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REVIEW

Myostatin and the skeletal muscle atrophy and hypertrophy signaling pathways

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Abstract Myostatin, a member of the transforming growth factor-β superfamily, is a potent negative regulator of skeletal muscle growth and is conserved in many species, from rodents to humans. Myostatin inactivation can induce skeletal muscle hypertrophy, while its overexpression or systemic administration causes muscle atrophy. As it represents a potential target for stimulating muscle growth and/or preventing muscle wasting, myostatin regulation and functions in the control of muscle mass have been extensively studied. A wealth of data strongly suggests that alterations in skeletal muscle mass are associated with dysregulation in myostatin expression. Moreover, myostatin plays a central role in integrating/mediating anabolic and catabolic responses. Myostatin negatively regulates the activity of the Akt pathway, which promotes protein synthesis, and increases the activity of the ubiquitin–proteasome system to induce atrophy. Several new studies have brought new information on how myostatin may affect both ribosomal biogenesis and translation efficiency of specific mRNA subclasses. In addition, although myostatin has been identified as a modulator of the major catabolic pathways, including the ubiquitin–proteasome and the autophagy–lysosome systems, the underlying mechanisms are only partially understood. The goal of this review is to highlight outstanding questions about myostatin-mediated regulation of the anabolic and catabolic signaling pathways in skeletal muscle. Particular emphasis has been placed on (1) the cross-regulation between myostatin, the growth-promoting pathways and the proteolytic systems; (2) how myostatin inhibition leads to muscle hypertrophy; and (3) the regulation of translation by myostatin.

Keywords Growth differentiation factor-8 · Muscle homeostasis · Muscle differentiation · mTOR · Translational machinery · Protein degradation

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Introduction

Skeletal muscle is the most abundant tissue in the vertebrate body. Its metabolism affects the metabolic balance of the entire organism and is the major body protein reservoir. It is a plastic tissue that continuously adapts in response to a variety of stimuli, including use and disuse. For example, mechanical load causes hypertrophy, while physical inactivity leads to atrophy. Importantly, loss of muscle mass is observed after musculoskeletal trauma, during aging, neuromuscular disorders and in many catabolic diseases, such as cancer, diabetes, renal failure, respiratory insufficiency, or sepsis.

Muscle mass is the result of a dynamic balance between protein synthesis and degradation [1–3]. This balance is coordinately regulated by two major branches of AKT

signaling pathways: the AKT (also known as protein kinase B)/mammalian target of rapamycin (mTOR) pathway that controls protein synthesis and the AKT/forkhead box O (FOXO) pathway that controls protein degradation. In the last few years, myostatin, a member of the transforming growth factor-β (TGFβ) superfamily, has emerged as a key regulator of skeletal muscle mass. Its deletion or loss of function induces muscle overgrowth, whereas its overexpression or systemic administration causes muscle atrophy. Importantly, as mature skeletal muscle cells respond to myostatin, the potential clinical applications of myostatin inhibitors are currently under study. Therefore, a better understanding of the molecular mechanisms that drive skeletal muscle hypertrophy upon myostatin inhibition/loss is required to investigate potential therapeutic approaches.

The identification of signaling factors that regulate skeletal muscle protein balance has provided the opportunity to determine how myostatin influences this process. A wealth of data revealed a cross-talk between myostatin and the intracellular AKT/mTOR signaling pathway and strongly supports the notion that myostatin affects the muscle protein balance by regulating protein synthesis and degradation. Much work has focused on investigating how myostatin inhibition results in skeletal muscle hypertrophy during development and in adult muscle, where myostatin inhibition might promote hypertrophy independently of satellite cells. Moreover, myostatin regulates the assembly of the translation pre-initiation complex in skeletal muscle cells, suggesting a potential control of translation efficiency. Myostatin also decreases Akt phosphorylation and signals through FOXO transcription factors to increase expression of atrophy-related genes (atrogenes) and induce atrophy, suggesting potential functional molecular link between myostatin signaling and the regulation of the proteolysis. These discoveries reveal a central role of myostatin in connecting the cellular signaling pathways regulating the anabolic and catabolic responses in muscle [4]. Given its central role, the purpose of this review is to highlight important outstanding questions about the regulation of opposite anabolic and catabolic signaling pathways by myostatin. We will discuss the known instances of cross-regulation between myostatin, the growth-promoting pathways and the proteolytic systems, with a focus on the mechanisms of muscle hypertrophy downstream of myostatin inhibition and the emerging role of myostatin in regulation of translation.

Myostatin: an important modulator of muscle homeostasis

Lessons from genetic invalidation of myostatin

Myostatin strongly inhibits muscle mass development. Indeed, knockout of the myostatin gene results in excessive muscle growth in mice [5]. Compared to wild-type animals, skeletal muscles from 5-month-old mice in which the myostatin gene was genetically ablated ($Mstn^{-/-}$) are much heavier (up to 260 % weight gain for the *pectoralis* muscle) due to increase of both muscle fiber number (hyperplasia) and cross-sectional area (hypertrophy). Naturally occurring myostatin gene mutations lead to a hypermuscular phenotype in mice, sheep, dogs, humans, and some cattle breeds [5–11]. Myostatin also plays a fundamental role in regulating adult muscle growth and size. For instance, myostatin knockout or inhibition in postnatal life enhances muscle development and increases muscle mass [12, 13].

The myostatin signaling pathway

The various components of the myostatin signaling pathway were defined by Rebbapragada et al. [14]. The mature C-terminal dimer of myostatin binds to one of the two activin type II receptors (ActRIIB to a greater degree than ActRIIA), which recruits, phosphorylates and thereby activates the activin type I receptors (ALK4 and ALK5) which in turn leads to the phosphorylation and activation of Smad2 and Smad3 [14, 15]. Phosphorylated Smad2 and Smad3 form a 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 [16]. A further level of complexity is that myostatin signaling is controlled by another member of the Smad family, the inhibitory Smad7 protein. After stimulation by myostatin Smad7 functions as a negative feedback inhibitor for the myostatin signal pathway [17, 18]. In fact, Smad7 inhibits myostatin gene expression, and also interferes with the formation of the Smad2/3–Smad4 complex leading to the inhibition of myostatin signaling.

Several studies have shown that inhibition of different components of the myostatin–ActRIIB–ALK4/ALK5–Smad2/3 pathway promotes muscle hypertrophy in adulthood [19, 20], (see in detail in section "Interplay between the myostatin and Akt/mTOR signaling pathways to control protein synthesis"). Interestingly, a recent study established that muscle hypertrophy of $Mstn^{-/-}$ mice depends on bone morphogenetic protein (BMP) signaling [21]. The authors showed that BMP signaling is dominant over myostatin signaling to control muscle hypertrophy. In fact, inhibition of BMP signaling caused muscle atrophy and abolished the hypertrophic phenotype of $Mstn^{-/-}$ mice, indicating that the hypertrophic phenotype caused by myostatin inhibition results from unrestrained BMP signaling.

Myostatin as a target of anabolic signals action in muscle

Interestingly, several studies have established a link between myostatin and anabolic signals (anabolic substances, resistance training) suggesting the involvement of myostatin in anabolic-mediated muscle hypertrophy.

Long-term administration (18 months) of growth hormone (GH) reduces myostatin levels in muscle of GH-deficient hypopituitary adult patients and this is associated with an increase in lean body mass [22]. Similarly, myostatin mRNA and protein levels are down-regulated in cultured skeletal muscle cells following incubation with GH. These data suggest that GH could exert its anabolic action by down-regulating myostatin. However, GH treatment in healthy men (1 month) failed to modify myostatin mRNA expression and lean body mass [23]. Although this discrepancy may reflect differences in the study populations and length of GH treatment, it also suggests that GH inhibitory effect on myostatin expression could be secondary to the muscle mass increase rather than direct.

Other studies have revealed a relationship between androgen anabolic effects and myostatin. Androgens negatively regulate myostatin expression in the androgen-dependent *levator ani* muscle in rats [24]. Moreover, androgens might regulate myostatin gene expression also indirectly. For instance, follistatin (FS), a negative regulator of myostatin, is up-regulated following testosterone treatment both in satellite cells and in muscle, suggesting that myostatin inhibition by FS may contribute to the hypertrophic effect of androgens [25]. Thus, androgen myogenic effect could, at least in part, be mediated through repression of myostatin expression and activity. On the other hand, Dubois et al. [26] identified multiple androgen response elements (ARE) in the myostatin promoter and demonstrated that myostatin expression is stimulated by androgens directly at the transcriptional level. Although this positive regulation is in apparent contradiction with the well-known anabolic action of androgens in muscle, myostatin induction by androgens could represent a counter-regulatory mechanism to avoid excessive hypertrophy. In support of this hypothesis, the same authors reported that muscle hypertrophy in response to androgens is augmented in $Mstn^{-/-}$ mice.

Consistent with the role of myostatin as a negative regulator of muscle size, several studies have shown that heavy-resistance strength training is associated with down-regulation of myostatin expression. For example, long-term resistance training or an acute bout of resistance exercise represses basal myostatin mRNA levels in rodents and humans [27–30]. These data suggest the existence of an inverse correlation between exercise-induced hypertrophy and myostatin mRNA levels. However, Willoughby et al. [31, 32] found increased muscle myostatin mRNA and serum myostatin protein levels after heavy resistance training in humans. The discrepancy among these in vivo studies may be related to the exercise modality and/or the timing of sampling. Indeed, Roth el al. took samples 48–72 h after the last training session to ensure a basal level of myostatin was examined when Willoughby et al. measured levels immediatly after the final training session. This could suggest that opposing changes in myostatin levels may occur during resistance exercise: an early increase in myostatin to promote cellular remodeling followed by a decreased basal expression, leading to muscle hypertrophy.

Whatever the mechanisms by which myostatin modulates muscle mass in response of anabolic stimuli, all together these data strongly suggest that myostatin could be a target of signaling cascades regulating skeletal muscle hypertrophy.

Myostatin: a critical role in atrophy

Findings from several groups indicate that myostatin gene expression is increased during physio-pathological conditions that lead to muscle loss. For example, myostatin protein up-regulation is observed during hypoxia-induced atrophy in rats, in hypoxemic patients with severe chronic obstructive pulmonary disease (COPD) and in human myotubes treated with hypoxia-mimicking agents [33]. This suggests a potential role of myostatin in COPD skeletal muscle dysfunction. Additionally, different studies comparing the muscle mass of humans with sarcopenia (age-related muscle loss), reported divergent results ranging from an inverse correlation between serum myostatin levels and loss of muscle mass [34], to no effect [35]. Whether myostatin is involved in age-related muscle loss is difficult to investigate in humans. However, there is some evidence that myostatin inhibition protect against "normal" muscle mass loss in aging animals. Indeed, $Mstn^{-/-}$ mice show reduced sarcopenia compared to wild-type littermates [36] and antibody-mediated myostatin inhibition attenuates muscle fiber atrophy, enhances muscle functional capacity, and reduces apoptosis in skeletal muscles from aging mice [37].

Other reports indicate that myostatin is not required in all conditions leading to atrophy. Indeed, myostatin content is unchanged in muscle atrophy following denervation or hind-limb suspension [38, 39]. It should be noted that variations in myostatin levels during the catabolic phase might confuse the interpretation of these findings. For example, in Carlson's study, myostatin mRNA was increased 24 h after hind-limb suspension; however, at later stages (when significant muscle atrophy was observed) myostatin mRNA level was not

significantly different between experimental and control animals, suggesting a transient up-regulation of myostatin mRNA level during muscle wasting [40]. In addition, the interpretation of this study is complicated by the fact that it used amounts of mRNA as an estimation of myostatin expression. This is a very indirect assessment and it is not possible to rule out the modulation of myostatin level and activity by translational or post-translational regulations [41].

In conclusion, the heterogeneity of these studies regarding the diversity of models, time course and stimulus strength further complicates the interpretation of the results. This complexity underscores the importance of further studies of clarifying the role of myostatin in muscle wasting to design novel therapeutic approaches. However, an important issue to address is whether myostatin is a requirement or merely plays a regulatory role in muscle atrophy progression.

Relevant to muscle wasting diseases, myostatin inhibition counteracts muscle atrophy in caveolin-3 deficient mice [42] and myostatin deletion protects against muscle loss induced by dexamethasone by blunting glucocorticoid-induced proteolysis compared to wild-type mice [43]. Furthermore, glutamine protective effect against glucocorticoid-induced atrophy in mice is associated with a decrease of myostatin expression [44]. Thus, myostatin seems to be an attractive therapeutic target against muscle wasting. A direct evidence of myostatin role in muscle atrophy was brought by Zimmers et al. [45] who showed that myostatin overexpression in adult mice induces muscle waste comparable to human cachexia syndromes. Importantly, muscle atrophy in these mice could be lessened by systemic administration of myostatin propeptide or FS, two myostatin inhibitors, indicating that the observed muscle wasting was caused by excess myostatin. Similarly, myostatin overexpression leads to muscle mass reduction in rats by inducing the down-regulation of muscle-specific genes (muscle structural genes and myogenic factors) [46, 47]. Collectively, these data suggest that myostatin plays a role in muscle atrophy. Nevertheless, the situation has been complicated by the study of McMahon et al. [48] reporting that muscle mass loss is more pronounced in $Mstn^{-/-}$ mice than wild-type controls during hind-limb suspension. This indicates that absence of myostatin does not protect against muscle atrophy and also suggests that the role of myostatin may be to inhibit hypertrophy rather than to induce atrophy. Collectively, these results also demonstrate that myostatin is likely not the primary cause of muscle atrophy. In conclusion, despite this progress in our understanding of the role of myostatin in muscle atrophy, mechanisms involved in regulation and actions of myostatin in all forms of muscle atrophy remain to be clarified.

Myostatin regulation of skeletal muscle growth

The role of myostatin in muscle biology is complex and includes the regulation of both number and size of skeletal muscle fibers.

Myostatin controls the biology of skeletal muscle cells

Satellite cell activation

Besides its role during muscle development, it is now clear that myostatin also regulates adult muscle growth and size. In adult organisms, skeletal muscle is mainly composed of skeletal muscle fibers that are terminally differentiated and unable to divide. Therefore, adult muscle growth is primarily due to increase of the protein content through activation of the AKT/mTOR pathway that regulates protein synthesis. However, accretion of new nuclei provided by muscle stem cells, called satellite cells, also seems to be required for post-mitotic myofiber growth.

During development, myoblasts derived from satellite cells proliferate and differentiate and then fuse with the existing myofibers to extend their size [49–51]. Some data have suggested that satellite cells might also contribute to muscle hypertrophy following myostatin loss. Indeed, the number of steady-state satellite cells per unit of muscle fiber and the proportion of activated satellite cells are significantly higher in $Mstn^{-/-}$ adult mice than wild-type animals [52]. Similarly, injection of myostatin shRNAs in rats increases the number of satellite cells by over twofold [53]. In vitro, satellite cells from $Mstn^{-/-}$ mice proliferate and differentiate faster than satellite cells from wild-type mice [52]. However, other studies came to an opposite conclusion and reported no difference in the number and proliferation rate of satellite cells from $Mstn^{-/-}$ and wild-type mice [54]. Consistent with these results, post-developmental inactivation of myostatin leads to hypertrophy without myonuclear accretion [55], and muscle hypertrophy induced by myostatin/activin A inhibition occurs also in $Sdc4^{-/-}$ or $Pax7^{-/-}$ mice in which satellite cell activity is deficient [56]. In vitro, cultured myotubes from $Mstn^{-/-}$ mice are larger than wild-type myotubes, whereas the number of nuclei remains constant [57]. These findings suggest that the larger myonuclear domain size (i.e., the cytoplasmic volume-to-myonucleus ratio) observed in the absence of myostatin is the result of cytoplasmic volume increase rather than of myoblast fusion. Finally, myostatin inhibition using soluble activin receptor type IIB in adult mice activates satellite cells; however, in this model muscle fiber hypertrophy precedes the incorporation of new nuclei [58]. Thus, myostatin inhibition might induce hypertrophy by increasing the cytoplasm/myonuclear ratio before activating satellite cells.

Cell proliferation and differentiation

Myostatin signaling modulates the expression of target genes, such as those involved in the control of myoblast proliferation and terminal differentiation. Incubation with recombinant myostatin can arrest cultured muscle cells (such as C2C12 myoblasts, primary bovine myoblasts and mouse satellite cells) in the G1 phase of the cell cycle [52, 59–61]. Conversely, myostatin inhibition, using a myostatin antisense plasmid, stimulates proliferation and differentiation of C2C12 myoblasts [62]. Myostatin anti-proliferative action is associated with up-regulation of p21 (cyclin-dependent kinase inhibitor) and the subsequent decrease of cyclin-dependent kinases (CDK2 and CDK4) and phosphorylated retinoblastoma protein (RB) levels [52, 59, 61] (Fig. 1). One mechanism by which myostatin down-regulates CDK4 activity and RB phosphorylation is through the regulation of cyclin D1, a known inhibitor of myogenesis. Moreover, myostatin promotes AKT inhibition, GSK-3β activation and cyclin D1 destabilization and this process is dependent on the activin receptor type IIB, but not on SMAD3 [63]. Myostatin has also been implicated in the negative regulation of satellite cell activation and self-renewal through PAX7 down-regulation [52] (Fig. 1).

In addition to premature cell cycle arrest, myostatin also controls the myogenic differentiation program. Myostatin regulates myoblast differentiation through inhibition of myogenic regulatory factors, such as Pax3, MyoD, and Myf5 [60, 64–66] (Fig. 1). Furthermore, the expression of dominant-negative SMAD3 is sufficient to rescue the activity of a *MyoD* gene promoter–reporter vector in C2C12 myoblasts incubated with recombinant myostatin. It has been reported the existence of a negative feedback mechanism between myogenic factors and myostatin as the myogenic factors MyoD and Myf5 induce the activation of the myostatin gene promoter [67]. Overexpression or addition of myostatin to C2C12 cells inhibits myoblast differentiation by down-regulating MyoD and myogenin gene expression via MEK/Erk1/2 pathway and that MEK/Erk1/2 MAPK may play a very important role in myostatin-mediated myogenic differentiation suppression [68]. Finally, myostatin inhibits AKT activation in human myoblasts and myotubes and siRNA-mediated inhibition of RAPTOR (regulatory-associated protein of mTOR), a component of the mTOR signaling complex 1 (mTORC1), contributes to myostatin-mediated inhibition of genes induced during muscle differentiation [20]. Therefore, it appears that myostatin's anti-differentiation effect is at least in part mediated by perturbation of AKT/mTORC1 signaling.

Taken together, these results demonstrate that myostatin acts through several signaling pathways to control the proliferation and differentiation of committed myoblasts.

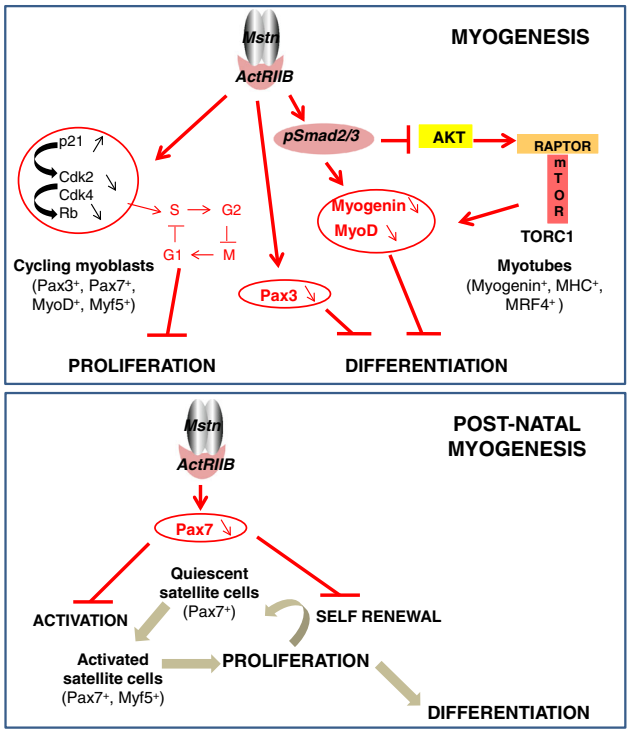

Fig. 1 Schematic illustration of myostatin control of skeletal muscle development and post-natal growth. Differentiation of skeletal muscle cells sequentially requires growth arrest followed by the onset of a timely expression of muscle-specific genes. Cell cycle withdrawal and each phase of differentiation are coordinated by activation of specific cyclins, cyclin-dependent kinases (CDK), CDK inhibitors (CDKI), and muscle regulatory factors (MRFs) that lead to the induction of specific muscle proteins, such as myosin heavy chains (MHC). *Upper panel* During the proliferation phase, myostatin can up-regulate p21 (a CDKI) and, as a consequence, the levels of CDK2 and CDK4 and phosphorylated RB decrease, leading to cell cycle arrest. Myostatin further inhibits differentiation by down-regulating MRFs (such as MyoD and myogenin) and the transcription factor PAX3. RAPTOR blockade also facilitates myostatin inhibition of muscle differentiation. *Lower panel* Myostatin also contributes to the inhibition of satellite cell activation and self-renewal in mature muscle cells by down-regulating the transcription factor PAX7

Regulation of cell survival function

Other data suggest a contribution of myostatin in tipping the survival/apoptosis balance in muscles in favor of cell survival [69, 70]. Transcriptomic and proteomic analyses showed that *Mstn*$^{-/-}$ mice exhibit changes in the expression of genes and proteins related to cell survival/apoptosis pathways. In fact, the down-regulation of some factors (e.g., alpha crystallin-related B6, heat shock protein 9A) and up-regulation of others (e.g., Dad1, survivin, TCTP, 14-3-3E) might be a signature of increased cell survival and anti-apoptotic processes. Moreover, the percentage of apoptotic nuclei, the activity of caspase 3, 8, and 9 and the abundance of their targets and regulators are reduced in

muscles from *Mstn*$^{-/-}$ mice compared to wild-type animals [70]. Similarly, acute antibody-directed myostatin inhibition reduces apoptosis in *tibialis* muscles of aged mice [37]. These findings raise the hypothesis that myostatin inhibition/loss might promote cell survival that will consequently limit the loss of muscle nuclei and increase the size of the nuclear-cytoplasmic domain, a key factor in muscle hypertrophy. However, studies performed in cultured myoblasts do not fully support the notion of myostatin as a direct regulator of muscle apoptosis [59, 60, 62]. In any case, additional experiments are needed to further elucidate the mechanism underlying hypertrophy induced by myostatin inhibition.

In conclusion, myostatin regulation of skeletal muscle size seems to involve on one hand its negative action on proliferation and differentiation of committed myoblasts, and in the other hand the regulation of protein turnover (see next chapter).

Myostatin functions at the crossroad between the protein synthesis and degradation signaling pathways in muscle

Interplay between the myostatin and Akt/mTOR signaling pathways to control protein synthesis

Signaling through the phosphatidyl-inositol 3-kinase (PI3K)/AKT/mTOR pathway controls protein synthesis and muscle hypertrophy in adults [71]. Expression of constitutively active AKT in skeletal muscle rapidly leads to muscle hypertrophy [72, 73]. The finding that mTOR inhibition antagonizes AKT growth-promoting effect suggests that AKT mainly acts via mTOR activation. mTOR forms two large complexes (mTORCs) together with RAPTOR (mTORC1) or RICTOR (mTORC2). mTORC1 directly regulates protein synthesis and is sensitive to rapamycin [74]. The effect of mTORC1 on the translation machinery and protein synthesis is mediated through inhibition of 4E-binding protein 1 (4E-BP1) and activation of the ribosomal protein S6 kinase (S6K) [75–77]. Thus, mTORC1 induces protein synthesis by phosphorylating and inhibiting 4E-BP1 and thereby relieving the repression of eukaryotic translation initiation factor 4E (eIF4E) and cap-dependent translation. It also regulates ribosome biogenesis by at least two different mechanisms: (1) phosphorylation of the ribosomal protein S6 (rpS6) by S6K1 that stimulates mRNA translation with a 5′-oligopyrimidine tract (5′-TOP) [78] and (2) modulation of ribosomal RNA (rRNA) synthesis [79]. Indeed, the mTOR/mTORC1 pathway seems to coordinate changes in translation efficiency (defined as protein synthesis per unit of RNA) and capacity (defined as the total ribosomal content per unit of tissue).

Several lines of evidence support the existence of a cross-talk between myostatin and the AKT/mTOR pathways. Transcriptomic and proteomic analyses found that the PI3K/AKT/mTOR pathway is up-regulated in the absence of myostatin in the mouse and cattle [69, 70]. In hypertrophied skeletal muscle of *Mstn*$^{-/-}$ mice, AKT/mTOR signaling components are more active than in wild-type animals [80, 81]. Subsequently, gain and loss of function studies have shown that myostatin inhibits the activation of the AKT/mTOR pathway. In neonatal rats, inhibition of myostatin through FS infusion increases muscle protein synthesis and this effect is correlated with S6K up-regulation and rpS6 phosphorylation [82]. Increased protein synthesis was also observed in mature wild-type mice treated with anti-myostatin antibodies and in *Mstn*$^{-/-}$ mice [83, 84]. Myostatin gene electrotransfer in adult muscle is associated with down-regulation of the AKT/mTOR signaling pathway, as evidenced by the decreased phosphorylation of AKT, rpS6, p70S6K and 4E-BP1, without effect on the muscle proteolysis pathway [47]. In vitro overexpression of myostatin attenuates myotube hypertrophy induced by IGF-1, whereas myostatin inhibition promotes AKT-dependent hypertrophy [80]. Incubation of cultured human myotubes with myostatin suppresses the AKT/mTOR signaling pathway, leading to inhibition of muscle differentiation and reduction of mature myotube size [20].

Several studies have addressed the molecular basis of the connection between myostatin and Akt. Inhibition of myostatin activity mediated by dominant-negative activin receptor type IIB overexpression promotes skeletal muscle hypertrophy via mTOR signaling [19]. Furthermore, inhibition of SMAD2 and SMAD3, the transcription factors downstream of myostatin, in skeletal muscle of adult mice also promotes muscle hypertrophy, and interestingly the mechanism of such regulation is partially dependent on mTORC1 signaling. In fact, inhibition of mTOR by rapamycin and by RNAi experiments partially inhibits the hypertrophic effect of blocking myostatin activity (about 35–40 %). This result is consistent with findings indicating that rapamycin does not prevent the stimulation of muscle protein synthesis induced by an anti-myostatin antibody [84]. Collectively, these studies indicate that myostatin and the AKT pathways cross-talk at different levels and suggest that myostatin could be a key signaling molecule in the control of protein metabolism.

Myostatin and the regulation of translation

Our study further provides important new insights concerning the link between myostatin and the translational machinery. Using an in vitro model of cultured satellite cells derived from *Mstn*$^{-/-}$ mice, we recently demonstrated

that translational factors are recruited to the 7-methyl guanosine cap binding complexes more efficiently in *Mstn*$^{-/-}$ cells than in control cells, whereas myostatin addition inhibits this process [57]. It is thus reasonable to hypothesize that myostatin inhibition leads to muscle hypertrophy by affecting the translation efficiency of specific muscle mRNAs. For instance, we have shown that myostatin deletion is associated with increased AKT, mTOR and rpS6 protein but not mRNA expression, suggesting that myostatin could control the translation of a specific subset of mRNAs that are part of a growth checkpoint in muscle cells. Alternatively, myostatin might act directly on some regulators of translation efficiency that, in turn, could play a role in the translation of specific mRNAs. Indeed, several genes encoding translation initiation and elongation factors are up-regulated in muscle of young *Mstn*$^{-/-}$ mice [69, 85]. In addition, overexpression of components of the translation machinery, such as eukaryotic translation initiation factor 3-subunit F (eIF3F) or eukaryotic translation initiation factor 2B-epsilon (eIF2Be), induces the same increase in cap-dependent translation and skeletal muscle hypertrophy as observed upon myostatin inactivation, suggesting that myostatin effect on protein synthesis could be indirect [86–88].

After translation initiation, the translation capacity is dependent on the level of functional ribosomes in the cell. Ribosomal gene transcription (rDNA transcription) is a major rate-limiting step in ribosome biogenesis. Evidence presented by Welle et al. [83] suggests that myostatin contributes to regulating the translation capacity. Specifically, protein synthesis and RNA content per muscle is higher in myostatin-deficient mice than in wild-type animals. Accordingly, the total RNA content, polysome formation and protein synthesis are all increased in cultured *Mstn*$^{-/-}$ myotubes [57]. Together, these data indicate that myostatin effects on protein synthesis may affect both ribosomal biogenesis and translation efficiency of specific mRNA subclasses (Fig. 2).

Myostatin and protein degradation signaling pathways

Myostatin's role in protein degradation is not well understood. Muscle atrophy is characterized by a decrease in the diameter and total protein content of myofibers and it is the result of reduced protein synthesis and increased protein degradation and turn over. In multiple cell types, FOXO transcription factors are key mediators of the catabolic response during atrophy and they coordinate the activation of the two most important cellular proteolytic mechanisms: the autophagy–lysosome and ubiquitin–proteasome systems. FOXO activity is suppressed by AKT-mediated phosphorylation, thus preventing the expression of atrogenes, such as MAFbx/Atrogin-1 and MuRF-1 (two critical

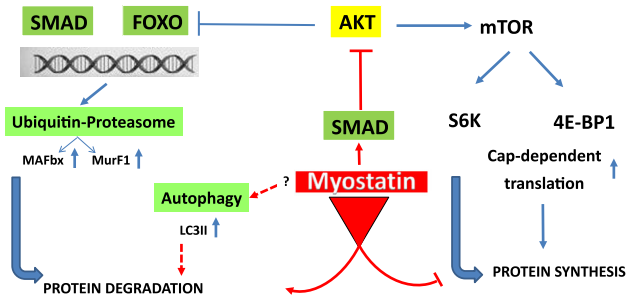

Fig. 2 Schematic illustration of myostatin roles in protein synthesis and degradation. First, the myostatin–SMAD pathway alters the activity of the protein kinase AKT, thereby inhibiting the assembly of the translation initiation complex and protein synthesis. AKT blocks FOXO nuclear translocation to inhibit the expression of MAFbx and MuRF1 and consequently protein degradation. Second, myostatin also suppresses the AKT pathway and signals through FOXO transcription factors to increase protein breakdown via the activity of the ubiquitin–proteasome system. Finally myostatin could inhibit also the autophagy–lysosome system

atrophy-related ubiquitin ligases) [89, 90]. There is evidence that myostatin could induce muscle atrophy partly by activating the ubiquitin–proteasome system. Indeed, McFarlane et al. [91] reported that in cultured C2C12 muscle cells, myostatin treatment blocks the IGF1/PI3K/AKT pathway and activates FOXO1, leading to increased expression of MAFbx and MuRF-1. Interestingly, FOXO1 can up-regulate myostatin expression, suggesting a synergistic feedback mechanism that amplifies the atrophic response [92]. Myostatin-mediated up-regulation of atrogenes has subsequently been described also in human myotubes [93, 94].

However, Trendelenburg et al. have reported that myostatin reduces the diameter of mature HuSkMC myotubes and down-regulates the AKT pathway, but does not induce the E3 ubiquitin ligases MuRF1 and MAFbx. In fact, these genes were down-regulated together with myogenin and MyoD, a result consistent with the anti-differentiation effect of myostatin [20]. These results challenge myostatin involvement in the inactivation of the AKT/mTOR axis and atrogene induction and suggest that, in post-differentiated myotubes, myostatin causes a decrease in fiber diameter without inducing the canonical "atrophy pathway". Indeed, myostatin could causes a decrease in muscle mass by inhibiting the expression of genes required for muscle maintenance.

Noteworthy, in muscles from *Mstn*$^{-/-}$ mice, MAFbx protein level and ubiquitinated myosin heavy chain are both reduced, suggesting a lower activity of the ubiquitin–proteasome system compared to wild-type muscles [95]. Conversely, Amirouche et al. [47] reported that myostatin overexpression in muscle did not alter the activity of the ubiquitin–proteasome system. Similarly, addition of recombinant myostatin to C2C12 myotubes decreased

protein synthesis without affecting the proteolysis rate [61]. In conclusion, the direct contribution of myostatin in triggering the expression of atrogenes and protein breakdown in vivo has not been precisely determined yet. Although in vivo studies do not support the notion that myostatin induces MAFbx or MuRF1 expression, several evidences indicate that once proteolysis is stimulated, myostatin inhibition can reduce proteolysis [43, 96, 97]. Few studies have explored the link between myostatin and the autophagy–lysosome system. In C2C12 cells, myostatin can stimulate autophagosome formation and the expression of several autophagy-related genes [98]. Furthermore, the recent study by Seiliez et al. [99] indicates that in vitro myostatin-mediated trout myotube atrophy is associated with an up-regulation of both the ubiquitin–proteasome and the autophagy–lysosome systems. Keeping in mind the complexity of the myostatin signaling in trout (due to the presence of four myostatin paralogs) [100], this hypothesized myostatin–autophagy link should be confirmed in primary muscle culture from mammals.

Combined, these results all suggest a link between myostatin, autophagy, and the ubiquitin–proteasome. Additional experiments are needed to elucidate the role of myostatin in the regulation of the AKT pathway and the cell proteolytic systems during muscle proteolysis (Fig. 2).

Conclusion

Over the last few years, myostatin has emerged as a key intermediary in the response of muscle to anabolic and catabolic stimuli. Myostatin negatively regulates the activity of the AKT pathway and acts as a repressor of translation initiation. Despite the evidence of myostatin requirement for the regulation of protein synthesis, myostatin role in triggering protein degradation is not obvious. Myostatin decreases AKT phosphorylation and signals through FOXO transcription factors to induce atrophy, but the mechanisms through which myostatin regulates the proteolytic systems need to be further investigated. Furthermore, to understand the myostatin effect on protein synthesis, it will be interesting to establish whether the particular regulation of the translational machinery by myostatin might involve mRNA-selective translation. Also, the effect of myostatin on translational capacity, such as regulation of rRNA biogenesis remains largely unexplored. This is crucial for the development of drugs or treatments to inhibit the myostatin signaling pathway.

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