studies could also be performed *in vivo* on the mighty mouse by surgically inducing an infarct and measuring markers of cardiac muscle remodeling and survivability. The studies would determine if myostatin has a protective or damaging effect on the cardiomyocytes after ischemic insult.

Myostatin suppress proliferation and differentiation of cardiomyoblasts *in vitro* as it does with skeletal muscle cells. BAMBI could be used to block its effects of myostatin in cardiomyoblasts. The advantage of BAMBI overexpression is that it is restricted to the expressing cells and is not secreted. This minimizes the potential for side effects. Delivering an overexpression plasmid using a recombinant Adenoassociated virus (AAV)-6 would infect only cardiac and skeletal muscle. If *in vitro* results are promising, these studies could be reproduced *in vivo*. Cardiac specific promoters such α-cardiac actin (18), could further limit BAMBI expression. This could prove to be very helpful, especially in treating pathological conditions of the heart.

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