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Myostatin: A Negative Regulator of Muscle Development and Maintenance

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ABSTRACT

Myostatin is a member of the TGFβ family which plays a major role in negative regulation of muscle development. Not only do mstn-/- mice display a dramatic increase in skeletal muscle mass, cattle harboring loss of function mutations in the myostatin gene also exhibit muscle overdevelopment associated to a shift in the contractile and metabolic features of muscle fibers. The occurrence of such mutations associated to increased muscle mass in humans has also been reported. Recent data clearly suggest that myostatin is also involved in muscle tissue maintenance in adults, in particular by activating pathways leading to proteolysis and satellite cell activity. As myostatin expression generally increases during muscle atrophy, some promising attempts have been made to improve the behavior of some muscle pathologies, such as myopathies, by targeting myostatin activity. These attempts have opened the way for novel pharmacological strategies focused on skeletal muscle diseases. Here we review the physiopathological consequences of changes in myostatin expression and their clinical interest. We also briefly address the myostatin molecular pathway by describing the knowledge which makes it possible to test the efficiency of pharmacological inhibition of this growth factor activity in muscle pathologies.

Keywords: degenerative diseases, muscle, muscle wasting, myostatin, therapy

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INTRODUCTION

Muscle development is the result of complex processes including myoblast proliferation, fusion into multinucleated myotubes and acquisition of contractile properties. These events are mainly regulated by muscle-specific basic Helix Loop Helix transcription factors, MyoD, Myf5, MRF4 and Myogenin. However, myostatin cloning in 1997 revealed the occurrence of a powerful negative regulator of muscle growth by the demonstration that myostatin gene knockout in mice leads to a dramatic and widespread increase in skeletal muscle mass (McPherron *et al*. 1997). Myostatin, also called GDF8, belongs to the TGFβ family and harbors the structural features of all members of this family. Natural myostatin mutations associated to the mh locus have been discovered in some cattle breeds (double-muscled) characterized by an important increase in skeletal muscle mass due to myofiber hyperplasia (Grobet *et al*. 1997; Kambadur *et al*. 1997; McPherron and Lee 1997; Smith *et al*. 1997). More recently, a mutation leading to a mis-splicing of a 108-base pair of the myostatin mRNA sequence has been identified in humans. This mutation leads to the synthesis of a severely truncated protein and is associated to muscle hypertrophy (Schuelke *et al*. 2004). Moreover, several studies have established that myostatin not only regulates muscle growth during development but is also able to modulate muscle regeneration and atrophy. As a consequence, attempts to modulate myostatin expression focus on a major interest aimed at developing new pharmacological strategies for treatment of diseases involving muscle wasting.

REGULATION OF MYOSTATIN EXPRESSION

In developing mice, myostatin is essentially expressed in skeletal muscle, as early as 9.5 days post coitum. However, in adult animals, although it is predominantly expressed in skeletal muscle, several studies have also detected myostatin expression in a range of other tissues, such as the mammary gland, eyes, gill filaments, spleen, ovaries, gut and brain. In addition, myostatin protein has also been detected in serum, thus indicating that this growth factor could target different tissues (Gonzalez Cadavid *et al*. 1998; Ji *et al*.

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Invited Mini-Review

Table 1 Myostatin binding proteins.

Localisation	Binding molecule	Myostatin form bound	Consequence of binding
Serum	Myostatin propeptide	Mature myostatin	Inhibits Myostatin receptor binding
Serum	GASP1	Mature myostatin and myostatin propeptide	Inhibits Myostatin activation
Serum	FLRG	Mature myostatin	Inhibits Myostatin receptor binding
Skeletal muscle	hSGT	Myostatin N-term signal peptide region	Inhibits Myostatin secretion and activation
Skeletal muscle	Titin cap	Mature myostatin	Inhibits Myostatin latent complex formation and secretion
Skeletal muscle	Follistatin	Mature myostatin	Inhibits Myostatin receptor binding

1998; Ostbye *et al*. 2001; Rodgers *et al*. 2001; Maccatrozzo *et al*. 2001). In agreement with this possibility, fat pad weight and lipid content significantly decrease in 12- or 32–week-old myostatin-null mice (Lin *et al*. 2002; McPherron and Lee 2002). Moreover, recent data also suggest that myostatin could be involved in early osteogenic processes (Hamrick *et al*. 2007).

Analysis of the highly conserved 5′-upstream regulatory region of the myostatin gene reveals the presence of several E boxes allowing MyoD-specific binding. This observation could explain the fairly muscle-specific pattern of myostatin expression. In addition, it suggests that MyoD control of myoblast withdrawal from the cell cycle could involve myostatin expression (Spiller *et al*. 2002). In parallel, responsive elements for nuclear receptors have also been identified (reviewed in Joulia-Ekaza and Cabello 2006), in particular for glucocorticoid receptors, suggesting that muscle wasting associated to treatments by this hormone or its derivatives could result from activation of myostatin expression (see below).

Interestingly, several FoxO boxes have also been characterized in the 5′-upstream regulatory region of the mouse myostatin gene, in agreement with the observation that FoxO1 expression stimulates myostatin promoter activity (Allen *et al*. 2006). This factor, expressed in skeletal muscle, belongs to the FoxO transcription factor family. Strikingly, FoxO1 seems to be involved in muscle protein degradation through activation of the ubiquitin ligase pathway during muscle atrophy.

Overall, it appears that myostatin expression is regulated by transcription factors involved in cell cycle withdrawal (MyoD), proteolysis (FoxO1) and muscle wasting (glucocorticoid receptor), thus suggesting that it probably plays an important role in muscle development and in muscle regeneration and/or atrophy.

THE MYOSTATIN PATHWAY

Myostatin is synthesized as a 376 amino-acid precursor protein, containing a signal sequence, a N-terminal propeptide domain, a proteolytic processing site and a C-terminal domain containing the conserved pattern of 9 cysteine residues – the cysteine knot – essential for TGFβ family members activity (McPherron *et al*. 1997). Proteolytic processing gives rise to the mature myostatin active in the form of a disulfide-linked dimer. Binding of myostatin to several proteins able to modulate its activation, secretion or receptor binding is observed in serum or in skeletal muscle (**Table 1**) (Thies *et al*. 2001; Hill *et al*. 2002; Nicholas *et al*. 2002; Hill *et al*. 2003; Wang *et al*. 2003; Amthor *et al*. 2004).

Members of the TGFβ family of growth factors exert their numerous effects through a family of serine-threonine kinase transmembrane heterotetrameric receptors. In the absence of a ligand, type I (RI) and type II (RII) receptors homodimerize at the cell surface. Binding of the active ligand to this dimer enhances transphophorylation of RI by RII in the GS sequence of the intracytoplasmic domain (**Fig. 1**). Expression of a dominant negative form of Act RIIB (Activin B type II Receptor) in mice mimics myostatin gene knockout in increased muscle mass, leading to the conclusion that the *in vivo* myostatin pathway probably involves ActRIIB receptors (Lee and Mcpherron 2001). In agreement with this possibility, *in vitro* binding studies have established that the active form of myostatin actually binds to ActRIIB. In turn, this receptor recruits ALK4 or ALK5 type I receptors to mediate myostatin signaling (Rebbapragada *et al*. 2003) (**Table 2**). These events are followed by phosphorylation of Smad 2 and Smad 3, inducing their translocation to the nucleus and their binding to specific DNA sequences leading to changes in the expression of targeted genes. The Co-Smad Smad 4 has been shown to potentiate this signaling, whereas Smad 7 and Smurf 1 play an inhibitory role. In addition, the inhibition

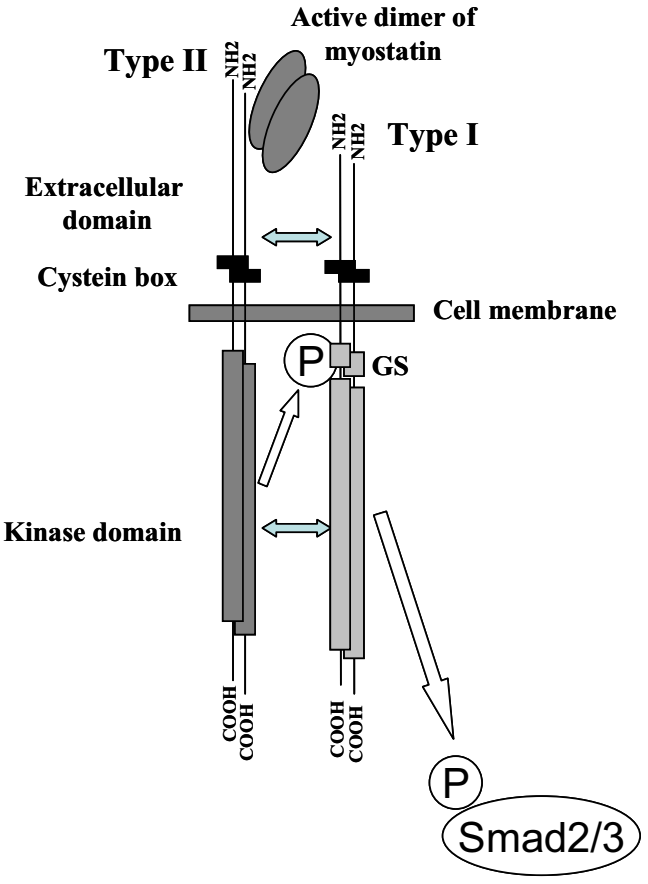

Fig. 1 Structure of the TGF beta family of transmembrane receptors. Binding of myostatin to type II receptors induces recruitment of type I receptors, transphosphorylation of RI by RII at the GS (Gly-Ser) rich sequence, leading to activation of Smad2/Smad3 transcription factors.

Table 2 Elements of the TGFβ ligand family pathway.

Ligand	Type II receptor	Type I receptor	R-Smad
Activins	ActRII	ActRIB/ALK4	Smad2
Myostatin	ActRIIB		Smad3
			Smad2
		TβRI/ALK5	Smad3
TGFβ	TβRII	ALK1	Smad1
		ActRI/ALK2	Smad5
			Smad8
	BMPRII	BMPRIA/ALK3	Smad1
BMPs	ActRII	BMPRIB/ALK6	Smad5
GDFs	ActRIIB	ActRI/ALK2	Smad8

of Smad 7 expression by myostatin establishes a negative regulatory feedback loop setting up fine-tuning of the cellular influence of this growth factor (Zhu *et al*. 2004).

MOLECULAR BASIS OF MYOGENIC MYOSTATIN INFLUENCE

At cell level, myostatin overexpression or addition of recombinant myostatin in culture media reduces myoblast proliferation by inducing their accumulation in the G0/G1 or G2 phases of the cell cycle. In addition, myoblasts fail to fuse into myotubes when exposed to differentiating conditions, a deficiency related to a general decrease in the expression of differentiation markers. Lastly, the myoblast apoptotic rate is inhibited by myostatin in both growing and differentiating conditions (Thomas *et al*. 2000; Rios *et al*. 2001, 2002; Joulia *et al*. 2003). *In vitro* studies including myostatin depletion by an antisens strategy, a more physiological approach, have led to the conclusion that myogenin and p21 are probably the major target genes of myostatin (Joulia *et al*. 2003). The latter data satisfactorily explain the accumulation of myoblasts in particular phases of the cell cycle (p21) and impaired differentiation (myogenin). Overall, all these studies clearly indicate that myostatin is involved in important processes of early events involved in muscle development by controlling proliferation and differentiation of precursor cells (myoblasts or satellite cells).

Furthermore, recent data clearly suggest that myostatin could also be involved in muscle tissue maintenance processes. In particular, McFarlane *et al*. (2006) have reported that myostatin positively influences expression of FoxO1, a transcription factor stimulating expression of ubiquitin-proteasome components, leading to protein degradation. On the other hand, as mentioned above, FoxO1 can enhance myostatin promoter activity (Allen *et al*. 2006). Consequently, activation of such a positive regulatory loop could induce an important proteolytic process able to explain the relationship between high myostatin expression levels and the muscle wasting observed in some chronic pathologies, as discussed below. Therefore, these data clearly suggest that myostatin is not only involved in muscle development, but also in the processes controlling adult skeletal muscle maintenance.

IN VIVO ABROGATION OR REDUCTION OF MYOSTATIN ACTIVITY STRONGLY INFLUENCES SKELETAL MUSCLE MASS

Since 1997, the development of myostatin gene-null mice has provided striking data concerning muscle growth. These mice are about 30% larger than heterozygous and wild-type littermates at 3-6 months of age. This overgrowth is essentially due to a widespread increase in skeletal muscle mass, with individual muscles weighing about 2-3 times more than those in wild-type littermates (McPherron *et al*. 1997). Subsequently, several attempts were made to inhibit the myostatin pathway without total suppression of its expression. Two types of genetically-modified mouse models were generated. In one approach, a myostatin protein lacking its normal cleavage site was overexpressed; this acts as a dominant negative form of the growth factor which can prevent formation of the active myostatin dimer (dnMS mice) (Zhu *et al*. 2000). In the other approach, mice overexpressed the myostatin prodomain under the control of a myosin light chain promoter, with the aim of inhibiting binding of myostatin to its receptor (Yang *et al*. 2001). Because myostatin activity was only partially inhibited in these models, phenotypic analyses established in both cases a significant increase in skeletal muscle mass, though considerably lower than that recorded in myostatin-null mice.

Interestingly, myostatin overexpression in adult rat muscle by electrotransfert led to a 10 to 20% reduction in muscle mass (Durieux *et al*. 1997). Reciprocal results were reported using an inducible tamoxifen myostatin invalidation in adult mice (Welle *et al*. 2007), thus demonstrating that this growth factor is not only involved in the early processes of muscle development, but also in the regulation of muscle mass increase and/or maintenance in adults. In another approach not using transgenesis, changes in myostatin availability were made by injections of myostatin-blocking antibodies in adult mice. This procedure also induced an increase in skeletal muscle size of 13 to 30%. Moreover, treatment with this antibody in adults did not induce side effects such as size and histological features of other organs or serum parameters (Whittemore *et al*. 2003). Interestingly, this last experiment established the possibility of inhibiting the negative myogenic influence of myostatin *in vivo* by means other than transgenesis, thus opening the way to new therapeutic strategies for pathologies involving muscle wasting.

Taken all together, these *in vivo* studies clearly established that absence of myostatin or reduction of its activity leads to enhanced muscle growth due to skeletal muscle fiber hypertrophy, in some cases associated to hyperplasia, depending on the experimental approach (McPherron *et al*. 1997; Zhu *et al*. 2000; Lee and McPherron 2001; Yang *et al*. 2001). Reciprocal results were obtained after muscle-specific overexpression of myostatin, leading to a significant reduction in the cross-sectional area of muscle fibers and in the number of nuclei per fiber (Reisz-Porszasz *et al*. 2003).

After characterization of several mutations occurring in the myostatin gene in double-muscled cattle inducing about a 20% increase in skeletal muscle mass associated to hyperplasia, identification of a natural mutation in a young boy exhibiting muscle hypertrophy at birth once more focused attention on myostatin. This mutation in the myostatin gene leads to the synthesis of a severely truncated protein, due to the appearance of a premature stop codon, the mutated mRNA accounting for 68.8% of the total amount of myostatin mRNA. Phenotypic observations revealed that decreased myostatin levels in humans induce a phenotype very close to that observed in mstn-/- mice, consisting of a strong increase in skeletal muscle mass associated to a decrease in fat accumulation (Schuelke *et al*. 2004).

INTER-RELATIONS BETWEEN MYOSTATIN EXPRESSION LEVELS AND SKELETAL MUSCLE PATHOLOGIES

Myostatin and glucocorticoid-induced muscle atrophy

Glucocorticoids are widely used in the treatment of chronic inflammatory illnesses, and administration of high doses generally leads to muscular atrophy in humans and animals; similarly, hypercortisolism is involved in muscle atrophy observed in Cushing's disease (Odebra *et al*. 1983; Dardevet *et al*. 1995; Auclair *et al*. 1997). As discussed above, the presence of glucocorticoid response elements in the myostatin promoter suggests that glucocorticoids could influence myostatin expression. In agreement with this possibility, administration of dexamethasone promotes a dose-dependent decrease in total body weight and skeletal muscle mass in rats, associated with dose-dependent upregulation of both myostatin mRNA and protein in muscle (Ma *et al*. 2003). Reciprocally, mstn-/- mice appear to be protected from glucocorticoid-induced muscle atrophy when compared to wild-type animals (Gilson *et al*. 2007). Along these lines, it has been recently shown in dexamethasone-treated rats that glutamine ingestion reduces glucocorticoid stimulation of myostatin expression and, consequently reduces muscle mass and weight loss (Salehian *et al*. 2006). All these data clearly indicate an important involvement of myostatin in glucocorticoid-induced muscle wasting.

Changes in myostatin expression during muscle wasting

Adult muscle growth is the result of recruitment of quiescent muscle cells – satellite cells – located along the fibers. Satellite cell activation induces their proliferation and fusion with the muscle fibers, providing them with more nuclei and consequently additional potentiality to synthesize muscle proteins. Numerous studies tend to conclude that myostatin expression levels are related to muscle wasting, thus suggesting that myostatin could target not only myoblasts during early myogenic processes, but also satellite cells by inhibiting their proliferation rate (Carlson *et al*. 1999; Wehling *et al*. 2000; McCroskery *et al*. 2003). This raises the possibility that the dramatic increase in muscle mass observed in myostatin-null mice could be due in part to stimulation of satellite cell activity. In agreement with this possibility, Dasarathy *et al*. (2004) have reported impairment of satellite cell proliferation and differentiation in relation to elevated levels of myostatin in a rat model of cirrhosis (portacaval anastamosis). Indeed, normal mice immobilization leading to muscle atrophy induces a significant reversible rise in muscle myostatin expression (Zimmers *et al*. 2002). Moreover, in chronic pathologies such as HIV infection in humans, a positive correlation has been established between serum levels of myostatin and the extent of muscle atrophy (Gonzalez-Cadavid *et al*. 1998). These data suggest that increased myostatin levels may contribute to muscle wasting during HIV infection or immobilization. Furthermore, a decrease in skeletal muscle mass has been observed in nude mice bearing myostatin-expressing tumors (Zimmers *et al*. 2002). Therefore, myostatin could be involved in the muscle loss encountered in other pathologies. However, although prolonged absence of myostatin in mice reduced aging-induced sarcopenia (Siriett *et al*. 2006), contrasting data have been published concerning myostatin levels in aging-induced sarcopenia in humans or rodents. (Kawada *et al*. 2001; Welle *et al*. 2002; Bauman *et al*. 2003), and the eventuality of influence by myostatin on these aging processes should be studied further.

In cattle, myostatin expression appears to decline following muscle injury, mRNA levels being reduced by 82.6% at day 5 after injury, and restored by day 10, in good correlation with myostatin protein levels. On the opposite, expression of the four muscle regulatory factors is induced while myostatin levels decrease, thus allowing muscle regeneration (Shibata *et al*. 2006). Regeneration efficiency in mstn-/- mice can be tested when these animals suffer muscle injury. In fact, lack of myostatin actually seems to improve their muscle repair processes when compared to wild-type littermates. Furthermore, *in vitro* experiments have established greater proliferation and earlier differentiation rates in mstn-/- satellite cells, underlining the importance of myostatin in muscle repair processes (McCroskery *et al*. 2005; Wagner *et al*. 2005).

All these data suggest that high myostatin levels could reduce postnatal growth and regeneration processes by inhibiting satellite cell activation. Furthermore, the regulatory loop occurring between myostatin and FoxO, leading to increased proteolysis, probably plays an important role in muscle wasting of different origins.

Myostatin: a promising therapeutic target in muscle dystrophies?

The strong influence of myostatin on muscle atrophy and regeneration raises the possibility of a new therapeutic target in muscle diseases such as muscle dystrophies. Several approaches have been developed in an attempt to improve mdx mice phenotype. These mice exhibit diaphragm rounds of degeneration followed by incomplete regeneration, which leads to extensive fibrosis and fatty replacement, similar to those encountered in human Duchenne and Becker muscular dystrophies. These approaches consist either of reducing myostatin activity – by injections of myostatin-blocking antibody or propeptide or transplantation of dnActRIIB-expressing myoblasts – or by establishing mstn-/-mdx mice. All these attempts have led to attenuation of the mdx phenotype, but some histological abnormalities typical of mdx mice have still been detected (Bogdanovitch *et al*. 2002; Wagner *et al*. 2002; Bogdanovitch *et al*. 2005; Benabdallah *et al*. 2005). Muscle mass and regeneration are also improved in the limb-girdle muscular dystrophy mouse model (scgd-/-), following injections of myostatin-blocking antibody, combined with a reduction in the extent of fibrosis. However, loss of myostatin activity in the late stages of the pathology does not provide positive results (Parsons *et al*. 2006). An innovative approach has also been developed in the limb-girdle muscular dystrophy 1C (LGMD1C) mouse model with intraperitoneal injections of the soluble myostatin receptor form (ActRIIB-Fc) (Ohsawa *et al*. 2006). This procedure enables interference with myostatin signaling reflected by the inhibition of the expression levels of p-Smad2 and p21. As a consequence, mice exhibit a significant increase in the myofiber cross-sectional area and improvement in muscle atrophy. Nevertheless, since ActRIIB is targetted by both myostatin and other growth factors belonging to the TGFβ family, injection of ActRIIB-Fc is likely to inhibit not only myostatin signaling but signaling of these factors as well (**Table 2**). This means the exact part played by myostatin inactivation in this improvement remains to be established, as does the influence of interference with ActRIIB signaling over time, and consideration given to potential side effects. To conclude, generation of double-deficient dy^{w}/dy^{w};mstn-/- mice for the purposes of ameliorating laminin-deficient congenital muscular dystrophy does not improve all aspects of muscle pathology, probably due to the absence of laminin α2; moreover it leads to important side effects, particularly an increase in pre-weaning mortality (Li *et al*. 2005).

CONCLUSIONS

Since its identification in 1997, myostatin appears to be a crucial regulator of skeletal muscle growth and maintenance. Reducing *in vivo* myostatin expression or activity promotes skeletal muscle growth or enhances muscle regeneration, suggesting a potential benefit for the muscle wasting encountered in some human pathologies. Characterization of molecules able to bind to myostatin has provided pharmacological targets to inhibit myostatin signaling without disruption of the myostatin gene, opening promising new prospects for treatment of human muscle pathologies. Recently, it has been suggested that myostatin-treated adipocytes positively influence metabolism, at least in part through increased glucose oxidation, thereby improving insulin sensitivity and resistance to obesity (Feldman *et al*. 2006). As a consequence, in addition to treatment of muscle wasting, targeted myostatin exposure may be of therapeutic benefit in human metabolic diseases.

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