Myostatin: Biology and Clinical Relevance

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Abstract: Myostatin is a negative regulator of muscle mass. Important advances in our understanding of the complex biology of this factor have revealed the therapeutic potential of antagonizing the myostatin pathway. Here we present the rationale for evaluating anti-myostatin therapies in human muscle-wasting disorders.

Key Words: Myostatin, Muscle, Adipose tissue, Rhabdomyosarcoma, Myostatin antagonism, Myostatin processing and signaling, therapeutic approaches.

MYOSTATIN PROCESSING AND SIGNALING PATHWAY

Myostatin, also called GDF8 (growth differentiation factor 8), is a secreted growth factor that belongs to the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors [1]. Like other TGF-β family members, myostatin is synthesized as a precursor protein that contains a signal sequence, an inhibitory N-terminal propeptide domain, and a C-terminal domain that is the active/mature ligand (Fig. **1**) [1]. The precursor undergoes proteolytic cleavage, folding and dimerization to form an active molecule [2-4]. Following proteolytic processing, the propeptide remains in contact with the C-terminal region via non-covalent forces in a latent complex. Importantly, this association makes the mature fragment of myostatin biologically inactive [5]. Latency could furthermore be maintained by association with other extracellular interacting proteins. For example, follistatin can bind to myostatin and inhibit its activity in receptor binding and signaling assays [5, 6]. In addition, myostatin is present in circulation as a part of a latent complex containing myostatin propeptide and/or follistatin related proteins FLRG and GASP [7, 8]. The fact that myostatin exists in latent complexes raises the important issue concerning the regulation of myostatin availability and function [9]. It has been established that members of the bone morphogenetic protein-1/tolloid (BMP-1/TLD) family of metalloproteinases can cleave *in vitro* the myostatin propeptide in the latent complex and can thereby activate latent myostatin, however, there is no data as yet supporting this mechanism *in vivo* [4].

Like other TGF-β superfamily members, myostatin signaling operates through a heterodimeric complex of transmembrane serine/threonine kinase receptors: the mature myostatin peptide binds to one of the two activin type II receptors (ActRIIB to a greater degree than ActRIIA), which recruits, phosphorylates and thereby activates the type I co-receptors (ALK-4/5) propagating signals along the Smad pathway [10, 11]. Myostatin acts through the receptor-associated proteins Smad2 and Smad3 [11-13]. Phosphorylated Smad2 and Smad3 form heterodimeric complex with the common mediator Smad4. These activated smad proteins function as the key intracellular mediators of signaling for myostatin as they translocate into the nucleus, and activate the transcription of the target genes through interaction with DNA and other nuclear factors [14, 15]. Recently, it has been shown that the inhibitory smad7 protein dramatically reduces myostatin-induced transcription and that myostatin can autoregulate its own expression through the induction of the inhibitory smad7 protein [16, 17]. Indeed, myostatin like many other ligands appears to act through the binding to activin type II receptors and type I co-receptors, however the question arises as to how specific signaling is achieved. A mechanism to achieve part of the selectivity of myostatin effects must be simply the restricted expression of the activin type II receptor and the appropriate type I co-receptor in a given cell type. In addition, cross-linking studies have shown that the mature myostatin peptide not only binds to the activin type II but also to ALK-4 and/or ALK-5 co-receptors [11]. Although multiple ligands have been previously shown to signal through a type II-I receptor complex consisting of ActRIIB and ALK-4 [18], this result reveals for the first time that ALK5 can mediate signals for a ligand other than TGF-β. Thus, the unique combination of ALK5 with activin type II receptors could ensure the specificity of myostatin signaling.

In addition to the canonical SMAD pathway, the mitogen-activated protein kinase (MAPK) pathway has been implicated in the transduction and regulation of myostatin signaling [19]. The activation of the p38 MAPK pathway enhances the myostatin-dependent transcriptional response. The molecular mechanisms that connect this SMAD-independent pathway to the myostatin receptor signaling complex remain elusive and might involve type I receptor-dependent mechanisms [19]. Therefore, the differential gene expression in response to myostatin signaling depends on the levels of expression of the myostatin receptor complex,

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All authors contributed to the research, development and writing of the contents presented within this work.

1389-5575/06 $50.00+.00

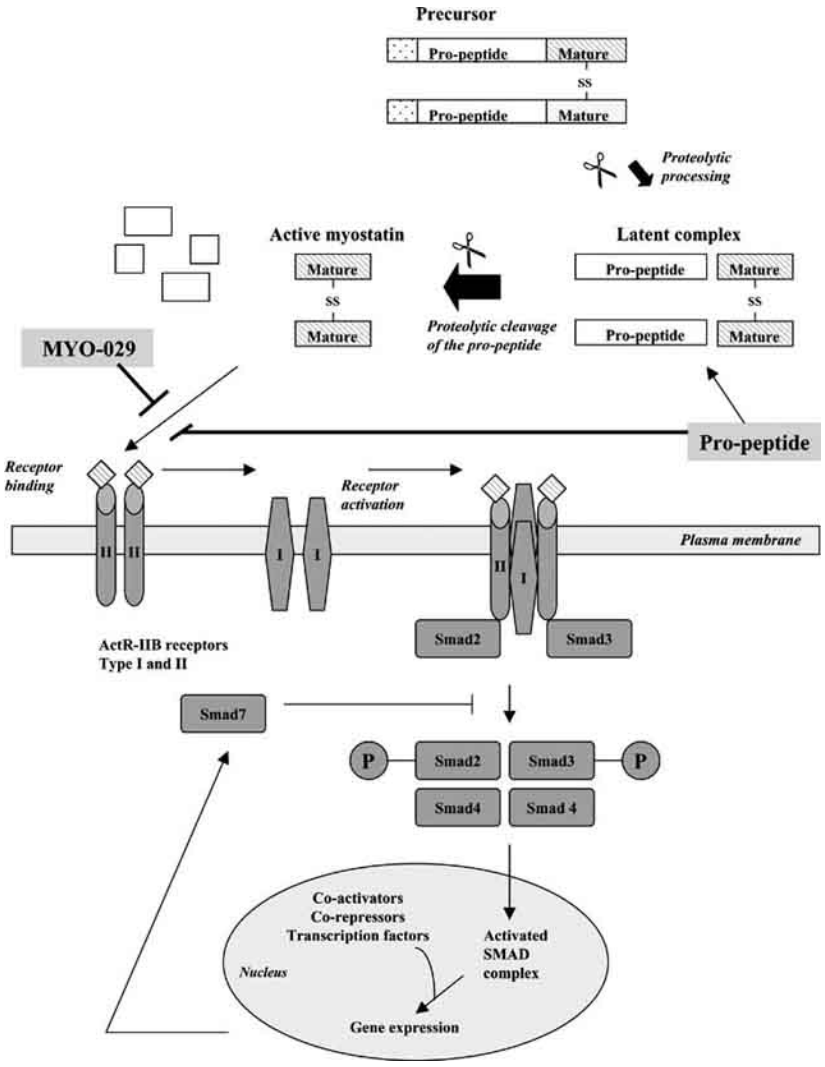

Fig. (1). Myostatin processing and signaling and therapeutic interventions.

Myostatin is synthesised as a precursor protein that is proteolytically processed to give rise to a C-terminal fragment, the propeptide which possesses receptor-binding activity and the disulfide linked C-terminal dimer, which is the active/mature myostatin ligand. Following proteolytic processing, the propeptide remains in contact with the C-terminal dimer via non covalent forces in a latent complex. Proteolytic cleavages of the propeptide are required to generate an active myostatin. Binding of myostatin to activin type II receptors leads to phosphorylation and activation of type I co-receptors, ALK-4 or ALK-5. The activated type I receptor phosphorylates SMAD2 and SMAD3, which bind the SMAD4 mediator to move into the nucleus and form complexes that regulate transcription in several ways, including interaction with DNA and other nuclear factors. SMAD7 represses signaling by other SMADs and down-regulates myostatin-induced transcription. Current therapeutic strategies for modulating myostatin signaling involve an inhibition of myostatin ligand binding to the heredodimeric receptor complex with anti-myostatin anti-body such as MYO-029 and stimulation of myostatin inactivation by proteins that form noncovalent links with myostatin such as the myostatin propeptide.

SMAD protein levels, the expression profile of cooperating transcription factors and the activation state of other signaling pathways. On the basis of this knowledge, many different strategies can be conceived to inhibit the biological effects of myostatin including antagonism of myostatin signaling by either specific competing ligands or dominant-negative receptors that inhibit signal transduction, myostatin antagonists (for example, neutralizing antibodies, follistatin or myostatin propeptide) that impede myostatin processing and activation. This review will focus on recent advances in these strategies and their potential therapeutic applications.

MYOSTATIN AND MUSCLE

In mice, myostatin is predominantly expressed in skeletal muscle tissues from the period of embryogenesis to adulthood suggesting a role for this factor in the control of muscle development and function [1]. The role of myostatin

in muscle comes from the phenotype of myostatin-deficient animals. Myostatin was first found to regulate muscle mass in mice from which the gene encoding myostatin has been knocked-out. The resulting "mighty mice" displayed muscle overgrowth due to both hyperplasia (increased number of muscle fibers) and hypertrophy (increased size of individual muscle fibers). These effects on muscle mass are persistent throughout the life of the animals. The phenotype of these mice suggested that myostatin functions as a negative regulator of muscle growth. Several mouse models also support this notion. For example, transgenic mice over-expressing the myostatin propeptide or follistatin or a truncated dominant negative form of ActRIIB (which lacks the intracellular kinase domain) exhibited muscle mass increases similar to myostatin knockout mice [10]. The opposite effect, namely, a decrease in muscle mass, has been reported in follistatin knockout mice [20] and in transgenic mice overexpressing myostatin in skeletal muscle [21]. Interestingly, the function of myostatin appears to have been conserved accross diverse species. Natural mutations in the myostatin gene have been identified in double-muscled animals such as the Belgian blue cattle [22] [23] [24]. The recent identification of a hypermuscular child with a loss-of-function mutation in the myostatin gene suggests that the function of myostatin is similarly conserved in humans [25]. In support of this, myostatin sequence has been highly conserved through evolution, among species ranging from zebrafish to humans [1].

In addition to the role of myostatin in muscle development, there are many reports that have highlighted the role of myostatin in adult animals. For example, administration of neutralizing myostatin antibodies to adult mice led to an increase in muscle mass and muscle force [26]. An increase in muscle mass has also been reported in mice having a postnatal deletion of the myostatin gene [27], and in mice injected with a mutant form of the propeptide resistant to cleavage by BMP-1/tolloid proteinases [4] or with a soluble form of the activin type IIB receptor [28].

Several reports in animal models have also shown losses of muscle mass associated with elevated myostatin mRNA or protein expression [29-31]. Likewise, administration of myostatin *in vivo* to adult mice produces the signs and symptoms characteristic of the muscle wasting syndrome, cachexia [5]. In addition, the muscle wasting observed in these mice can be partially reversed by systemic delivery of the myostatin propeptide or follistatin in the mice indicating that the observed muscle wasting was caused by excess myostatin [5]. Epidemiological studies have reported high serum levels of myostatin in HIV-infected men with muscle wasting [32]. Increased myostatin expression has also been observed in several muscle atrophy settings including prolonged bed rest in young men [33], chronic disuse atrophy in older patients [34] and age-related muscle wasting (sarcopenia) [35]. Taken together, these results demonstrate that changes in myostatin expression inversely correlate with changes in muscle mass and indicate that targeting the myostatin pathway may provide a clinical benefit in the treatment of muscle wasting disorders such as muscular dystrophy, cachexia and sarcopenia.

The role of myostatin in muscle biology is complex and involves two aspects: regulation of muscle fiber number as well as muscle fiber size. The ability of myostatin to inhibit the proliferation and differentiation of muscle cell lines *in vitro* is central to the mechanism regulating fiber number [2, 13, 36-39]. The role of myostatin as a potential regulator of adult fiber size is underscored by the discovery that myostatin can inhibit the activation and self renewal of satellite cells [40] which are stem cells resident in skeletal muscle, responsible for adult muscle cell growth and new muscle protein production [41]. Recently, myostatin absence has been shown to result in improved muscle healing through enhanced regeneration and reduced fibrosis [42].

MYOSTATIN AND ADIPOSE TISSUE

The physiological function of myostatin is not restricted to skeletal muscle since, in absence of myostatin, mice also show a reduction in both fat accumulation and abnormal glucose metabolism [43] [44]. Elegant studies in two mouse models of obesity have shown that loss of myostatin prevents an age-related increase in adipose-tissue mass and improves the glucose metabolism [44]. Recently, the possibility that myostatin could regulate body composition by modulating the commitment and/or differentiation of mesenchymal multipotent cells is highlighted by the observation that myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T1/2 cells in culture [45]. As skeletal muscle function is important for the maintenance of normal glucose function [46-48], it is also likely that the effects of myostatin mutation in adipose tissue may reflect an indirect effect of the lack of myostatin signaling in skeletal muscle. A direct effect of myostatin on adipogenesis cannot yet be excluded as myostatin can block differentiation of adipogenic cell lines *in vitro* [5, 11, 49]. Much remains to be learned concerning the mechanisms by which myostatin exerts its effect on adipose tissue and glucose homeostasis.

Descriptions of an increased muscle mass with the lack of adipose-tissue mass in myostatin-null mice have raised the question of whether myostatin might be a target for increasing skeletal muscle mass in patients with muscle-wasting diseases as well as for suppressing the development of obesity and diabetes. In this regard, it has recently been shown that, contrary to wild-type mice, transgenic mice expressing specifically the myostatin propeptide in skeletal muscle did not develop obesity and insulin resistance [50]. In addition, there is evidence of metabolic interaction between muscle and adipose tissue. Compared to wild-type mice, the propeptide transgenic mice maintain normal serum concentration of insulin, leptin, and resistin and increase adiponectin secretion on the high-fat diet. Therefore, the propeptide transgene has an impact on insulin and several adipocyte hormones. These findings highlight the importance of metabolic interactions between muscle and adipose tissue and how the absence of myostatin signaling could prevent obesity and insulin resistance. Although myostatin presents an attractive target for the development of muscle therapeutics, its role on fat metabolism presents challenges that must be considered in pre-clinical and clinical drug development program.

MYOSTATIN IN RHABDOMYOSARCOMA CELLS

The role of myostatin has been explored in cell lines derived from rhabdomyosarcoma (RMS), a malignant soft-

tissue tumor committed to the myogenic lineage, but arrested prior to terminal differentiation. These tumor cells show increased production of active myostatin, an increase that is correlated with their non-muscle-differentiating phenotype. This tumor-derived myostatin can function in an autocrine manner since specific down-regulation of the myostatin protein restores terminal myogenic differentiation and normal cell cycle withdrawal in RMS cells, including RD human rhabdomyosarcoma cells [39]. In addition, myostatin can also control cell cycle progression in RMS cells since its inhibition improves proliferation of RD cells [39] and conversely exogenous myostatin has been shown to inhibit the proliferation of the rhabdomyosarcoma RD cell line [37]. Although the precise signaling pathways mediating the intracellular response to myostatin are not known, it is likely that the SMAD pathway in conjunction with the p38 MAPK pathway are involved. In support of this, it has recently been shown that myostatin can induce the activation of these two downstream pathways in the human rhabdomyosarcoma cell line, A204 [19]. In addition, the Smad proteins are expressed in embryonal rhabdomyosarcoma RD cells at high levels and are functional in a TGF-β signaling pathway [51]. These findings suggest that approaches interfering with this myostatin autocrine loop could have anti-tumoral applications. Administration of myostatin could antagonize the proliferation of tumoral cells. However, such approach could have undesirable side effects since it has been recently reported that systemic administred myostatin causes muscle and fat wasting in adult animals [5]. Another approach could be based on inhibition of myostatin activity allowing cell cycle withdrawal and induction of the myogenic program in rhabdomyosarcoma although the ability of myostatin to signal in an autocrine mode could limit the effectiveness of antibody therapies in solid tumor. In addition, it is possible that rhabdomyosarcoma-derived myostatin might function in a paracrine manner by acting on the proliferation and/or differentiation of cells in adjacent tissues. A better understanding of the mode of action of myostatin in these tumoral cells will aid for developing anti-rhabdomyosarcoma therapy.

PROOF OF CONCEPT FOR MYOSTATIN ANTAGONISM IN MUSCLE DYSTROPHY

A recent important advance in the validation of myostatin as a therapeutic target is that, *mdx* mice, a mouse model for human Duchenne Muscular Dystrophy (DMD), develop an increased muscle mass and strength as a result of injection of neutralizing antibodies to myostatin or the myostatin propeptide [12, 52]. The same phenotype is observed in *mdx* mice lacking myostatin [53]. Furthermore, the muscles of these mice also have a better muscle architecture and show decreased fibrosis (the replacement of muscle by fat and connective tissue) indicative of muscle regeneration. Collectively, these findings have demonstrated the efficacy of antibody anti-myostatin, of propeptide at improving muscle growth and strengh in mice with DMD and encouraged the development of human therapies to block myostatin.

In contrast to the therapeutic potential of inhibiting myostatin observed in *mdx* mice, a recent report has shown that elimination of myostatin did not ameliorate the dystrophic phenotype in *dy* mice (which have a laminin-deficient congenital muscular dystrophy) but increased postnatal lethality [54]. There is evidence that in this genetic background lack of myostatin promotes muscle regeneration and formation, but at the expense of fat formation, and does not reduce muscle pathology. This underscores the need to carefully consider the reduction in fat in development of strategy based on myostatin elimination. Since brown fat is important for neonatal humans and mice to maintain body temperature, elimination of myostatin should be envisioned after birth or in forms of myopathies with later onset. Such strategy would have to attenuate the consequence of fat reduction.

Muscle cell transplantation therapy is actually envisioned as a potential treatment for Duchenne Muscular Dystrophy (DMD) or as a vehicle for the autologous delivery of functional dystrophin loci to dystrophic muscle. The success of this approach is, however, greatly compromised by the limited muscle regeneration in *mdx* mice and in DMD patients. A significant increase in the extent of muscle repair leading to the formation of more dystrophin positive fibers was observed both in *mdx* mice carrying a dominant negative form of myostatin receptor (dnActRIIB) transplanted with normal myoblasts and in *mdx* mice transplanted with nondystrophic dnActRIIB myoblasts [55]. This improved success of myoblast transplantation in *mdx* mice by blocking the myostatin signaling provides the rationale for exploring the potential of combination cell-based therapy with pharmacological blockade of myostatin signal.

If targeting the myostatin pathway does turn out to be an effective strategy for treating human diseases, a number of considerations have to be taken into account. This kind of treatment cannot be a complete cure for human Duchenne Muscular Dystrophy (DMD) because the genetic cause of the disease would not be eliminated. The injection of antibodies to myostatin or of a stabilised myostatin propeptide would have the important advantages that no immune or toxicity problems would arise, and no genetic risks caused by viruses. In the next future, efforts must be continued to developp orally bioavailable small molecule inhibitors of myostatin such as for example soluble forms of the myostatin receptor or of the propeptide which would target the myostatin before its binding to the receptor. In addition, the possibility to use myostatin inhibition in human clinical trials raises many important questions about the long-term effects on muscle of myostatin blockade as well as the potential risk of side effects on other tissues such as adipose tissue. Since myostatin can regulate muscle progenitor cells there is a risk that long-term inhibition of myostatin might lead to an accelerated depletion of muscle regenerative capacity in the setting of a chronic muscle disease. However, it has been recently shown that increased muscle mass and strength are maintained in senescent myostatin-null mice and in aged *mdx* mice lacking myostatin compared to their counterpart mice (wt and *mdx*, respectively) [56]. This indicates that the prolonged absence of myostatin does not have negative effects in mice. Future long-term clinical studies will determine whether the inactivation of myostatin for a long time in DMD patients would also not produce any side effects.

Recently a recombinant human antibody engineered to neutralize myostatin, MYO-029, was developed by New Jersey pharmaceutical company Wyeth and currently undergoes human testing. The aim is to perform a PhaseI/II trial to study MYO-029 in adult patients with muscular dystrophy. This initial safety trial will focus on adults with Becker-, facioscapulohumeral- and limb-girdle muscular dystrophy.

Thereby, myostatin is a potential therapeutic target designed to alleviate the secondary defects in muscle dystrophy but not to correct the primary ones. In addition to muscle diseases, blocking myostatin actions could also have some therapeutic value in various muscle wasting settings including cancer and ageing.

CONCLUSIONS

The pursuit of the efforts to understand the complex role of myostatin in muscle is important for elaborating new therapies for regulating muscle mass in human disease. In this context the molecular mechanisms involved in activation of myostatin *in vivo* must received much attention. The extensive knowledge surrounding the mechanisms regulating myostatin activation and activity will focus drug discovery efforts on the proteases, that cleave myostatin, on the specific competitive ligands or soluble forms of the myostatin receptor that block myostatin interaction with its receptor, as therapeutic targets. The next several years promise to be filled with new and exciting data as anti-myostatin therapies will be evaluated for clinical efficacy in disease-related muscle wasting. However, it should be taken into consideration the possibility that inhibitors of myostatin action would be also used to improve muscle performance in athletes.

ACKNOWLEDGMENTS

Work in the authors's laboratories is supported by grants from the Institut National de la Recherche Agronomique (INRA), the Ligue Nationale contre le Cancer, Comité des Pyrénées Orientales et comité de l'Hérault and the Association Française contre les Myopathies (AFM).

ABBREVIATIONS

TGF-β	=	Transforming growth factor-β
GDF8	=	Growth differentiation factor 8
BMP-1/ TLDfamily	=	Bone morphogenetic protein-1/tolloid family of proteinases
FLRG	=	Follistatin like related gene
GASP	=	Growth and differentiation factor-associated serum protein
SMADs	=	Family of transcription factors that mediate TGF-b signals. The term SMAD is derived from the founding members of this family, the Drosophila protein MAD (Mothers Against Decapentaplegic) and the Caenorhabditis elegans protein SMA (Small body size)
MAPK	=	Mitogen-activated protein kinase
RMS	=	Rhabdomyosarcoma
AIDS	=	Acquired immuno-deficiency syndrome
DMD	=	Duchenne Muscular Dystrophy
ActRIIB	=	Activin type II B receptor

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Received: October 17, 2005 Revised: January 06, 2006 Accepted: January 09, 2006