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Clinical, Agricultural, and Evolutionary Biology of Myostatin: A Comparative Review

Buel D. Rodgers and Dilip K. Garikipati

Department of Animal Sciences and the School of Molecular Biosciences (B.D.R.), Washington State University, Pullman, Washington 99164; and Department of Biochemistry (D.K.G.), University of Washington, Seattle, Washington 98195

The discovery of myostatin and our introduction to the "Mighty Mouse" over a decade ago spurred both basic and applied research and impacted popular culture as well. The myostatin-null genotype produces "double muscling" in mice and livestock and was recently described in a child. The field's rapid growth is by no means surprising considering the potential benefits of enhancing muscle growth in clinical and agricultural settings. Indeed, several recent studies suggest that blocking myostatin's inhibitory effects could improve the clinical treatment of several muscle growth disorders, whereas comparative studies suggest that these actions are at least partly conserved. Thus, neutralizing myostatin's effects could also have agricultural significance. Extrapolating between studies that use different vertebrate models, particularly fish and mammals, is somewhat confusing because whole genome duplication events have resulted in the production and retention of up to four unique myostatin genes in some fish species. Such comparisons, however, suggest that myostatin's actions may not be limited to skeletal muscle *per se*, but may additionally influence other tissues including cardiac muscle, adipocytes, and the brain. Thus, therapeutic intervention in the clinic or on the farm must consider the potential of alternative side effects that could impact these or other tissues. In addition, the presence of multiple and actively diversifying myostatin genes in most fish species provides a unique opportunity to study adaptive molecular evolution. It may also provide insight into myostatin's nonmuscle actions as results from these and other comparative studies gain visibility in biomedical fields. (*Endocrine Reviews* 29: 513–534, 2008)

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Abbreviations: CHO, Chinese hamster ovary; FLRG, follistatin-like related gene; GDF, growth/differentiating factor; IGFBP, IGF binding protein; LAP, latency-associated peptide; LTBP, latent TGFβ binding proteins; MSTN, myostatin; PEMC, porcine embryonic myogenic cells; REE, resting energy expenditure; rt, rainbow trout; T-cap, titin-cap.

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I. Introduction

SINCE ITS INITIAL discovery in 1997 by Alexandra McPherron and Se-Jin Lee (1), myostatin and the myostatin-null phenotype have intrigued different scientific and pop culture communities. The now very well known "double muscling" phenotype popularized by the generation of myostatin knockout mice (Fig. 1, B and C) was already well known among animal scientists and other biologists interested in livestock production because similar phenotypes, indeed the term "double muscling" itself, had been characterized in many cattle breeds (Fig. 1A) and in the Texel sheep. The genetic basis for the phenotype in these animals, however, was only revealed with myostatin's discovery and with studies utilizing cattle that subsequently followed (2–5). The field has grown considerably over the past decade and has generated over 500 scientific articles while to date, 1137 and 642 core nucleotide and protein records, respectively, have been deposited into GenBank. To put this into perspective, this is approximately twice the number of comparable sequence records for another potent and more readily recognized regulator of skeletal muscle growth, IGF-I.

Such scientific and cultural interest is not surprising when one considers the obvious and potential impact of manipulating myostatin production and/or bioactivity in the clinic or on the farm, which has likely helped spur interest with the general public as well. Indeed, a survey of web sites using the Yahoo search engine and the keyword "myostatin" identifies

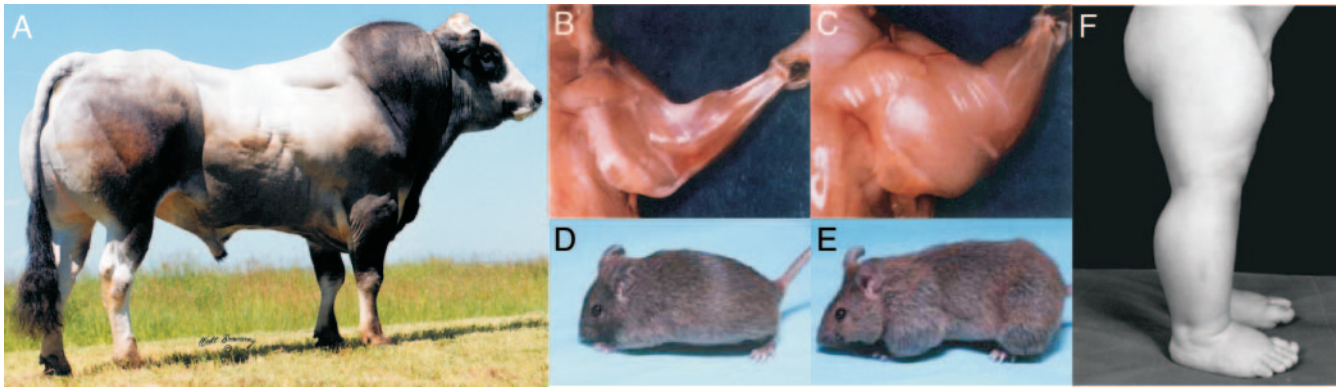

FIG. 1."Double muscling" and the myostatin-null phenotype. A, Muscle hypertrophy in Piedmontese cattle breeds is due to a missense mutation within the third exon of the bovine myostatin gene (4). [Picture reproduced with permission from the North American Piedmontese Cattle Association (NAPA, www.piedmontese.org).] B and C, Forearm musculature of wild-type (B) and myostatin "knock-out" (C) mice. [Reprinted by permission from Macmillan Publishers Ltd: Nature (Ref. 1), copyright 1997.] D and E, Wild-type (D) and follistatin transgenic (E) mice (31). [Copyright 2001 National Academy of Sciences, U.S.A.] F, Leg musculature of a 7-month-old infant boy with a null mutation within the splice donor site on exon 1 of the myostatin gene (26). [Copyright 2004 Massachusetts Medical Society. All rights reserved.]

nearly 270,000 sites. Many of these sites are not associated with scientific journals or institutions, but rather with companies that market nutritional supplements that presumably block or neutralize myostatin. Most of these products—Musclegen (Fareplant, Inc., Westerville, OH), MyoStim (Champion Nutrition, Inc., Concord, CA), Myo-Blast (Cytodyne Technologies, Inc., Manasquan, NJ), Anabol X (Pinnacle, Inc., Central City, NE), *etc*.—are composed of sulfated polysaccharides isolated from a brown marine plant, *Cystoseira canariensis*, that have been reported to bind myostatin and other cytokines (6). However, a thorough assessment of their neutralizing activity (7) suggests that they are completely ineffective *in vivo* even when administered at very high doses (1200 mg/d). Nevertheless, the pursuit of perfect form—the archetypal Vitruvian Man—whether for commercial or biomedical reasons or even for less scrupulous reasons (*e.g.*, performance enhancement) will no doubt continue to fuel interest in myostatin research.

A thorough review of myostatin biology, from a purely biomedical perspective, was published in 2004 by Se-Jin Lee (8). The goals of the current review, however, are to highlight recent advances in the field and to discuss, from a comparative perspective, how studies using a diverse array of vertebrate models have influenced our understanding of muscle biology, basic evolutionary processes, and the pursuit of novel therapies for treating muscle growth disorders.

II. Myostatin-Null and Transgenic Phenotypes

A. Murine models

Myostatin is a highly conserved member of the TGFβ superfamily and possesses all of the structural components common to the family: nine invariant cysteine residues, an "RXXR" furin-type proteolytic processing site, and a bioactive C-terminal domain (8). Its expression in mammals is limited primarily to skeletal muscle, which in mammals appears to be the principal target tissue. The muscle mass of myostatin-null cattle, sheep, mice, and humans is dramatically increased and produces a phenotype often referred to as "double muscling" (Fig. 1C). Indeed, the mass of individual muscles from myostatin knockout mice, appropriately named "Mighty mice," is often double that of comparable muscles from wild-type mice (1). Enhanced muscle growth in these animals is due to increases in both cell number, or hyperplastic growth, and cell size, or hypertrophic growth, which results in larger and heavier myofibers. Another hypermuscular phenotype was also described at approximately the same time as the mighty mouse: the "Compact" mouse. This line was generated by artificially selecting for high carcass protein content and resembled Mighty mice in many ways (9). Although linkage mapping identified only a single affected locus (*Cmpt*), maximum likelihood analysis suggested that the phenotype was not due to a single gene despite the fact that a 12-bp deletion in the myostatin gene was eventually proven responsible (10). However, subsequent mapping identified several modifying loci (11, 12) including a region that contains myogenin, a myogenic regulatory factor involved in the differentiation and maturation of skeletal muscle, which likely explains the differences between the two murine models. As expected, administering myostatin has the opposite effect and can induce muscle atrophy and a cachectic state as in transgenic mice overexpressing myostatin (13) or in mice receiving Chinese hamster ovary (CHO) cell transplants stably expressing myostatin (14).

B. Domesticated species

Similar phenotypes have also been described in some domestic breeds of cattle including the Piedmontese (Fig. 1A), Belgian Blue, and Marchigiana, all of which possess mutant alleles for myostatin (2–4). The Piedmontese is marketed as the "Myostatin Breed" because its standard and the North American Piedmontese Cattle Association's registry require proof of at least one mutant myostatin allele, which may be the first cattle registry based on a particular genotype (www.piedmontese.org). Meat from Piedmontese scores high in palatability studies and is particularly tender, more so than the other breeds (15–17). By contrast, meat from the double-muscled Texel sheep (see below) is very tough (18, 19). Thus, enhanced musculature itself does not necessarily impact

meat palatability because other genetic factors clearly contribute.

An 11-nucleotide deletion in the coding sequence of the C-terminal bioactive domain and the introduction of premature stop codons resulting from the frame-shift are responsible for enhanced muscularity in the Belgian Blue (2). This coincidentally maps to the previously characterized muscular hypertrophy (mh) locus. Double muscling in the other two breeds results from a transversion mutation (G to T) in Marchigiana myostatin (20), which again introduces a stop codon, and a missense mutation in the Piedmontese that converts a critical cysteine to tyrosine (3, 4). This particular cysteine is necessary for proper folding of mature myostatin (see *Sections III.A and B*) and is required for full bioactivity (21). A two-nucleotide deletion in the canine myostatin gene has also been described in whippets, a sight hound used in dog racing (22, 23). Dogs heterozygous for the mutation are more muscular than wild-type dogs and even run faster, which the authors suggest is the first indication of a performance-enhancing polymorphism associated with the myostatin gene. Unfortunately, dogs homozygous for the mutation are often referred to as "bully whippets" due to their extreme muscularity and are frequently destroyed by breeders.

Texel sheep are also double muscled due to a mutation in the myostatin gene. Several quantitative trait loci mapping studies for muscle, carcass, and/or fat traits identified markers close to the myostatin gene (18, 19, 24, 25). Fine mapping ultimately identified an interval that included the myostatin gene (25). Unlike all the other mutations discussed, double muscling in the Texel is due to a G to A transition mutation in myostatin's 3′ untranslated region rather than in the coding sequence. The mutation introduces a target site for specific microRNAs, mir1 and mir206. Both are highly expressed in skeletal muscle, whereas mir1 is also expressed at appreciable levels in cardiac muscle. Binding of these particular microRNAs perturbs myostatin translation and results in lower circulating levels, which in turn contributes to skeletal muscle hypertrophy. A survey of human single nucleotide polymorphism databases identified approximately 2500 polymorphisms that introduce identical microRNA binding sites in the 3′ untranslated regions of other genes. Conversely, another 2500 single nucleotide polymorphisms that destroy existing binding sites were also identified. Thus, identifying the source of the Texel's muscle hypertrophy presents a unique model system for investigating the contribution of microRNA to phenotypic variation.

C. Humans

A splice site mutation on the first intron-exon boundary of the human myostatin gene was recently described in a young boy (Fig. 1F) (26). Skeletal muscle hypertrophy was immediately evident at birth and was confirmed by ultrasonography. The child's exceptional strength was also evident because at just 4.5 yr of age, he could suspend two 3-kg dumbbells with both arms extended. Mechanistically, this mutation removes the 5′ small nuclear RNA pairing region used by the spliceosome. The first intron is not removed during pre-mRNA processing, which results in the expression of mature transcripts that include in-frame stop codons. Translation is therefore terminated prematurely and well before the bioactive domain. Several physical exams suggest that the child is developing normally and is healthy and fit. Cardiac muscle expresses activin receptors and is sensitive to myostatin's negative effects (see *Section IV.B*). However, all assessments to date indicate normal cardiac performance. Although such claims should be expressed with a modicum of skepticism, this study is the first to suggest that the successful manipulation of myostatin production and/or bioactivity in a clinical setting could have many beneficial outcomes without deleterious side effects.

D. Fish

Myostatin-null zebrafish have been described in three studies, and although the results are far from conclusive, they suggest that myostatin's effects may extend beyond skeletal muscle. Injection of antisense morpholinos was reported to enhance the rate of somite formation in developing embryos, the size of individual somites and whole embryos, and the gene expression of some myogenic regulatory factors, but it did not specifically influence myogenesis itself or muscle mass (27). None of these effects were seen in transgenic zebrafish overexpressing a dominant-negative myostatin, although a minor increase in muscle cell number was noted, but only in female fish (28). Changes in somitogenesis were also not noted in the developing Mighty Mouse (1). A third study (29) reported to have generated "giant zebrafish" by injecting double-stranded myostatin RNA from a distant and unrelated species (tilapia). Overall growth enhancement appeared to continue well past the effective half-life of the treatment and even in juvenile fish. Each of these studies was performed before the discovery of a second class of fish myostatin paralogs (see *Section IV.A*), and it is therefore difficult to determine which if any myostatin transcript was affected. Nevertheless, these studies indicate that the biological actions of myostatin in fish may not be limited to the growth and development of skeletal muscle, but may influence these processes in many tissues. Muscle growth is more limited in zebrafish than in other fish species that possess "indeterminate" growth (30). Thus, this particular model may be better suited for investigating the divergent actions of myostatin in nonmuscle tissues (see *Section V.B*).

III. Cellular Actions of Myostatin

A. Proteolytic processing and regulated bioactivity

Several myostatin binding proteins have been identified and include follistatin (31), follistatin-like related gene (FLRG; also known as follistatin like-3, FSTL-3, and follistatin-related peptide or FLRP) (32), growth/differentiation factor-associated serum protein (GASP)-1 (33), and titin (T)-cap (34). T-cap is a sarcomeric protein that binds the N-terminal domain of titin where it helps regulate the cytoskeletal organization of muscle cells. T-cap also binds myostatin presumably in the golgi and prevents its secretion. The N-terminal peptide that results from proteolytic processing of pro-myostatin also binds myostatin with high affinity, and

like follistatin, FLRG and GASP-1, can prevent receptor binding and activation (31). Such interactions commonly occur between proteolyzed fragments of TGFβ superfamily members (35), and although the exact mechanism of ligand activation has yet to be determined for myostatin, it appears to strongly resemble that of its superfamily siblings. This includes removal of the signal peptide from prepro-myostatin and proteolysis of pro-myostatin at the furin/PACE cleavage site (Fig. 2). This separates the bioactive domain from the N-terminal latency-associated peptide (LAP), which binds to the disulfide-linked myostatin dimer. The two proteins are then secreted as a small latent complex where they enter the circulation or potentially bind to the extracellular matrix forming a large latent complex. Proteolytic cleavage of LAP then releases myostatin from circulating and extracellular complexes (see Ref. 8 for a detailed review of processing events and proteases). Thus, myostatin bioactivity is not mediated *per se* by increased synthesis or release from skeletal muscle, but by three independent proteolytic events, of which the latter two may be regulated.

For most TGFβ superfamily ligands, the formation of large latent complexes requires the binding of TGFβ LAP peptides to latent TGFβ binding proteins (LTBP) (35). Anderson *et al.* (36) recently identified a large extracellular pool of unprocessed myostatin in skeletal muscle that may be mediated in part by the association of myostatin with LTBP-3. Coexpression of LTBP-3 and myostatin in a human kidney epithelial cell line (293T) sequestered myostatin within the extracellular matrix and attenuated its bioactivity, whereas the ectopic expression of LTBP-3 in mouse skeletal muscle increased fiber cross-sectional area. Another matrix-associated protein, decorin, also binds myostatin at a 1:1 ratio and with relatively high affinity (~10–20 nM Kd), but not LAP, and prevents receptor activation (37). The spatiotemporal expression patterns of both myostatin and decorin in rat skeletal muscle are similar and are consistent with decorin's ability to sequester myostatin outside the cell (38). This small leucine-rich proteoglycan binds TGFβ as well and regulates collagen fibrillogenesis and myoblast proliferation (39). Myostatin and TGFβ may help to maintain fibrosis in some forms of muscular dystrophy because both factors induce myogenic cells to differentiate into myofibroblasts (40–42). Myostatin also stimulates fibroblast proliferation *in vitro* as well as the secretion of TGFβ1 from mouse C2C12 myoblasts (42). Stably overexpressing decorin in myoblasts accelerates the differentiation rate, whereas gene transfer of decorin *in vivo* has similar effects on skeletal muscle regeneration (43), actions that are mediated by decorin sequestration of myostatin and TGFβ as well as the up-regulation of follistatin (42). Crossing *mdx* mice, models for Duchenne muscular dystrophy, with Mighty mice ameliorates much of the dystrophic phenotype including fibrosis (44). Furthermore, skeletal muscle from Mighty mice regenerates more readily than wild-type muscle and has reduced fibrosis after notexin- or laceration-induced muscle injury (42, 45). These studies indicate that myostatin may associate with the extracellular matrix independent of LAP and suggest that neutralizing the coregulatory relationship between myostatin and TGFβ with decorin could aid in the clinical treatment of some muscular dystrophies.

The nullifying effects of LAP and follistatin on myostatin bioactivity have been demonstrated quite conclusively using *in vitro* and/or *in vivo* systems, with LAP receiving the most attention. Both proteins bind myostatin and prevent receptor binding and activation *in vitro*, whereas transgenic mice overexpressing LAP or follistatin develop hypermuscularity (Fig. 1) similar to that seen in the Mighty Mouse (31, 46–49). Conversely, muscles of follistatin knockout mice are smaller (50), which is consistent with increased myostatin activity and/or muscle growth inhibition. The muscle mass of follistatin transgenic mice (Fig. 1, D and E), however, is bigger than that of LAP transgenics (31), suggesting that ligands in addition to myostatin may serve to inhibit skeletal muscle development. Potential candidates include activin and growth/differentiating factor (GDF)-11, both of which bind follistatin (51, 52), as well as activin type 2 receptors (Acvr2 and Acvr2b, also known as ActRIIa and ActRIIb), which also mediate myostatin's effects (31, 48). These results suggest that double muscling alone does not maximize the tissue's growth potential. Indeed, transgenic Mighty mice overexpressing follistatin have even greater muscle mass than follistatin transgenics alone (53), which produces muscles four times larger than those in wild-type mice (*i.e.*, "quadruple muscling"). The effects were dose dependent because muscles of follistatin transgenic mice with only a single myostatin gene were smaller than follistatin transgenics lacking both myostatin genes, but larger than those with two functional copies (*i.e.*, FS-Tg/mstn$^{-/-}$ > FS-Tg/mstn$^{-/+}$ > FS-Tg/mstn$^{+/+}$). The biggest differences were seen in offspring from myostatin-null mothers, which the authors suggest could be due to the lack of maternal transfer of myostatin or

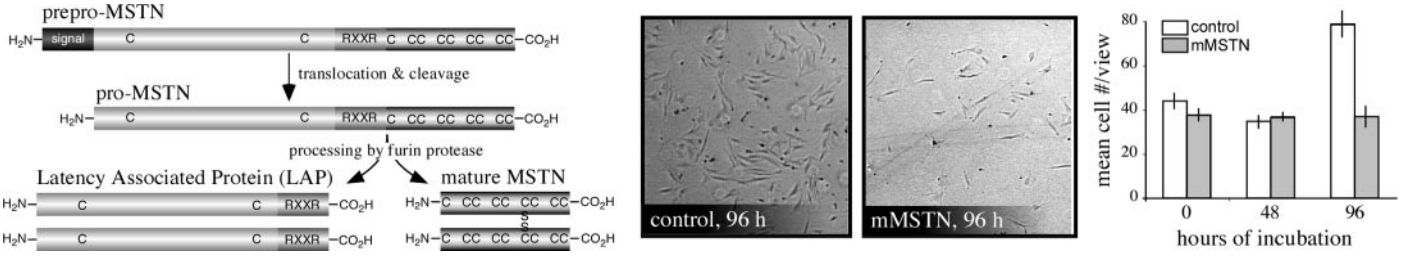

FIG. 2. Proteolytic processing of mature myostatin (MSTN) and conserved bioactivity. *Left*, Pro-myostatin is cleaved by a furin class protease at a conserved RXXR (R, arginine; X, any amino acid) epitope, and the resulting peptides dimerize via disulfide linkages at the indicated cysteines producing mature myostatin. The dominant-negative LAP sequesters myostatin dimers in a latent complex and can prohibit receptor activation. *Right*, Primary myosatellite cells from rainbow trout were incubated in 100-mm dishes with 50 ng/ml mouse myostatin (R&D Systems, Minneapolis, MN) or equimolar amounts of bovine serum albumin, and myostatin was added at 0 and 48 h. At each time point, cells were manually counted in each of six views. Mean values for each time point are shown (histogram), as are representative images.

a myostatin-regulated factor into the fetal circulation. Activin receptors are expressed throughout the placenta (54, 55), so these results could just as likely be due to the lack of placenta stimulation or even to the repartitioning of energy because the myostatin-null phenotype is also associated with reduced adiposity (1, 56–58). The results are nevertheless intriguing and suggest that normal skeletal muscle is even more plastic than originally presumed and once again that multiple factors contribute to the negative regulation of muscle development.

B. Regulation of myoblast proliferation, differentiation, and quiescence

Myostatin appears to prevent myoblast hyperplasia in mammals by inhibiting cell cycle progression past the G_1 and G_2 stages (59–61). 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 (62–64), although the teleological significance of this particular effect may appear controversial because conflicting data suggest that myostatin initiates cell cycle withdrawal, which is a necessary prerequisite for differentiation (59, 60, 64, 65). However, studies with primary myosatellite cells (also known as "skeletal muscle stem cells" located below the sarcolemma and basal lamina) from myostatin-null mice suggest that myostatin-stimulated cell cycle withdrawal is accompanied by cellular quiescence (65, 66) rather than differentiation. This explains the apparent discrepancy and supports earlier studies indicating that myostatin is a myoblast survival factor (60). A model for myostatin action in mammals suggests that in the absence of other myogenic regulators, myostatin inhibits myoblast hyperplasia by stimulating cell cycle withdrawal and delays differentiation by inducing cellular quiescence. Recent studies further suggest that myostatin-induced cellular quiescence is reversible and is associated with reduced expression of the myogenic regulatory factors Pax-3, Myf-5, and MyoD (67). It is therefore inaccurate to describe myostatin's actions as solely inhibitory. Indeed, myostatin initiates the first and necessary step in the differentiation process, cell cycle withdrawal, and prevents apoptosis of the quiescent cells. Its negative effects on myofiber hypertrophy are due in part to the inhibition of myosatellite cell activation, proliferation, and/or renewal because the fusion of these cells with existing myofibers is largely responsible for postnatal muscle growth (68). These cells are more abundant in skeletal muscle of myostatin-null mice, which proliferate more rapidly than those isolated from wild-type mice (65). Myostatin also inhibits protein synthesis in differentiated C2C12 myotubes (61). These studies together suggest that myostatin ultimately limits skeletal muscle size by inhibiting the hyperplastic growth of developing myoblasts and thus, the number of cells that eventually differentiate into mature myofibers, and by reducing myofiber protein synthesis and myosatellite cell renewal, both of which inhibit the hypertrophic growth of mature muscle.

Cell culture studies have used recombinant myostatin generated in both prokaryotic and eukaryotic expression systems and have generated similar, but not identical results. Recombinants from both systems inhibit myoblast proliferation *in vitro*; however, those generated in bacteria are functionally active only at very high concentrations (2–10 μg/ml, 100–500 μM) (59, 61, 63, 65, 69, 70). By contrast, myostatin generated in CHO cells is biologically active at concentrations 100- to 1000-fold lower (14, 46, 71). The production of biologically active myostatin in bacteria is actually quite surprising because the formation of disulfide-linked dimers (Fig. 2) requires an oxidizing environment that does not exist in bacterial cytoplasm. Myostatin recombinants generated in prokaryotic expression systems are therefore monomeric unless denatured and refolded in the presence of an appropriate oxidizing agent, which itself does not guarantee proper folding. Indeed, most of the studies using recombinant myostatin generated in bacteria attempted to refold the peptides. Proper folding is critical to biological activity because double muscling in Piedmontese cattle results from the production of misfolded peptides (3, 4) that lack a residue critical to the formation of the cysteine knot structure common to all TGFβ superfamily proteins (72–75). It appears, therefore, that only a very small fraction of myostatin generated in bacteria is biologically active because such preparations are primarily composed of either nonfunctional monomers or misfolded dimers. Whether or not the growth inhibitory effects of bacterial recombinants are mediated through Acvr2 or Acvr2b, by preventing endogenous myostatin from binding to other proteins (*e.g.*, LAP) or by cross-reacting with receptors for other TGFβ superfamily ligands is not known. The use of myostatin generated in bacteria, which is commercially available from many different sources, has helped to define basic cellular responses and has been validated to some degree by studies with recombinant myostatin generated in eukaryotic cells, which is also commercially available. However, the continued use of bacterial recombinants could ultimately prove misleading especially when used *in vivo*, with tissues other than skeletal muscle (see *Section VI*) or when trying to define mechanisms of myostatin action.

C. Receptors and signaling

All TGFβ superfamily ligands signal through membrane-bound heteromeric serine-threonine kinase receptor complexes composed of two type 1 and two type 2 receptors (76). Ligand binding to type 2 receptors recruits type 1 and both autophosphorylate via transinteractions with one another's intracellular kinase domains. The signaling pathway to the nucleus is short and quick because specific receptor (R)-Smads are recruited to the complex and are phosphorylated by type 1 receptors. The R-Smads then oligomerize with appropriate co-Smads and translocate into the nucleus. This complex directly binds promoter elements and initiates or represses gene transcription. Myostatin bioactivity is mediated by activin receptors, specifically Acvr2 and Acvr2b. Cross-linking studies and radioreceptor assays indicate that although myostatin binds both, it binds the latter with slightly higher affinity (31). Skeletal muscle mass was increased by 125% in transgenic mice overexpressing dominant-negative Acvr2b (31) and by 60% just 2 weeks after three ip injections of a soluble form of Acvr2b's extracellular do-

main (48). Mice homozygous for deactivating mutations in Acvr2 have pectoralis and triceps muscles that are 27–40% larger than the same muscles from wild-type mice (48). These muscles are just 20–26% larger in mice with mutant Acvr2b receptors, suggesting that Acvr2 may play a more important role in regulating myostatin's actions, at least in these muscles. The relative distribution of each receptor in different skeletal muscles or even within a specific muscle is not known. Thus, the contribution of each receptor may be equally relative and may differ between individual muscles or even fiber types. Myostatin activation of either receptor recruits the type I receptors activin like kinase-4 or -5 (77), which in turn phosphorylate Smads 2 and 3. These transcription factors oligomerize with Smad4 and eventually regulate gene transcription, including the expression of Smad7 and c-ski. This particular Smad is an inhibitory Smad because it sequesters Smad4 in the cytoplasm and prevents it from binding to the Smad2/3 complex (77, 78). C-ski is a corepressor that stabilizes the inactive Smad2/3/4 complex on Smad/ski binding elements (79, 80). It also appears to inhibit Smad2 and Smad3 signaling in part by directly blocking histone deacetylase activity as well as their association with a transcriptional coactivator (81, 82). Nuclear localization of c-ski is required for the differentiation of myoblasts because it enhances myogenin transactivation through direct interactions with MyoD/MEF2 heterodimers (82), which is in direct opposition to myostatin's negative effects on differentiation. These studies together suggest that in skeletal muscle, Smad7 and c-ski serve as intracellular negative feedback mechanisms for myostatin or other TGFβ superfamily ligands that signal via Smads 2 and 3.

Myostatin signaling is not limited to canonical Smad pathways because it curiously interacts with mitogenic pathways as well. Myoblast proliferation and cell cycle progression are stimulated by IGF-I, a potent mitogen for many different cell types including myoblasts. IGF-I also stimulates myoblast differentiation and the associated cell cycle arrest (83). The mechanisms of IGF-I's ability to stimulate these normally diametrically opposed cellular activities is currently under dispute and may include the local production of IGF binding protein (IGFBP)-3 (see *Section III.D*) (84–87). Nevertheless, IGF-stimulated myoblast differentiation is mediated by increased activity of the Cdk2 inhibitor p21 (88–90). Myostatin also stimulates myoblast cell cycle withdrawal and activates p21 (59, 60, 65), but unlike IGF-I it inhibits rather than stimulates differentiation. Myostatin also activates Akt (58, 91, 92) and the MAPKs extracellular signal-regulated kinase (Erk)-1/2 (93) and c-Jun N-terminal kinase (JNK) (94). The significance of cross-talk between myostatin and IGF signaling is not known, nor is it known how myostatin activates these pathways while simultaneously arresting the cell cycle. However, myostatin and IGF-I are both survival factors, and thus myostatin activation of these particular aspects of a mitogenic pathway is likely related to its ability to maintain cellular quiescence in growth-arrested cells. Indeed, blocking Erk-1/2 and JNK activation similarly blocks myostatin's ability to inhibit differentiation (93, 94).

D. Conserved action and novel insights from comparative models

Most *in vitro* studies of myostatin's actions were performed using C2C12 myoblasts. This immortalized mouse cell line was established from myosatellite cells originally isolated from dystrophic skeletal muscle (95). Although the cells maintain full myogenic potential and are capable of differentiating into contractile myotubes, they can also differentiate into osteogenic cells when exposed to bone morphogenic protein-2, another TGFβ superfamily member (96). Nevertheless, results from these studies are consistent with those using primary porcine embryonic myogenic cells (PEMC). They suggest that the C2C12 model is physiologically relevant and that myostatin's growth inhibitory actions, which have been described *in vivo* with mice, cattle, sheep, dogs, and humans (see *Section II*), are likely conserved in eutherian mammals. Myostatin and TGFβ are both expressed in PEMC (97), and both inhibit proliferation in a dose-dependent manner (98). Their expression is reduced in differentiated cells (97), whereas the cytokines also stimulate the gene expression and secretion of IGFBP-3 and -5 (98). As their names imply, the IGFBPs bind the IGFs with high affinity and in doing so can either attenuate or potentiate their action depending on the tissue, the differentiation status of the specific cell type, and the location of binding (*i.e.*, circulation or extracellular) (99). IGFBP-3 and -5, however, inhibit myoblast proliferation in both IGF-dependent and -independent manners (85, 100–103), the latter effects being potentially mediated by the nuclear localization and functional interaction with a subunit of RNA polymerase II (103). Nuclear localization of IGFBP-3 also occurs in PEMCs and increases in response to TGFβ stimulation (myostatin was not tested) (104). Furthermore, immunoneutralization of IGFBP-3 blocks myostatin's antiproliferative effects in these cells (85). An emerging model of myostatin action therefore suggests that IGFBP-3 and -5 partly mediate the cytokine's effects and that the extracellular sequestration of IGF-I and the nuclear localization of IGFBP-3 and possibly IGFBP-5 are involved.

Myostatin genes, putative promoters, and/or cDNA have been cloned in several avian species including turkey (*Meleagris gallopavo*), chicken (*Gallus gallus*), pigeon (*Columba livia*), duck (*Anas platyrhynchos*), goose (*Anser anser*), and quail (*Coturnix chirensi*) (4, 105). Initial studies with avians, for that matter with most nonmammalian vertebrates, attempted to correlate changes in myostatin expression to key periods of skeletal muscle development or to physiological responses to catabolic insult (see *Section V.B*). Although primarily descriptive, such studies were the first to suggest that myostatin's growth inhibitory actions may be conserved in skeletal muscle of avians and fish. Recent studies, however, are beginning to assess not only myostatin's actions in different vertebrates, but the myostatin attenuating actions of LAP as well. Polymorphisms in the chicken myostatin gene are associated with differences in body weight (106), whereas the immunoneutralization of myostatin in 3-d-old embryos has a small, but significant and positive effect on both body weight and muscle mass (107). Administration of polyclonal antibodies against LAP *in ovo* decreased thigh and leg

weights of post-hatch chickens, providing the first functional evidence that LAP inhibits the biological activity of myostatin in a nonmammalian vertebrate (108). Chicken embryonic myoblasts and primary myosatellite cells are both sensitive to myostatin's suppressive effects (109, 110), although myosatellite cells isolated from chicken pectoralis major are more responsive to myostatin than those from biceps femoris (110). It is unknown whether this differential response is due to tissue sensitivity *per se* (*i.e.*, receptor binding) or to tissue-specific expression of myostatin binding proteins that could potentially influence bioavailability. Nevertheless, these results suggest that muscle sensitivity to myostatin may differ between fiber types and/or muscle groups. Indeed, the degree of muscle growth enhancement in myostatin-null animals can vary considerably between muscle groups (1). The use of primary chicken embryonic myoblasts as well as myosatellite cells will therefore prove invaluable in determining the underlying mechanisms involved. Myostatin gene expression increases as primary chicken myosatellite cells differentiate into mature myotubes (111), suggesting a positive role for myostatin at some point in the differentiation process. Targeted ablation of myostatin transcripts using RNA interference reduces myostatin protein and mRNA by 55 and 75%, respectively, and concomitantly delays, rather than stimulates, differentiation (112). Silencing myostatin in differentiating cells ultimately produced myotubes that were morphologically distinct from control cells. These data further suggest that myostatin's growth inhibitory actions are natural to the differentiation process. They also once again illustrate the utility of the primary chicken myosatellite cell culture model.

Skeletal muscle growth in mammals and avians is primarily limited to increases in cell size, rather than to increases in cell number. Although undifferentiated myosatellite cells contribute to hypertrophic growth after muscle injury or exercise, their numbers remain relatively constant after birth (113, 114). Most fish species, however, have abundant myosatellite cells because hyperplastic growth accounts for approximately 50% of the changes in growing fish skeletal muscle (115). Primary myosatellite cells are readily isolated from adult fish and are easily cultured *in vitro* (116–121). These cells proliferate and fully differentiate into mature multinucleated myotubes and express skeletal muscle-specific markers that are recognized by mammalian antisera (122–124). We are now exploiting this underutilized model system to determine whether myostatin's actions are conserved in more distant vertebrates. Indeed, recombinant mouse myostatin (MetaMorphix, Inc., Beltsville, MD) inhibits the proliferation of primary myosatellite cells isolated from rainbow trout (Fig. 2). The recombinants were generated in CHO cells and were used at concentrations equivalent to the physiologically relevant concentrations used with mammalian systems (14, 46, 71). Thus, myostatin's most fundamental biological action, the growth inhibition of proliferating skeletal myoblasts, appears to be widely conserved among several, if not all, vertebrate classes. Extensive studies are nevertheless needed to determine the degree of conservation and the underlying mechanisms involved as several aspects of myostatin biology in the fishes appear to be quite different from those in mammals. Some of these differences question whether myostatin's role in fish, and possibly mammals as well, is truly limited to skeletal muscle. They also illustrate the need for both *in vitro* and *in vivo* assessment of myostatin action in lower vertebrates.

IV. Evolution of the Myostatin/GDF-11 Gene Subfamily

A. Phylogenetic analysis

Although the most influential and defining functional studies have been performed with medically important model systems, a surprising number of myostatin sequences have been described in different vertebrate species not normally associated with biomedical research. These naturally include commercially important mammalian species, although many nondomesticated species are also represented, most of which are bony fish (*Osteichthyan*, not *Chondrichthyan*). In fact, after accounting for the redundancy of sequences for a particular species, the numbers of fish and mammalian species with CoreNucleotide sequences in GenBank are about the same. Interest in fish, however, is not purely academic. Over 20,000 fish species are currently known, and many are potential candidates for culture. Assuming that myostatin's actions are conserved in these species, the successful manipulation of myostatin expression and/or bioactivity could significantly impact the aquaculture industry, if not revolutionize it. Skeletal muscle in fish constitutes a far greater percentage of total body weight than it does in mammals, and seemingly minor improvements in animal growth can disproportionately enhance product yield. Many technological barriers, however, still exist within the aquaculture industry due in many instances to a poor understanding of fish biology. In fact, many cultivars have not been heavily selected for commercially important traits such as growth, and none have been selected to the degree of most domestic mammalian species. Thus, the potential impact of enhancing muscle growth by disrupting myostatin's actions is likely far greater in fish.

A zebrafish homolog was the first fish sequence to be described (4), although sequences from commercially important species were first described at the fourth International Symposium on Fish Endocrinology in 2000 (125, 126). The following year could accurately be described as the "Year of the Fish" because 16 myostatin homologs were described from 12 different species (127–133). Thus, some fish species expressed two myostatins from distinctly different loci. These additional genes were presumed by some to be ancestors of GDF-11, a structurally similar and highly conserved TGFβ superfamily sibling. However, the different fish myostatins lacked epitopes characteristic of mammalian GDF-11 peptides. Indeed, GDF-11 homologs have since been described in zebrafish (134) and can be identified by BLAST searches of different fish genomic databases. Thus, the additional myostatins arose independently and after the divergence of myostatin and GDF-11. This was subsequently supported by an extensive phylogenetic analysis of the entire myostatin/GDF-11 subfamily (135).

Alignments of myostatin amino acid sequences from multiple vertebrate species indicate that the proteins are fairly

well conserved overall, particularly within related taxa (132). Sequences are best conserved within the carboxy-terminal bioactive domains (88–100%) and differ significantly in their respective LAP domains (50–90%). It is unknown, however, whether the intermolecular interactions that occur between mammalian myostatin and LAP peptides also occur in other vertebrates. Subtle, yet notable, differences within the bioactive domains of fish myostatin (MSTN)-1 and -2 orthologs exist and include methionine for lysine and histidine for arginine substitutions in the carboxy-terminal domains of fish MSTN-2 proteins. The former substitution occurs in a region hypothesized to have contributed to enhanced musculature in domesticated and wild bovids (4, 132, 136). Myostatin's actions are mediated by binding to activin receptors (31, 77), and perturbations in activin signaling increases muscle mass (14, 31, 48). The carboxy-terminal domain of activin is nearly identical to that of myostatin and is critical to receptor activation (137, 138). Thus, differences within this domain could potentially impact bioactivity. Future functional studies are therefore needed to determine whether MSTN-1 and -2 proteins bind Acvr2 or Acvr2b equally or to other receptors.

Three of the original cloning studies that reported novel fish myostatins constructed neighbor-joining trees to evaluate myostatin phylogenies (127, 128, 133). Each of these studies sampled only a limited number of sequences and employed only elementary methods, a Poisson-corrected distance matrix calculated from amino acid sequences, that are known to underestimate distances with increased sequence divergence (139). By contrast, a more rigorous model-based approach was later conducted and included substantially more sequences and outgroups (135). This study also analyzed nucleotide sequences aligned to an inferred amino acid alignment, which allowed for a more detailed exploration of clade membership and timing of gene copy duplication. The analysis identified two myostatin sister clades, MSTN-1 and MSTN-2, within the teleosts that arose from a well-characterized genome duplication event (D1 in Fig. 3) in the fish lineage (140, 141). This likely occurred before the divergence of teleosts or recently thereafter because MSTN-2 homologs have been identified in two separate teleost Superorders: *Acanthopterygii* and *Ostariophysi*. The recent tetraploidization of salmonids (142) resulted in an additional duplication, and the production of two more paralogs (D2 in Fig. 3). Barring losses within specific taxa, this study indicates that all fish should possess at least two myostatin genes, whereas salmonids should have four, namely MSTN-1a, -1b, -2a, and -2b. Indeed, all four genes were identified in rainbow trout (143, 144) and recently in Atlantic salmon (145).

These analyses revealed that the vast majority of previously described fish myostatin genes were actually MSTN-1 orthologs despite names that suggested otherwise. The former nomenclature was extremely misleading and, for the most part, was based on the order by which each gene was identified rather than true phylogenetic relationships. This resulted in MSTN-1 orthologs inaccurately being named MSTN-2 and vice versa. A small consortium of comparative biologists in the field, including the current authors, therefore proposed a standardized nomenclature for the entire myostatin subfamily that is based solely on the phylogenies

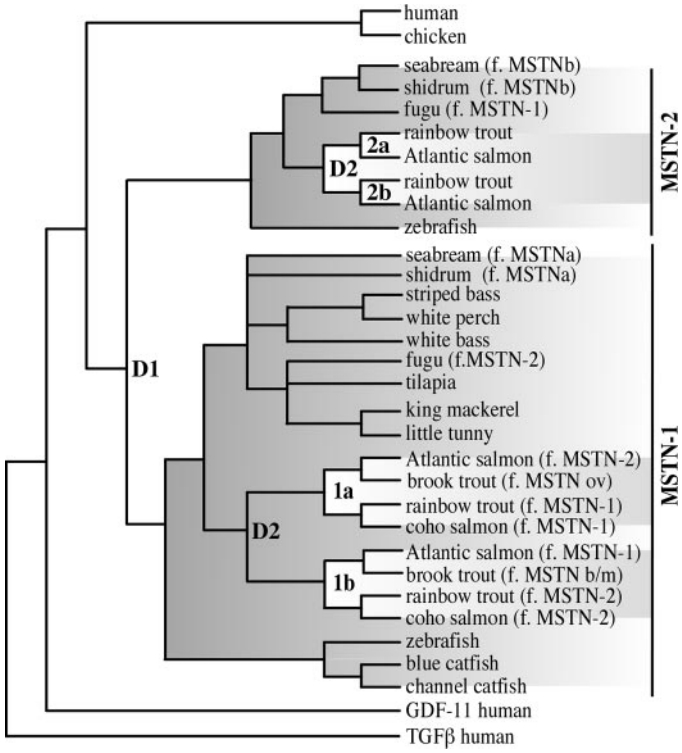

FIG. 3. Phylogenetic relationship of vertebrate myostatin (MSTN) homologs. The tree was constructed from previously published Maximum Likelihood and Bayesian Inference analyses (135, 144, 145). Clades for the two teleost fish paralogs, MSTN-1 and MSTN-2, are *shaded*. Within each clade are the additional salmonid paralogs (1a, MSTN-1a; *etc.*), which are indicated by *reverse shading*. Genome duplication events within bony fish and salmonid lineages are indicated as D1 and D2, respectively. The nomenclature for the fish homologs has been revised (146). Thus, the former (f.) names are indicated in *parentheses* (ov, ovarian; b/m, brain/muscle; GDF, growth/differentiating factor; TGF, transforming growth factor).

described (146). A description of the specific genes whose names have been changed as well as their respective GenBank accession numbers is included in Table 1. The nomenclature revisions also included the adoption of "myostatin" as the official gene name, rather than "GDF-8" (a known alias), and all of the recommendations were ultimately accepted by the Mouse Genomic Nomenclature Committee and the Human Genome Organization (HUGO) Gene Nomenclature Committee, which subsequently revised their databases. This change was also propagated to other public databases including those managed by the National Center for Biotechnology Information (NCBI) and Ensemble.

Putative homologs of myostatin have also been described in two invertebrates, the fruit fly (*Drosophila melanogaster*) (147) and bay scallop (*Argopeten irradians*) (148), and in two chordates, the urochordate sea squirt (*Ciona intestinalis*) (148) and the cephalochordate amphioxus (*Branchiostoma belcheri tsingtauense*, also known as lancelets) (149). The notion that these genes could represent ancestral forms of vertebrate myostatin and GDF-11 genes is intriguing because putative homologs for GDF-11 have not been specifically identified in any of these species. However, their coding sequences are only minimally conserved with other vertebrate myostatin or GDF-11 homologs (~35–40%), and the genomic organization

TABLE 1. Revisions to fish myostatin gene nomenclature

Species	MSTN-1			MSTN-2		
	Suffix	Alias	GenBank accession no.	Suffix	Alias	GenBank accession no.
Atlantic salmon	a/b	2/1	AJ344158/AJ297267			
Blue catfish			AY540992			
Brook trout	a/b	ov/bm	AF313912/AF247650			
Channel catfish			AF396747			
Coho salmon	a/b	1/2	AY434465/AF394687			
Fugu		2	AY445322		1	AY445321
King mackerel			AF317667			
Little tunny			AF317666			
Rainbow trout	a/b	1/2	AF273035/AF273036	a/b		DQ417326/DQ417327
Seabream		a	AF258448		b	AY046314
Shidrum		a	AF316881		b	AY059386
Striped bass			AF290910			
Tilapia			AF197193			
White bass			AF197194			
White perch			AF290911			
Zebrafish			AY258034			AY687474

Alias, Former name; ov, ovarian; bm, brain/muscle.

of each gene also substantially differs (see *Section V.A*). It is difficult, therefore, to determine whether these genes are true myostatin/GDF-11 ancestors or whether they are simply novel TGFβ superfamily homologs. More in-depth phylogenetic analyses that include myostatin and other TGFβ superfamily genes from basal vertebrate groups and from representative chordates will help determine the true identity of the ancestral gene as well as the timing of gene duplication.

B. Impact of natural and artificial selection

Four studies have investigated the impact of selection pressures on myostatin coding sequence polymorphisms and have discovered evidence for naturally occurring positive selection that may have influenced the evolution of modern bovids (136) and potentially salmonids (145). As with many gene families, multiple duplications have provided genetic materials for such evolutionary processes as differential selection, pseudogenization, subfunctionalization, and neofunctionalization, all of which contribute to functional divergence (150–155). Liberles *et al.* (156) and Tellgren *et al.* (136) identified several instances of positive (diversifying) selection in bovids, some of which corresponded to similar regions in fish myostatins (132). Analysis of nonsynonynmous/synonymous nucleotide substitution rates indicated that selection occurred during the divergence of modern bovids and, therefore, was not a product of artificial selection among domesticated species. However, Pie and Alvares (157) found insufficient evidence for selection in these lineages, although the differing results are likely due to differences in analytical methods. Where positive selection has been found, early counting methods (158, 159) or maximum likelihood methods (160) were employed and have been criticized for not robustly adjusting for codon usage bias (*e.g.*, counting methods) (157, 161) or for their susceptibility to producing false-positives (157, 162). However, similar methods also identified evidence for positive selection among salmonid myostatins (145), supporting the conclusion that strong evolutionary forces have helped influence the functionalization of myostatin in different vertebrates and quite possibly the functional divergence in salmonids.

As predicted by Kerr *et al.* (135), four myostatin genes were identified in rainbow trout (rtMSTN-1a, -1b, -2a, and -2b) (143, 144). These genes are differentially expressed (see *Section V.B*), possess clade-specific polymorphisms, and have distinct promoter regions. The Atlantic salmon MSTN-2 orthologs were also recently identified (145), and our lab has cloned orthologs of all four genes from many salmonid groups, including the genus *Thymallus* (grayling), which belongs to a subfamily separate from the more readily recognized *Salmoninae* subfamily (trout and salmon), and in every taxa, MSTN-2b is a pseudogene (see *Section V.A*). Ostbye *et al.* (145) recently calculated the nucleotide substitution rates of a very small subset of salmonid myostatins and determined that the MSTN-1 genes, most likely MSTN-1a, evolved under strong positive selection, whereas MSTN-2b evolved under relaxed selection. Expanded sampling of salmonid lineages will provide a well-resolved myostatin gene family phylogeny, particularly by incorporating coding and noncoding sequences with rigorous likelihood-based analyses. This will provide a robust framework for the analysis of selection patterns in terms of coding regions as well as branch location. The MSTN-2b paralogs have clearly become nonfunctional, although it is not known whether this event predates lineage diversification or if it occurred more recently. Nevertheless, further analysis of myostatin gene phylogenies will help explain overall patterns of functional divergence, particularly as the salmonid genes are currently diverging, and will provide a highly unique opportunity to investigate the underlying mechanisms of molecular evolution in "pseudo real-time".

V. Functional Divergence and Comparative Genomics of Myostatin

A. Genomic organization

Myostatin genes have been mapped in several vertebrate species, albeit to different degrees of resolution, and are located on chromosomes 2 in humans (2q32.2), chimps (2B), cattle (2q14-q15), and sheep, 1 in mice (C1.1), 9 in rats (9q22), 15 in pigs, 18 in horse, 37 in dogs, and 7 in chicken (7p11) (2,

3, 5, 10, 12, 19, 163–166) (see also Entrez Gene at www.ncbi.nlm.nih.gov/sites/entrez?db=gene). The fish MSTN-1 and -2 genes have also been cloned in zebrafish (28, 135) and map to chromosomes 9 and 22, respectively (see Entrez). They are also located on separate chromosomes in the green-spotted pufferfish (*Tetraodon nigroviridis*), chromosomes 2 and 3, which share more duplicated genes than any other chromosome pair, and are putative paralogs themselves (167). These data are consistent with the phylogenetic distribution of the two fish myostatin clades and further suggest that MSTN-1 and -2 arose from an early genome duplication event that occurred approximately 350,000,000 yr ago (140, 141). Ostbye *et al.* (145) recently mapped Atlantic salmon MSTN-1a and -1b to separate linkage groups indicating that these paralogs, and likely the MSTN-2a and -2b paralogs as well, arose from the recent (25,000,000–100,000,000 yr ago) tetraploidization of the salmonid genome (142), which again is consistent with the well-described phylogenies (135, 144).

In all vertebrate clones to date, the genomic organization includes three similarly sized exons separated by two intervening introns whose sizes appear to be conserved (~2 kb) in birds and mammals (Fig. 4). Intron sizes are smaller among salmonid MSTN-2 genes (only rainbow trout shown), which is consistent with an increased susceptibility to relaxed selection because the probable development of null mutations within coding regions is greater among genes with smaller noncoding regions. Both rainbow trout and Atlantic salmon MSTN-2b paralogs contain in-frame stop codons in their first exons and lack a 51-bp cassette from the second exon that is common to all other fish MSTN genes. It is unknown whether similar indels and polymorphisms exist in myostatin genes from other salmonid species. However, these data suggest that only three of the four myostatin genes are functionally active in modern salmonids because rtMSTN-2b appears to be a pseudogene. At first glance, the putative *Drosophila* myostatin/GDF-11 homolog, myoglianin, appears to possess a genomic organization similar to the fish MSTN-1 genes (147). It also contains three exons and is approximately the same size. However, the ratio of coding to noncoding sequence is significantly different because myoglianin has smaller introns and a disproportionately large first exon that is almost the same size as the entire coding sequence for each fish gene. The putative amphioxus myostatin/GDF-11 gene differs considerably from other known and putative ho-

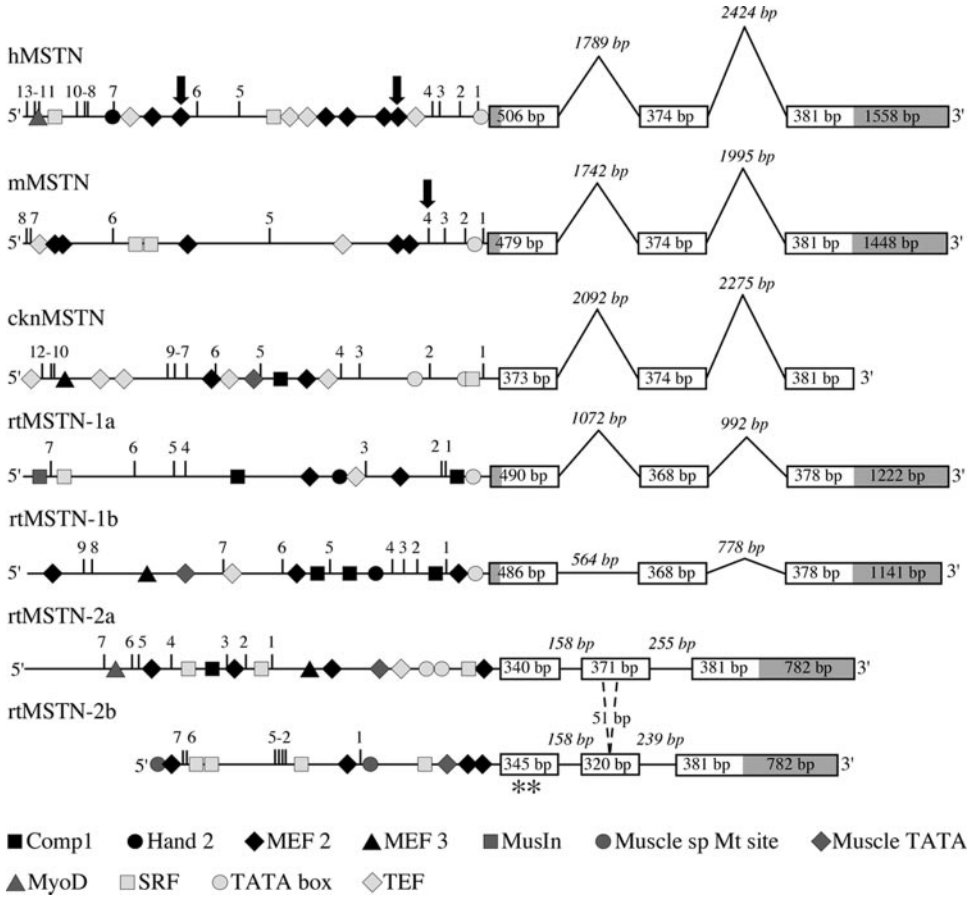

FIG. 4. Comparative mapping of vertebrate myostatin genes and promoters. The genomic structure and organization of human (h), mouse (m), chicken (ckn) and rainbow trout (rt) myostatin (MSTN) genes are shown. Exons are *boxed* with open reading frames in *white* and untranslated regions in *gray* (if known). Exon sizes are indicated *within the boxes*, intron sizes in *italics*. Two in-frame stop codons within rtMSTN-2b are indicated by *asterisks*, and a 51-bp cassette missing from the second exon is indicated by *dashed lines*. The locations of putative *cis* elements within each gene's promoter region (−2.4 and −1.5 kb for rtMSTN-2a and -2b, respectively; −2 kb for others) are indicated by the *shapes in the key*; only known myogenic elements are shown. Putative E-boxes are *numbered* and motifs determined to be functionally active in the human and mouse promoters are indicated by *arrows*.

mologs because it is much larger, over twice the size of fish MSTN-1 genes, and contains five exons of variable sizes (149). Amphioxus is the closest extant relative of vertebrata and is an important model for understanding the evolution of all chordates including vertebrates. If the amphioxus gene is indeed a true ancestor, then substantial changes in the genomic organization must have occurred subsequent to the rise of modern vertebrates. Further studies are therefore needed to clarify the phylogenetic relationship between the vertebrate and nonvertebrate homologs.

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, whereas the codons flanking the second and third exons are highly conserved among all vertebrate species (Fig. 5). A previously identified consensus sequence (143) for the codons flanking the first and second exon, MAT(E/K) - PXXI (X= any amino acid), was slightly different from a recently described consensus (144) that incorporated the rtMSTN-2 proteins, MAT(E/K) *vs.* MA(T/K). By contrast, the consensus between the second and third exons, (G/E)(E/D)GL - XPFϕ (ϕ + hydrophobic amino acid), is consistently found in all vertebrate myostatin homologs to

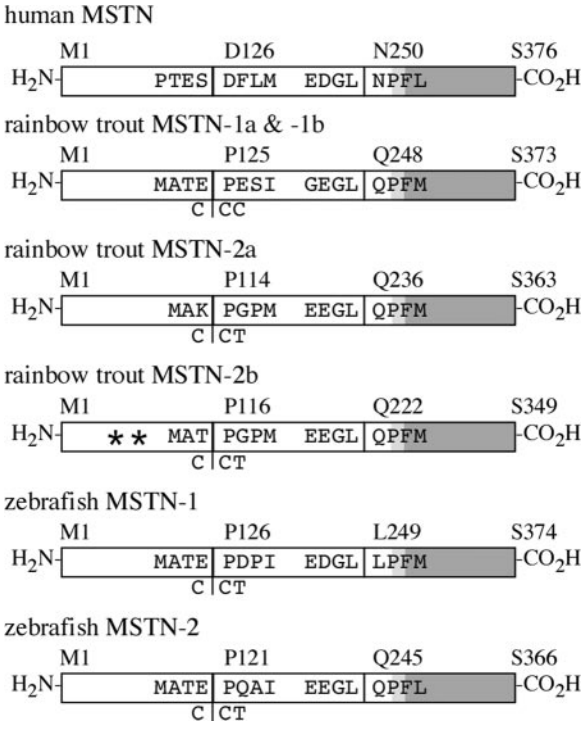

FIG. 5. Comparative mapping of myostatin coding domains. For each gene, *sequential boxes* represent coding domains from each of three exons. The first amino acid coded by each exon and the last of each protein are shown above. The sequences adjacent to each exonic boundary are shown *inside the boxes*. In all 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. In-frame stop codons in the rainbow trout myostatin (MSTN)-2b paralog are indicated with *asterisks*. The location of each RXXR proteolytic processing domain is indicated in *light gray*, and the highly conserved domain that is eventually processed into the mature myostatin dimer is in *dark gray*.

date (Fig. 5). Furthermore, the mature myostatin peptide, as well as the precluding RXXR (Fig. 2) proteolytic processing site, is encoded by the third exon in all vertebrate homologs. The high level of conservation among vertebrate myostatin genes is therefore reflected not only in coding sequences, but in overall gene organization as well.

Coding and noncoding regions of a genome evolve at different rates (168), and as a result, alignments of noncoding regions are often difficult to interpret and sometimes to even perform. Indeed, homologous promoters with similar functions in different species often do not align. However, subsequence analysis can identify consensus sequences of known motifs within noncoding regions including gene promoters. Such analyses identified a rich distribution of putative muscle-specific transcription factor binding sites in the promoters of different vertebrate myostatin genes (Fig. 4), including some of those previously determined to be functionally active in humans, mice, and cattle (169–172). Earlier studies indicated that myostatin transactivation is stimulated by the activated glucocorticoid receptor and MEF2 in humans (169) and by MyoD and Myf5 in mice and cattle (170, 171). More recent studies indicate that FoxO1 and Smad2, -3, and -4 also directly stimulate myostatin gene expression (172). Although glucocorticoids appear to mediate some stress-induced increases in mammalian myostatin gene expression (173, 174), stress and/or glucocorticoids were reported to have the opposite effect in two fish species (175, 176) and in chickens (177). These reports were among the first indications that myostatin promoters have significantly diverged in mammals. The value of *in silico* comparisons, however, rests in the ability to identify such evolutionary changes in promoter organization and to predict possible differences in activity. Functional significance of these differences, however, must be directly assessed. Nevertheless, gene expression patterns can be correlated to changes in promoter structure. For example, myostatin genes that are readily expressed in skeletal muscle, all except for the rtMSTN-2 genes, contain several E-box motifs within the proximal half of the promoter. These motifs are potential binding sites for basic helix-loop-helix (bHLH) transcription factors and are necessary for transactivation *in vitro* and *in vivo* (170). Many of these factors, including MyoD and Myf5, heterodimerize with E-proteins to promote myogenesis by stimulating the transactivation of genes necessary for muscle cell determination, differentiation and, maturation (178). The zebrafish MSTN-2 promoter was similarly analyzed and was found to also lack E-box motifs in this region, whereas the zfMSTN-1 promoter contained 6 (135). This is consistent with other fish myostatin genes because the MSTN-1 orthologs are all highly expressed in skeletal muscle, whereas the MSTN-2 orthologs are not (see *Section V.B*).

The structure and distribution of putative *cis* elements in the rainbow trout gene promoters revealed both clade- and gene-specific differences (Fig. 4). Although the rtMSTN-1 promoters are structurally similar and share putative Comp1, HAND2, MEF2, MusIn, SRF, and TEF-1 binding sites, they differ significantly from both rtMSTN-2 promoters, which themselves are also quite different. This does not suggest that these specific elements are biologically active, but rather that the promoters of these closely related genes

are indeed diverging. Mutations in the rtMSTN-2b promoter would be inconsequential because the altered expression of a pseudogene would not be phenotypically expressed. The expression of fish MSTN-2 genes is far more limited than that of the MSTN-1 genes (see *Section V.B*) and appears to occur mostly in the brain. Thus, selective pressures have profoundly influenced the evolution of myostatin gene promoters independent of their effects on coding sequences. This resulted in the divergence of MSTN-1 and -2 expression patterns, which occurred after the *Actinopterygian-Sarcopterygian* split (140, 141), as well as after the presalmonid diversifying tetraploidization (142) because the promoters and expression patterns of the salmonid MSTN-1 genes are similar, but not identical.

Sequence differences in noncoding regions can also contribute to functional divergence by mechanisms qualitatively different from those in coding regions and are based on two fundamental hypotheses (179): changes in *cis* regulatory elements 1) are more likely to have phenotypic consequences, and 2) are more sensitive to selection pressures. Environmental factors and physiological responses greatly and rapidly influence gene transcription, whereas changes in protein structure occur more slowly. Mutations in *cis* elements can also be codominant (180, 181) and are thus more sensitive to natural selection because these changes are often expressed in heterozygotes, whereas similar changes in coding regions are usually recessive (182, 183). Thus, polymorphisms in orthologous gene promoters contribute to functional divergence by substantially changing gene expression patterns that ultimately affect fitness. Indeed, differences in promoter activity are inherently indicative of functional divergence because altered expression in different tissues or development stages similarly alters function. Although the precise function for each fish myostatin paralog is not known, they are clearly diverging because the gene expression patterns are very different. Further analysis of myostatin function and molecular evolution will provide a unique opportunity to better understand fundamental mechanisms of evolutionary change. They may also help understand novel functions for myostatin in other vertebrates, including mammals, because recent studies indicate that myostatin expression in mammals is more diverse, and more similar to fish, than originally presumed.

B. Differential gene expression

Surprisingly little is known about myostatin expression in developing mammalian embryos. It is first detected in the myotome compartment of developing mouse somites (1) and presumably continues in developing myogenic cells. By contrast, many studies have both qualitatively and quantitatively assessed myostatin expression in developing embryos of different fish species (27, 28, 127, 130, 133–135, 143, 144, 175, 184–187) and in chickens (188–190). The most comprehensive of these studies used extensive RNA panels and gene-specific "real-time" assays to correlate expression levels of all myostatin genes in rainbow trout and zebrafish to key ontological events (143, 144, 187). All transcripts were detected in unfertilized and newly fertilized embryos. This is consistent with maternal deposition and with the expression of MSTN-1 and -2 genes in other fish species and even in chicken embryos (188–190). It also suggests that myostatin plays a significant role during early development, but not during gastrulation because all studies to date report a rapid decline in myostatin expression during this stage. Expression levels of all MSTN-1 genes in both fish species progressively increased during somitogenesis, which is consistent with myostatin's myogenic role in mice. However, myostatin is also expressed in many developing chicken tissues and in most adult fish tissues (see below). Further studies are clearly needed to determine whether this dynamic regulation of myostatin expression occurs in mammalian embryos and whether myostatin is also expressed in developing mammalian tissues other than skeletal muscle. If conserved, the different fish model systems, particularly zebrafish, will prove invaluable to elucidating myostatin's different developmental functions.

Initial studies suggested that in adult mammals, myostatin expression was limited primarily to skeletal muscle (1), although subsequent studies identified low levels of expression in mammary gland (191) and heart (192). By contrast, many nonmammalian vertebrates do not share this limited expression pattern because myostatin mRNA and/or protein is expressed in most fish tissues (28, 125, 129, 131, 133, 175, 184, 185, 187, 193, 194) and in many different developing chicken tissues as well (188, 195, 196). In fish, the MSTN-1 genes are widely expressed, whereas MSTN-2 expression is more limited and occurs mostly in the brain (127, 135, 144, 145, 187, 197). Expression of the former is also dynamically regulated during development, whereas MSTN-2 expression changes very little (127, 144, 187). These data strongly suggest that in fish, myostatin's actions are not restricted to the negative regulation of skeletal muscle growth and development, but may additionally influence these processes in many other tissues through the differential expression of each paralog. The similarly ubiquitous expression pattern in chickens suggests that myostatin's more limited expression pattern in mammals evolved more recently. A more thorough analysis of myostatin's tissue-specific expression pattern in other avians is needed to determine whether limited expression in general is unique to mammals. Nevertheless, the comparative analysis of myostatin expression suggests that myostatin originally functioned as a general differentiation factor, which is consistent with the wide distribution of activin receptors in nonmammalian vertebrates (198–201), and recently adopted a more specialized role in mammals.

Despite what appears to be a mostly skeletal muscle phenotype in the Mighty Mouse, growing evidence suggests that myostatin may influence other mammalian tissues as well. Indeed, the initial report by McPherron *et al.* also identified myostatin expression in adipose tissue (1), although this and possibly other phenotypes were likely overshadowed by the extreme nature of the enhanced musculature. The ubiquitous expression pattern of myostatin in fish and chicken together with its expression in mammalian adipose tissue, hearts, and mammary glands suggests that its expression and in turn, its function, may not be as limited in mammals as originally presumed. In fact, Helterline *et al.* (187) identified myostatin expression in mouse spleens, and a recently published genomewide survey of the mouse brain transcriptome iden-

tified several regions where myostatin expression occurs (202, 203). A "neuroinformatic pipeline" for assessing *in situ* expression and localization of neural transcripts was recently developed by the Allen Brain Institute and is available to the general public (www.brain-map.org). Using this three-dimensional tool, myostatin expression was mapped to many brain regions that are clearly distinguishable from GDF-11 (Fig. 6). Myostatin is expressed evenly throughout the hippocampal formation and the cerebral cortex, including the cortical plate. Within the olfactory areas, myostatin expression is confined to the piriform and cortical amygdalar areas. By contrast, GDF-11 is primarily expressed in the main olfactory bulb, the thalamus, and dorsal nuclei of the cerebral cortex above the cortical plate. Analysis of expression levels and density within specific brain regions further demonstrates distinctly different spatial expression patterns for each gene (Fig. 7). It is therefore highly unlikely that either expression pattern was influenced by cross-hybridization of probes because both patterns are unique. The functional significance of myostatin in the brain is currently unknown. However, GDF-11 appears critical to proper neural development (see *Section VI.C*) (204), and thus myostatin may possess overlapping and compensatory roles. Recent studies also indicate that myostatin influences cardiac muscle growth processes (91, 192, 205–208) and adipogenesis (57, 209, 210) in mammals. Thus, some of myostatin's more ubiquitous functions may not have been entirely lost in mammals.

C. Alternative processing

Processing of pre-mRNA involves the removal of intronic sequences and modifications to the 5′ and 3′ ends. This is coordinated by small nuclear RNA that directs the spliceosome to target sequences within the primary transcript. Alternative splicing, the generation of multiple transcripts from a single unprocessed pre-mRNA, occurs with poorly conserved splice sites and is influenced by *cis* and *trans* elements (sequences within the transcript and their binding proteins, respectively) that either enhance or silence spliceosomal activity. It also explains how over 60,000 transcripts are generated from approximately 30,000 genes in most vertebrates and is the primary source of protein complexity (211–213). Alternative splicing of myostatin transcripts has been described in rainbow trout (144) and in developing chicken embryos (189). Although the precise functional significance remains to be determined for both animal models, preliminary evidence suggests that in rainbow trout, it enhances myostatin's effects in the brain, whereas in the chicken it helps control bioavailability.

Both intact and truncated myostatin transcripts are expressed in chicken embryos (189). The latter lacks the coding sequence for the C-terminal mature peptide. Translation of this transcript would therefore block myostatin bioavailability because only the LAP domain would be produced. In rainbow trout, MSTN-2a and -2b are lowly expressed in most tissues except for brain where rtMSTN-2a is highly expressed (144). Nearly all tissues express mostly unprocessed tran-

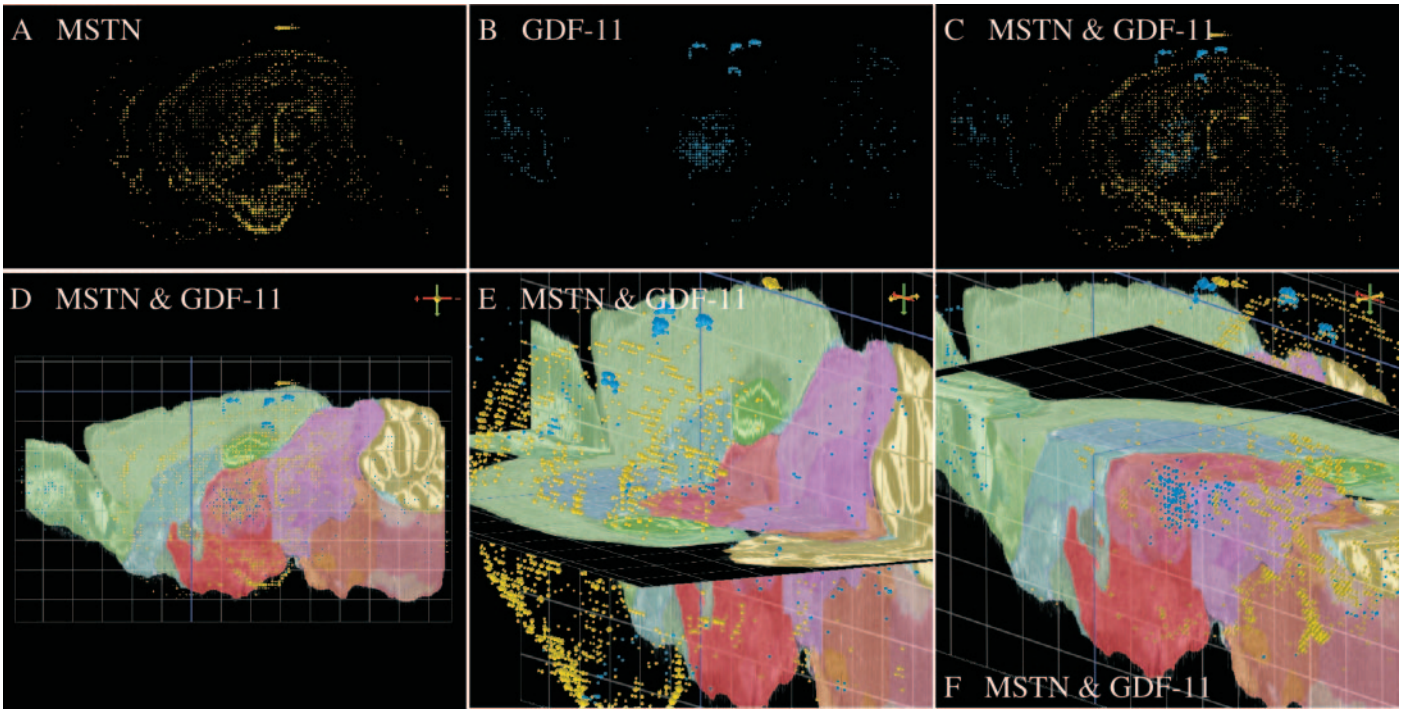

FIG. 6. Differential localization of myostatin and GDF-11 expression in mouse brains. Expression of both genes was determined by the Allen Brain Atlas project (202, 203) using *in situ* hybridization. Brains from adult (56-d-old) male C56/B57 mice were hybridized with gene-specific probes as described (www.brain-map.org). Three-dimensional expression patterns were reconstructed using Brain Explorer 1.4 and sagittal section data files, both of which are available at the indicated website. A and B, Individual expression patterns of myostatin (MSTN) and GDF-11 expression, respectively. C, Overlay of A and B. D, Combined expression patterns on top of saggital nissl (*left to right*) with color-coded anatomical features (see website for key). E and F, Three-dimensional images of combined expression patterns with sagittal and horizontal nissls. Image and nissl orientation is indicated by the color-coded compass in *upper right* of panels D–F.

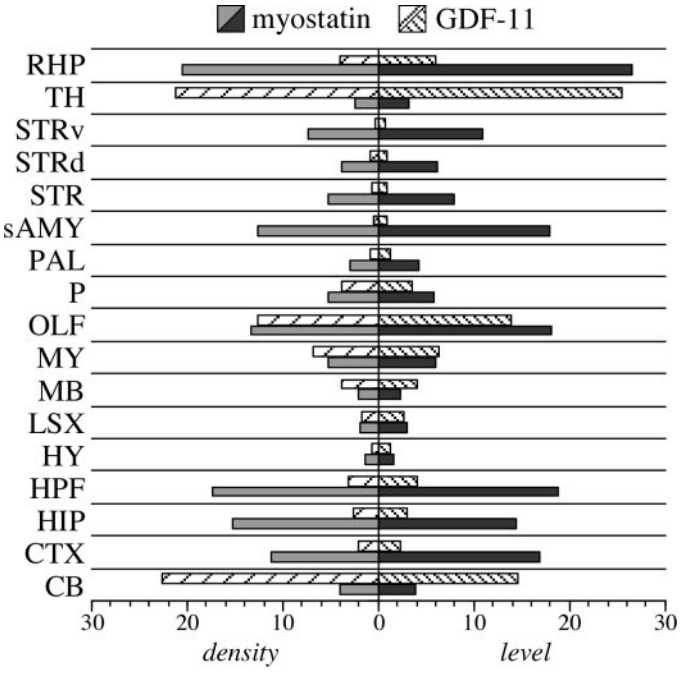

FIG. 7. Expression levels of myostatin and GDF-11 in different brain regions. Expression of both genes was determined by the Allen Brain Atlas project (202, 203) using *in situ* hybridization and gene-specific probes. Levels of expression were replotted from data available at www.brain-map.org and are scaled to normalized densities (number of expressing cells/anatomical space) and intensities (total level of expression/anatomical space) of 0 to 100. GDF, Growth/differentiation factor; RHP, retrohippocampal region; TH, thalamus; STRv, ventral striatum; STRd, dorsal striatum; STR, striatum; sAMY, striatum-like amydalar nuclei; PAL, pallidum; P, pons; OLF, olfactory bulb; MY, medulla; MB, midbrain; LSX, lateral septal complex; HY, hypothalamus; HPF, hippocampal formation; HIP, hippocampal region; CTX, cerebral cortex; CB, cerebellum.

scripts that retain both introns, which themselves contain several in-frame stop codons. By contrast, rtMSTN-2a, but not -2b, is fully processed in the brain. Tissue-specific alternative processing is rare and is mediated by *cis* recognition of tissue-specific proteins that bind to repeating motifs in the pre-mRNA (214). It occurs most often in the brain where it is commonly mediated by Nova proteins that act as splice enhancers or silencers, depending on their proximity to the splice site, and is required with transcripts possessing poorly conserved splice sites (215–218). Nova proteins recognize YCAY motifs and are known to enhance the alternative splicing of two neurotransmitter receptors, $GABA_ARg2$ and GlyRa2, and to silence the splicing of some Nova transcripts themselves, specifically Nova-1 (219–222). Both rtMSTN-2 genes lack conserved splice sites, although rtMSTN-2a has almost twice the number of expected YCAY motifs throughout and three times the expected motifs in the second intron alone. Whether Nova regulates the alternative splicing of rtMSTN-2a remains to be determined. Nevertheless, a comparative analysis of rtMSTN-2a and -2b processing provides a perfectly controlled system to investigate the basic mechanisms responsible for tissue-specific pre-mRNA processing.

As the mature myostatin peptide is encoded entirely by the third exon, the introduction of alternative transcription start sites in either intron of rtMSTN-2b, or any other salmonid MSTN-2b, could presumably result in the expression of a mature myostatin. Therefore, the inability to remove expressed introns contributes to the pseudogenization of rtMSTN-2b as it prohibits the "accidental" translation of an otherwise silenced gene. This is particularly important as the rtMSTN-2b gene still possess a functionally active promoter. Contributions from noncoding regions to the functional divergence of duplicated genes or to the subfunctionalization of a particular allele are not necessarily limited to gene promoters. Gene expression and pre-mRNA processing are both regulated by protein:DNA interactions that depend upon highly specific binding sites. Thus, alterations in such binding sites over time could not only influence gene function, but ultimately organismal complexity and speciation as well.

VI. Novel Actions

A. Adipose tissue

Myostatin is minimally expressed in adipose tissue, and myostatin-null animals have less total and im body fat than wild-type animals (1, 56, 223–225). Increases in muscle mass have long been known to similarly increase resting energy expenditure (REE), which in turn can reduce fat free mass (226) and is inversely correlated to negative outcomes of patients with type 2 diabetes mellitus (227). Thus, the reduced adiposity in myostatin-null animals could simply be due to the caloric draw from enhanced musculature. Circulating levels of leptin, an adipokine and satiety factor that controls body fat, are reduced rather than elevated in these animals (56, 228). This suggests that the increased REE is indeed responsible. However, myostatin has been shown to directly influence the cellular physiology of three different adipocyte culture systems with conflicting results. It inhibits the differentiation of 3T3-L1 preadipocytes (209, 229) and primary preadipocytes from cattle (230) or humans (231). It also down-regulated the expression of several adipogenic markers and transcription factors in these cells, including peroxisome proliferator-activated receptor γ, C/EBPα, aP2, and leptin.

By contrast, myostatin promotes adipogenesis in a multipotent mesenchymal cell line (C3H10T1/2) that can differentiate into many cell lineages including muscle-like cells (210, 232). Although the latter results are consistent with reduced adiposity in myostatin-null animals, the studies' physiological relevance is somewhat questionable in light of the consistent results obtained with primary cells and with a very well-established cell line (3T3-L1). Transgenic mice overexpressing myostatin specifically in adipocytes fail to develop obesity when fed a high-fat diet and respond better to glucose tolerance tests than do obese nontransgenic mice (232). Their adipocytes, however, express preadipocyte markers, suggesting that adipogenic determination may be stimulated by myostatin, but not differentiation. Reduced adiposity in myostatin-null animals is, therefore, likely due to the indirect effects of enhanced musculature on REE rather than to direct effects on the adipocytes themselves. In fact, myostatin fails to influence lipolysis *in vitro* or fat mass *in vivo* when administered pharmacologically to wild-type or obese mice (229).

McPherron and Lee (56) directly tested myostatin's ability to influence the development of obesity by crossing the Mighty

Mouse with two murine models of obesity and insulin resistance: the agouti lethal yellow (A^y) and leptin-deficient obese ($Lep^{ob/ob}$) mice. Introducing a myostatin-null background into either strain increased muscle mass, suppressed adiposity (as indicated by reduced mass of different fat pads), and vastly improved glucose tolerance. Similar results were also observed in transgenic mice overexpressing LAP and fed a high-fat diet (57, 58). Thus, enhancing muscle mass by blocking myostatin bioactivity and/or bioavailability can prevent the development of obesity and insulin resistance. These results do not, however, prove that myostatin's effects are mediated entirely by increasing REE because the myostatin-null environment could have influenced the development of preadipocytes *in utero* or even the *de novo* synthesis of triglycerides in already differentiated adipocytes. Several additional questions also remain unanswered. In particular, will disrupting myostatin production and/or bioavailability be similarly beneficial in animals that are already obese and displaying signs of insulin resistance? Regardless, these results are extremely exciting and potentially introduce a new therapeutic target for treating obesity and type 2 diabetes mellitus.

B. Cardiac muscle

Cardiac expression of myostatin has been documented in sheep (192), chickens (195, 196), mice (192), rats (205), and different fish species (129, 130, 143, 144, 187). In developing chicken hearts, myostatin expression is detected early and progressively increases until morphogenesis is complete, suggesting a functional role for myostatin in the development of cardiac as well as skeletal muscle (195). High levels of myostatin expression in primary mouse cardiomyoblasts are correlated with a low proliferative index, whereas recombinant myostatin inhibits the growth of these cells, as well as rat H9C2 cardiomyoblast growth, without inducing apoptosis (205, 233). Myostatin also suppresses cardiomyocyte hypertrophic growth responses, specifically protein synthesis, induced by either phenylepinephrine (205) or IGF-I (208). Recent studies with Akt transgenic mice (234), *in vitro* models of cyclic stretch, and IGF-I-stimulated cardiomyocytes (208) all suggest that myostatin not only regulates some cardiac muscle growth process, but that it may function as a cardiac chalone (235) just as it does in skeletal muscle (8). 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.

Myostatin expression in hearts is also elevated in several pathophysiological conditions. In response to a myocardial infarction, myostatin immunostaining steadily increases in the peri-infarcted region and persists for over 1 month (192). Increased expression also occurs with chronic swim training in rats (236) and with volume-overloaded chronic heart failure (207, 237). These changes are similar to those occurring in damaged or regenerating skeletal muscle (174, 238–241) and suggest that myostatin may also influence cardiac muscle repair. Whether or not myostatin's role in this process is beneficial or detrimental remains to be determined. Two studies report conflicting results on the effect of a myostatin-null environment on heart size (233, 242); however, both studies indicate that basic cardiac output parameters (ventricular volume measurements, ejection fraction, *etc.*) are at least normal. A more in-depth analysis of myostatin's effects on cardiac function is definitely warranted. These preliminary studies are nevertheless promising and suggest that repairing damaged cardiac muscle by blocking myostatin's actions could have highly beneficial clinical outcomes.

In mammals, myostatin is clearly expressed in developing and adult cardiac muscle and is capable of manipulating different cardiac muscle growth processes. It is unknown, therefore, why a significant cardiac phenotype has never been described for any myostatin-null animal. Cardiac tissue lacks myoprogenitor cells analogous to the myosatellite cells found in skeletal muscle and may be less sensitive to myostatin's inhibitory effects. Thus, blocking myostatin's actions or removing the gene altogether would have very little effect, except under circumstances when myostatin expression is elevated. Several questions remain. Can other cardiac factors compensate for myostatin or the lack of it? Does developing cardiac muscle express factors or mechanisms that block myostatin's actions (*e.g.*, follistatin, FLRG, *etc.*)? Do myostatin-null hearts function better or worse when challenged (*i.e.*, stress test)? Most importantly, will blocking myostatin's actions help regenerating cardiac muscle after an infarction?

C. Brain

Although myostatin is expressed in different brain regions of different vertebrates, its function in the brain is unknown. 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. It is more widely expressed in mammalian embryos (52, 243) and is secreted by neuroprogenitor cells and fully differentiated neurons of the olfactory epithelium where it inhibits neurogenesis by inducing cell cycle arrest of the neuroprogenitors (204). In the retina, GDF-11 limits the number of ganglion, amacrine, and photoreceptor cells by controlling neuroprogenitor cell competence (244). Thus, GDF-11 functions as a negative autoregulator, or chalone, of neural tissue, which mirrors myostatin action in mammalian skeletal muscle. It is therefore possible that myostatin has similar functions in the brain, albeit in different regions (Figs. 6 and 7), and that compensatory changes in GDF-11 expression and/or availability prohibit the development of neural phenotypes in myostatin-null animals. Fish brains maintain a large number of hyperplastic neuroprogenitor cells that can be easily isolated and even cultured *in vitro* (245, 246). *In vivo* models for neuroprogenitor cell proliferation and differentiation have also been developed in goldfish and zebrafish (247–250). These underutilized comparative model systems may therefore prove valuable in distinguishing the neural functions of myostatin and GDF-11.

VII. Final Thoughts: Implications for Biomedical, Agricultural, and Evolutionary Sciences

The most apparent biomedical application for manipulating myostatin action is clearly to enhance muscle growth, although the use of novel "myostatin-blocking" technologies need not be limited to the treatment of skeletal muscle pathologies—injury,

sarcopenia, wasting/cachexia, some forms of muscular dystrophy, *etc*.—because exploiting such technologies may prove beneficial in treating survivors of a myocardial infarction as well. Several of these technologies have already been developed, including immunoneutralizing antisera (251–253), a soluble form of Acvr2b's extracellular domain (48) and a quiver of myostatin binding proteins (see *Section III.A*), and each successfully stimulates skeletal muscle hypertrophy in wild-type mice and, in some instances, in a murine model for Duchenne muscular dystrophy (44, 253–256). The indirect effect of neutralizing myostatin's actions on adiposity suggests that these technologies could also be useful in treating obesity and type 2 diabetes mellitus. A promise of "more muscle, less fat" is an attractive marketing campaign and one that these technologies could potentially deliver. Thus, blocking myostatin's actions for cosmetic purposes is not just probable, but possibly inevitable as the biomedical successes gain visibility among the general public.

Double-muscled cattle like the Belgian Blue and the Piedmontese frequently require cesarean delivery due to large calf size. Piedmontese meat is particularly tender (16, 17); nevertheless, the high cost of veterinary care associated with parturition has prevented the widespread acceptance of these and other double-muscled breeds in most cattle production markets. Crossing myostatin-null breeds with normal-muscled breeds or even immunoneutralizing cows before breeding, as has been done with mice (252), could address issues of growth rate and product yield in many animal production industries. It is unknown, however, whether the potential loss of im fat would potentially offset these commercial gains. These issues are not a concern with egg-laying vertebrates. Thus, myostatin technologies may have a greater commercial impact on domestic fish and fowl production, especially because the mass ratio of muscle to nonmuscle tissues in fish is greater than in any other vertebrate class. Such commercial gains are predicated on the assumption that blocking myostatin actions will have no adverse side effects on nonmuscle tissues, many of which express multiple myostatin genes. A more thorough assessment of myostatin's nonmuscle actions in fish is therefore needed to determine whether blocking its actions is both feasible and commercially beneficial.

Comparative model systems will also help identify novel functions for both myostatin and GDF-11 in ways pertinent to agriculture and medicine as their more basic and conserved actions are defined. The subset of salmonid myostatin genes is a particularly useful model for investigating the functional divergence of duplicated alleles because differences in coding and noncoding sequences have contributed to their evolution in a manner that influences gene expression, transcript processing, protein structure, and pseudogenization. Determining the underlying mechanisms involved presents a unique opportunity to investigate competing evolutionary models—double-recessive *vs.* duplication-degeneration-complementation (150, 257)—that potentially explain the molecular basis of fundamental evolutionary processes. Lack of such mechanistic understanding is an important problem because, without it, we cannot understand how perceivably minor changes in gene structure and function can significantly impact phenotypic differences between species and/or speciation itself. Thus, a growing knowledge of myostatin molecular genetics and bioactivity, in different tissues and in different organisms, has the potential to impact science as well as society.

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Address all correspondence and requests for reprints to: Buel D. Rodgers, Ph.D., Department of Animal Sciences, 124 ASLB, Washington State University, Pullman, Washington 99164. E-mail: danrodgers@wsu.edu

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