Myostatin: a modulator of skeletal-muscle stem cells

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Abstract
Myostatin, or GDF-8 (growth and differentiation factor-8), was first identified through sequence identity with members of the BMP (bone morphogenetic protein)/TGF-β (transforming growth factor-β) superfamily. The skeletal-muscle-specific expression pattern of myostatin suggested a role in muscle development and homoeostasis. The hypermuscular phenotypes observed upon inactivation of the myostatin gene in multiple species, including mice, cattle and most recently a human subject, have confirmed the role of myostatin as a negative regulator of skeletal-muscle development. Myostatin binds with high affinity to the receptor serine threonine kinase ActRIIB (activin type IIB receptor), which initiates signalling through a smad2/3-dependent pathway. In an effort to validate myostatin as a therapeutic target in a post-embryonic setting, a neutralizing antibody was developed by screening for inhibition of myostatin binding to ActRIIB. Administration of this anti-myostatin antibody to adult mice resulted in a significant increase in both muscle mass and functional strength. Importantly, similar results were obtained in a murine model of muscular dystrophy, the mdx mouse. Unlike the myostatin-deficient animals, which exhibit both muscle hypertrophy and hyperplasia, the antibody-treated mice demonstrate increased musculature through a hypertrophic mechanism. These results validate myostatin inhibition as a therapeutic approach to muscle wasting diseases such as muscular dystrophy, sarcopenic frailty of the elderly and amyotrophic lateral sclerosis.

Introduction
Myostatin, or GDF-8 (growth and differentiation factor-8), was identified through sequence identity with members of the BMP (bone morphogenetic protein)/TGF-β (transforming growth factor-β) superfamily [1]. The skeletal-muscle-specific expression pattern of the myostatin transcript suggested a role in muscle development and homoeostasis. The hypermuscular phenotypes observed upon inactivation of the myostatin gene in multiple species, including mice, cattle and most recently a human subject, have confirmed the role of myostatin as a negative regulator of skeletal-muscle development. Recent efforts to understand the molecular mechanism by which myostatin exerts its inhibitory effect on muscle growth and differentiation have led to experimental approaches which have demonstrated muscle mass increases in adult animals through myostatin inhibition. These experiments indicate that the myostatin signalling pathway may be a potent therapeutic intervention point in the treatment of muscle wasting diseases, such as muscular dystrophy, sarcopenic frailty of the elderly and ALS (amyotrophic lateral sclerosis).

The developmental role and mechanism of myostatin activity
Targeted deletion of the GDF-8 gene in mice results in a >200% increase in skeletal-muscle mass, resulting from both hypertrophy and hyperplasia of muscle fibres [1]. This observation defined the role of GDF-8 as a negative regulator of skeletal-muscle development and led to the name myostatin. Recent studies indicate that the enhanced muscular phenotype resulting from myostatin deficiency is maintained throughout life, as aged myostatin null mice continue to exhibit normal muscle of increased size and strength [2]. Mutations in the myostatin gene have also been shown to result in increased musculature in pigs, cattle and most recently in a human subject [3–8]. The ‘double muscled’ cattle breeds, Belgian Blue and the Piedmontese, both have genetic alterations in their coding regions for the myostatin gene. The Belgian Blue has a frameshift mutation that results in a complete absence of the mature myostatin protein, while the Piedmontese breed possesses a single point mutation in an invariant cysteine residue of the mature region, leading to the production of an improperly folded protein [3,5,6,9]. Importantly, the intermediate hypermuscular phenotype observed in heterozygote Belgian Blues and myostatin mice suggests that increases in muscle mass can be achieved through only a partial inactivation of myostatin activity. Recently, the first case of a myostatin-deficient human has been reported [10]. Genetic sequence analysis of the child, who was noted to be exceptionally muscular at birth, revealed a point mutation in the myostatin gene.
This mutation occurs at a key nucleotide in the first intron, resulting in misspliced myostatin mRNA and undetectable levels of myostatin protein. The child at 4.5 years of age continues to demonstrate exceptional musculature and strength, but is otherwise healthy. These data clearly define the role of myostatin as an important negative regulator of skeletal-muscle development.

The developmental time point at which myostatin inhibition occurs, or the mechanism by which that inactivation takes place, results in hypermuscular animals with distinct phenotypes. Increases in fibre number (hyperplasia) and/or increases in fibre size (hypertrophy) have been observed. Transgenic mice overexpressing myostatin inhibitors driven by a muscle-specific promoter/enhancer exhibit muscle mass increases resulting from both fibre hypertrophy and hyperplasia in a manner analogous to the myostatin null animals [11]. In contrast, both the Piedmontese cattle and a transgenic mouse overexpressing the corresponding cysteine mutation, under the control of a ubiquitous promoter, exhibit hypertrophy without hyperplasia [12,13]. Adult animals treated with neutralizing antibodies also manifest their increased musculature through a hypertrophic mechanism [14]. A conditional knockout strategy targeted to generate postnatal, muscle-specific ablation of myostatin results in a mixed mechanism of muscle mass increase. Depending upon the individual muscle examined, the increase in mass occurs though pure hypertrophy (tibialis) or though a combination of hypertrophy and hyperplasia (gastrocnemius) [15]. Taken together, these results indicate that myostatin acts to control both the differentiation and proliferation of skeletal muscle throughout embryonic development and continues to regulate muscle homeostasis in the adult.

Regulation of myostatin activity and the signalling pathway

In a manner similar to TGF-β, but unlike most other members of the BMP/GDF superfamily, myostatin is secreted as a latent complex [11,16,17]. After cleavage of the N-terminal signal sequence by the signal peptidase and a separate proteolytic processing event that cleaves the 38 kDa propeptide from the 12 kDa C-terminal domain, the molecule is secreted in a biologically inactive configuration in which the propeptide region remains non-covalently associated with the mature C-terminal dimer. Thus the myostatin propeptide region serves two functions; first, to help guide the proper folding and dimerization of the mature C-terminal peptide and secondly, to regulate the biological activity of the C-terminal dimer through the formation of a latent complex. Although the mechanism of activation of this latent complex is underdetermined, tissue specific factors may be responsible for the generation of the active species and the subsequent inhibitory activity of myostatin in skeletal muscle. Myostatin has been shown to circulate as an inactive complex in both human and mouse serum [18]. In addition to the propeptide, which in a purified form can potentely inhibit the biological activity of the mature myostatin dimer, FLRG (follistatin-related gene) and GASP-1 (growth and differentiation-associated factor-associated serum protein-1) have been shown to exist in complex with myostatin in serum [18,19]. Both FLRG and GASP-1 bind to the active C-terminal dimer of myostatin and inhibit its biological activity. Interestingly, although follistatin has also been demonstrated to bind tightly and potently inhibit the biological activity of myostatin and other members of the TGF-β superfamily, and is know to be present in high levels in serum, it was not found to be associated with circulating myostatin in this proteomic analysis. Although the precise functions of both FLRG and GASP-1 are unknown, their ability to bind to the C-terminal dimer make them attractive candidates for the termination of signalling responses initiated upon activation of the myostatin latent complex. Additionally, since GASP-1 contains domains which have homology with a protease inhibitor and is capable of binding the myostatin propeptide, it could further protect the myostatin latent complex from activation by non-specific proteases and preserve the tissue specific action of myostatin in muscle.

In vitro studies with members of the BMP-1/tolloid family of metalloproteases provide evidence for a potential in vivo mechanism of tissue-specific activation of the myostatin latent complex. Mutation of the myostatin sequence at Asp76 results in a propeptide molecule that is resistant to myostatin cleavage [17]. In vivo administration of this mutated propeptide in mice results in muscle mass increases, while control experiments with the native propeptide do not. In vitro, treatment of the myostatin propeptide with all four members of the BMP-1/tolloid family of proteases results in cleavage at residue Asp76, but the mutated version is protease resistant. Furthermore, similar protease treatment of the myostatin latent complex results in activation of myostatin activity as assessed by in vitro signalling assays. The skeletal-muscle-specific expression pattern of one member of this family of BMP-1-like family of metalloproteases, Tll-2, suggest its potential as a candidate for the site-specific activator of myostatin latency in vivo [20].

Through a number of cross-linking experiments performed with transiently overexpressed receptors and complementation assays in receptor deficient cell lines, the specific components of the myostatin signalling pathway have been defined [21]. The mature myostatin C-terminal dimer has been shown to bind with high affinity to the ActRIIB (activin type IIIB receptor) and, to a lesser extent, to the highly related receptor ActRII [11,21]. Myostatin binds in an ordered sequence to a heterodimeric serine/threonine receptor complex. The dimeric ligand binds tightly to a type II receptor, ActRIIB, and initiates the intracellular signalling cascade by recruitment of a low affinity type I receptor, either ALK-4 (activin-like kinase-4) or ALK-5, into the signalling complex. The myostatin-induced formation of this heteromeric receptor complex enables the constitutively active ActRIIB kinase domain to transphosphorylate the type I receptor component ALK-4/5 in the membrane-proximal, intracellular GS region, resulting in activation of the serine/threonine kinase domain. The activated type I receptor kinase
phosphorylates the receptor-regulated smads 2 and 3, allowing them to interact with the co-smad, smad 4, and translocate to the nucleus. This smad complex binds directly to DNA and interacts with nuclear co-activators or co-repressors to regulate transcription of myostatin target genes [22]. These studies demonstrate that myostatin initiates signalling through a type II–I receptor complex consisting of ActRIIB and ALK-4 or ALK-5 and induces a smad 2/3 dependent intracellular signalling cascade. Myostatin has also been shown to signal in a smad-independent manner through a mitogen-activated protein kinase pathway, although the mechanistic details of this activation remain to be determined [23].

Although the myostatin receptors are expressed in many tissues and cell lines, it is important to remember that myostatin exists in vivo as a latent complex, which must be activated before receptor binding can occur and the subsequent signalling cascade can be initiated to induce myostatin-specific responses. Therefore some caution must be used in overinterpretation of in vitro experiments done on cell lines treated with purified recombinant, active myostatin. Myostatin has been shown to have direct effects on the proliferation and/or differentiation of numerous muscle cell lines and adipocytes [21,24–28]. The effect on muscle fibre number is likely to result from the activity of myostatin on myoblast proliferation [24,26,27,29] and/or differentiation [28] during development while the effects on fibre size appear to be mediated through the action of myostatin on muscle satellite cells. Myostatin inhibits proliferation through an up-regulation of p21 and decreases in the levels of both Cdk2 (cyclin-dependent kinase 2) and phosphorylated Rb (retinoblastoma protein), resulting in cell cycle inhibition [24,29]. A similar effect on proliferation has been observed in both satellite cells in culture and through direct analysis of satellite cells from myostatin deficient mice. The effect on differentiation appears to occur through down-regulation of the myogenic differentiation factors MyoD, Myf-5 and myogenin [27,28]. Understanding the regulation of myostatin activity and the specific components of the myostatin signalling pathway have been critical to the identification of targets and approaches to inhibit myostatin activity in post-developmental and ultimately useful therapeutic settings.

**Approaches to myostatin inhibition and therapeutic potential**

Although genetic studies have clearly established the significance of myostatin in muscle development, experiments utilizing systemically delivered myostatin inhibitors were needed to establish its ability to increase muscle mass in a postnatal setting. Knowledge gained from studies on the regulation of myostatin activity and the mechanism by which it initiates its intracellular signalling pathway have been critical to the development of unique approaches to inhibit myostatin activity and have enabled these proof of concept experiments to be performed in vivo. Administration of myostatin neutralizing antibodies to adult mice results in a 23–30% increase in muscle mass and a 21–26% increase in muscle strength [14]. Two other mechanistically similar approaches that act through sequestration of the activated myostatin C-terminal dimer, systemic administration of a protease resistant form of the myostatin propeptide and a soluble version of the ActRIIB receptor, also lead to muscle mass increases in adult mice [17]. Interestingly, while treatment with a mutated version of the myostatin propeptide leads to muscle mass increases of 18–27%, similar to that observed in the antibody treated mice, administration of the soluble form of the ActRIIB receptor resulted in even larger muscle mass increases of up to 39%. This result suggests that additional ligands capable of binding to ActRIIB may contribute to the limitation of muscle growth caused by myostatin. The demonstration of an increase in muscle mass and improvement of muscle function in adult animals treated with myostatin inhibitors suggests that it may be worthwhile to target this signalling pathway for therapeutic intervention in the treatment of muscle wasting diseases.

Treatment of dystrophin-deficient mdx mice, a model of Duchenne muscular dystrophy, with a myostatin neutralizing antibody resulted in a 35% increase in skeletal-muscle mass, a proportional increase in strength, and an improvement in the histological appearance of the dystrophic diaphragm [30]. A genetic study involving a cross between the mdx mice and myostatin null mice resulted in a 45–100% increase in the weight of individual muscles of the Mstn−/−/mdx, when compared with the same muscles of the Mstn+/+/mdx mice [31]. Transgenic animals overexpressing either the myostatin propeptide, follistatin or a dominant negative form of the myostatin receptor, ActRIIB, under the control of a muscle-specific promoter, all exhibit large increases in skeletal-muscle mass [11,32]. Particularly striking are the muscle mass increases of up to 110% in the dominant negative ActRIIB lines and up to 327% in the follistatin transgenics, again suggesting that inhibitory approaches utilizing more promiscuous inhibitors may lead to muscle mass increases greater than through inhibition by myostatin-specific neutralizing agents. Additional studies involving treatment of mdx mice with either the myostatin propeptide or the myostatin-binding protein, follistatin, produce beneficial effects similar to those observed in the antibody treated mdx model [33,34]. It should be noted, however, that a recent study involving the genetic cross of a laminin-α-2 deficient dy/dy mouse and the myostatin knockout mouse indicate that the genetic absence of myostatin is not able compensate for the severity of muscle damage induced in this particular model of muscular dystrophy [35]. We have obtained additional positive results in a second neuromuscular disease model namely, the SOD-1 (superoxide dismutase 1) model of ALS. In this model, there is substantive and progressive loss of anterior horn motor neurons, muscle wasting and ultimately death. Addition of a myostatin neutralizing antibody has had a number of potent effects although there is no impact on longevity as has been found for other putative treatments in this model. However, at mid stage disease (84 days), we found a significant reversal of muscle atrophy, increased muscle grip strength and a
significant increase in M-cadherin positive satellite cells. Our interpretation is that the myostatin antibody is leading to an activation of the satellite cell pool that become incorporated into myofibres and partially reverse the disease progress, but ultimately become denervated and atrophic. Further preclinical and clinical studies are warranted.

In addition to the potential of myostatin inhibition in the treatment of muscular dystrophies, the consistent demonstration of increases in muscle mass and strength in animal models provide a rationale for the treatment of other muscle-wasting diseases such as sarcopenic frailty of the elderly and cachexia, a wasting syndrome observed in patients with chronic diseases such as sepsis, cancer and AIDS. Although myostatin inhibition has not been tested in models of cachexia, evidence for myostatin involvement in this wasting state comes from studies involving overexpression of myostatin in mouse models [36,37]. When myostatin was systemically overexpressed by implantation of a stable cell line engineered to secrete high levels of myostatin protein, a muscle wasting phenotype accompanied by fat loss, similar to that observed in cachetic diseases, was evident [36]. Although purified myostatin C-terminal dimer has been shown to have direct effects on adipocyte cell lines in vitro and early reports on tissue-specific expression have demonstrated low levels of myostatin transcripts in adipose tissue [1,21], experiments involving injection of active myostatin protein into mice resulted in decreased muscle mass without affecting fat mass (J.F. Tobin, personal communication). These experiments suggest that the in vivo effect of myostatin on fat may be indirect and secondary to the effects on muscle mass.

Despite questions concerning the direct role of myostatin in fat, there is substantial genetic evidence to suggest that myostatin inhibition could be useful in the treatment of metabolic disorders. In addition to maintaining increased musculature over wild-type animals, myostatin null mice do not accumulate fat as they age [2,38]. More notably, myostatin inhibition has not been tested in models of obesity and metabolic disease will provide information on the mechanism by which myostatin may affect glucose homeostasis and a rationale for the use of particular forms of myostatin inhibition for the treatment of Type 2 diabetes.

Conclusions

Since the identification of myostatin as a negative regulator of skeletal-muscle development, numerous experiments have been performed to validate the myostatin signalling pathway as a critical modulator of muscle mass in the adult animal. Inhibition of the myostatin signalling pathway has great potential for the treatment of a variety of muscle-wasting diseases, including muscular dystrophy, sarcopenic frailty of the elderly, cachexia and potentially ALS. Although many aspects of the mechanisms by which myostatin regulates muscle and fat mass remain to be determined, it is clear that myostatin plays a major role in regulating the metabolic state of the animal and at least in part via stem cell activation. Pharmacological manipulation of the myostatin pathway could also lead to the development of additional therapeutic approaches in a variety of pathologies.

References


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