INVITED REVIEW:
Inhibitors of myostatin as methods of enhancing muscle growth and development

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ABSTRACT: With the increasing demand for affordable, high-quality meat, livestock and poultry producers must continually find ways to maximize muscle growth in their animals without compromising palatability of the meat products. Muscle mass relies on myoblast proliferation during prenatal or prehatch stages and fiber hypertrophy through protein synthesis and nuclei donation by satellite cells after birth or hatch. Therefore, understanding the cellular and molecular mechanisms of myogenesis and muscle development is of great interest. Myostatin is a well-known negative regulator of muscle growth and development that inhibits proliferation and differentiation in myogenic cells as well as protein synthesis in existing muscle fibers. In this review, various inhibitors of myostatin activity or signaling are examined that may be used in animal agriculture for enhancing muscle growth. Myostatin inhibitors are relevant as potential therapies for muscle-wasting diseases and muscle weakness in humans and animals. Currently, there are no commercial myostatin inhibitors for agriculture or biomedical purposes because the safest and most effective option has yet to be identified. Further investigation of myostatin inhibitors and administration strategies may revolutionize animal production and the medical field.

Key words: fiber hypertrophy, muscle mass, myoblast proliferation, myostatin

INTRODUCTION

Myostatin (MSTN), also known as growth/differentiation factor-8, is a transforming growth factor β (TGF-β) family member that regulates skeletal muscle development by limiting proliferation and differentiation of muscle precursor cells (McPherron et al., 1997). Among vertebrates, MSTN sequences contain 3 exons and are highly homologous, indicating a conserved function. Expression can first be detected in the myotome of developing somites and continues to be expressed in skeletal muscle throughout adulthood (Lee and McPherron, 2001; Castelhano-Barbosa et al., 2005). Myostatin is translated as a precursor protein that is subjected to 3 proteolytic processing events to yield the active, mature MSTN peptide. Initially, a signal peptidase removes the amino(N)-terminal signal sequence. Pro-MSTN dimerizes by a disulfide bond near the carboxy(C)-terminus and is systematically cleaved at the RXXR site by a calcium-dependent serine protease known as furin (Fig. 1; Lee and McPherron, 2001), which is concentrated within the trans-Golgi network (Molloy et al., 1994, 1999). This generates the N-terminal propeptide with N-linked glycosylation and a C-terminal receptor-binding domain (McFarlane et al., 2005). The latent MSTN complex forms as the propeptide noncovalently binds the C-terminal region via a critical peptide sequence, and this prevents MSTN from binding to the target receptor. Members of the bone morphogenetic protein-1/tolloid (BMP-1/TLD) family are metalloproteinases that can cleave the propeptide and release mature MSTN (Wolfman et al., 2003). Inability to cleave the propeptides from the latent complex has been shown to increase muscle growth in adult mice (Wolfman et al., 2003). Mature MSTN dimers bind to activin receptor IIB (ActRIIB), which then signals sequential phosphorylation of activin receptor-like kinase

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4 (ALK4) or activin receptor-like kinase 5 (ALK5) type I receptor and Sma- and Mad-related protein (Smad) 2/3 (Rebbapragada et al., 2003). After Smad2/3 recruits Smad4, the complex translocates to the nucleus and inhibits transcription of myogenic regulatory factors (MRF) to block myogenic precursor cells from exiting the cell cycle and differentiating into myofibers (Liu et al., 2001; Langley et al., 2002; Huygens et al., 2004). High expression of \textit{MSTN} inhibits \textit{Paired box 7} (\textit{Pax7}) expression through extracellular signal-related kinase 1/2 (\textit{ERK1/2}) signaling; therefore, myogenic precursor cell proliferation and satellite cell renewal are both inhibited (McFarlane et al., 2008). Furthermore, protein synthesis is downregulated through inhibition of insulin-mediated Akt phosphorylation by Smad2/3. The unphosphorylated tuberous sclerosis 1/2 (\textit{TSC1/2}) complex continues to suppress the mechanistic target of rapamycin (\textit{mTOR}) to prevent protein synthesis (Trendelenburg et al., 2009). Inhibiting Akt phosphorylation also increases forkhead box O1 (\textit{FoxO1}) activity, which induces the ubiquitin proteolytic system to result in protein degradation (McFarlane et al., 2006).

\textbf{Figure 1.} Myostatin is produced as an inactive peptide that homodimerizes near the C terminus via a disulfide bond to form promyostatin. The furin-type protease cleaves promyostatin at an Arg-X-X-Arg (RXXR) site to create the N-terminal and COOH(C)-terminal fragments. The C-terminal fragments remain bound by the disulfide bond but are also noncovalently bound to the N-terminal fragments to generate the latent myostatin complex. The latent complex cannot bind to the target receptor due to association with the N-terminal propeptides. Bone morphogenetic protein-1 (BMP-1)/tolloid proteinases cleave the propeptides at an arginine (R) residue to release them from the latent complex. The mature C-terminal myostatin dimer can bind to activin receptor IIB (ActRIIB).
Inhibitors of myostatin

Myostatin signals in both an endocrine and a paracrine manner. Lee et al. (2016b) used the Cre-Lox recombination system to generate mice that did not have MSTN production in posterior muscles (quadriceps and gastrocnemius) but did have production in anterior muscles (pectoralis and triceps). These mice had significantly reduced concentrations of circulating MSTN. Interestingly, the posterior muscles had highly significant increases in muscle weight in both sexes with a majority of the increase due to hyperplasia and, to a lesser extent, fiber hypertrophy. Although the paracrine role of MSTN is more pronounced, the posteriorly located muscles were significantly smaller in the Cre-Lox MSTN mice when compared with MSTN-knockout mice. This suggests that endocrine actions of MSTN are still notable.

INHIBITION OF MYOSTATIN

Myostatin is a strong negative regulator of myogenesis and skeletal muscle accretion (Lee and McPherron, 2001). Injections of MSTN into the circulation of adult mice resulted in severe muscle loss, further solidifying its role as a negative regulator of muscle size (Zimmers et al., 2002). Studies that investigated inhibition of MSTN revealed large increases in skeletal muscle mass by both hypertrophy and hyperplasia (McPherron et al., 1997; Elashry et al., 2009; Rose et al., 2009). Interestingly, MSTN-knockout animals have greater numbers of type II fibers and fewer numbers of type I fibers (Girgenrath et al., 2005). With the increasing demand for larger muscle mass in production animals, downregulation of MSTN activity has gained interest as a potential method for achieving greater muscle yield. Antagonists of MSTN and various mutations have been identified that dramatically impact muscle growth in animals (McPherron et al., 1997; Lee and McPherron, 2001). Furthermore, alternative splicing of the MSTN gene reveals unique inhibition mechanisms that exist in avian species (Shin et al., 2015). Myostatin inhibition is also being assessed for medical purposes, such as repairing injury, sarcopenia, cachexia, and muscular dystrophy (Hamrick et al., 2010; Murphy et al., 2010; Fakhfakh et al., 2011). Although MSTN is mostly expressed in skeletal muscle, expression has been observed in other tissues, including adipose tissue, cardiac muscle, and the brain (McPherron et al., 1997; Sharma et al., 1999; McPherron and Lee, 2002; Sundaresan et al., 2008); therefore, caution must be taken when investigating and using inhibitors of MSTN.

Natural Mutations

Mutations in the MSTN gene that disrupt the reading frame or splicing result in little or no production of the mature C-terminal peptide. First reported by McPherron and Lee (1997), 2 cattle breeds were determined to possess mutations in the coding region of MSTN, which is responsible for their characteristic double-muscled phenotype. Belgian Blue cattle have an 11-nucleotide deletion in exon 3, creating a shift in the reading frame and a protein devoid of the bioactive region. Piedmontese cattle have a missense mutation in exon 3 that replaces a cysteine with a tyrosine to result in loss of function. This cysteine residue is invariant for all TGF-β family members and is involved in the formation of the intramolecular cysteine knot structure, which contains a total of 9 cysteines (Mittl et al., 1996; Galat, 2011). Notably, Piedmontese cattle have higher expression of MSTN mRNA and precursor protein in the skeletal muscle but decreased expression of mature MSTN (Berry et al., 2002). Therefore, this mutation prevents proteolytic processing into mature MSTN and results in a loss of biological activity. Meat from these 2 breeds has a lower total fat content, reduced marbling, and increased numbers of type II fibers, but tenderness is increased due to a higher number of muscle fibers by uninhibited proliferation during development (Kambadur et al., 1997; Wegner et al., 2000; Wheeler et al., 2001) as well as decreased collagen content (Bailey et al., 1982; Fiems, 2012). Cross-breeding Piedmontese and Belgian Blue with other cattle breeds improves carcass characteristics (Short et al., 2002; Domingo et al., 2015); however, reproductive issues, such as narrower birth canals and increased calf size, are major obstacles diminishing the popularity of the breeds (Kambadur et al., 1997; Kolkman et al., 2012). The MSYN F94L mutation, which is detected in high frequency in Limousins, is associated with increased muscle mass but not reduced fertility and dystocia (Vankan et al., 2010). Integration of the F94L mutation or other MSTN mutations (O’Rourke et al., 2012) that result in increased muscle mass without compromising reproduction could be used to improve carcass yield from beef cattle.

Certain breeds of sheep with increased muscle mass have also been characterized as possessing MSTN mutations. Texel sheep originating from the Netherlands are a heavy-muscled breed that produces a lean carcass. Genetic mapping revealed that the MSTN allele in Texel sheep have a G to A transition in the 3′-untranslated region, which produces a target site for microRNA (miRNA) in the skeletal muscle. Translational inhibition of MSTN by the miRNA (miR-1 and miR-206) causes increased muscling in these sheep (Clop et al., 2006). Meat from Texel sheep is very tough, which hinders their profitability (Walling et al., 2004; Johnson et al., 2005). Moreover, a single base deletion was identified in the MSTN coding region of Norwegian white sheep that results in a premature stop codon and a double-muscled phenotype (Boman et al., 2009).
Myostatin mutations associated with athletic performance and human health are documented. In Bully whippets, a 2-bp deletion in exon 3 of MSTN results in a premature stop codon and loss of function of the normal protein to cause a double-muscled phenotype in dogs. Even whippets with 1 copy of the mutation are significantly more muscular and faster than their wild-type counterparts (Mosher et al., 2007). This mutation increases the numbers of fast-twitch muscle fibers, which explains their faster speeds during short distances. A child with muscle hypertrophy was determined to have 2 copies of a G to A transition in the noncoding region of the MSTN gene. This particular mutation disrupted splicing of the precursor mRNA and generated a protein that was unable to be processed into the mature form. Analysis of the child’s serum revealed that the MSTN propeptide was absent; therefore, mature MSTN was also absent. The child possessed extraordinary strength and could suspend two 3-kg dumbbells with his arms fully extended at 4.5 yr old. Moreover, his mother was a professional athlete and possessed 1 copy of the mutation (Schuelke et al., 2004). However, reduced MSTN activity switches muscle from an aerobic to an anaerobic energy metabolism, decreasing the oxidative capacity and increasing fatigability (Mouisel et al., 2014).

**FOLLISTATIN**

Follistatin (FST) is a natural antagonist of several TGF-β family members including MSTN (Fig. 2; Nakamura et al., 1990; Fainsod et al., 1997; Amthor et al., 2004). This glycoprotein was originally discovered in porcine ovarian follicular fluid, and it inhibited synthesis of FSH from the pituitary gland (Phillips and de Kretser, 1998). During chicken embryo development, MSTN and FST are expressed in similar regions that give rise to skeletal muscle, and FST protein binds to MSTN with high affinity. Additionally, FST inhibits MSTN binding to ActRIIB, allowing for upregulation of key MRF, such as Pax3 and MyoD (Amthor et al., 2004).

Overexpression of FST using a skeletal muscle-specific myosin light chain promoter in mice significantly increased muscle mass by 2- to 3-fold through hyperplasia and hypertrophy (Lee and McPherron, 2001). By contrast, FST-knockout mice have reduced muscle mass at birth and several other defects that lead to death within a few hours, indicating that FST regulates activity of many TGF-β members (Matzuk et al., 1995). When the FST transgene was present in MSTN-null mice, muscle mass quadrupled, which suggests that FST inhibited other regulators of muscle metabolism.
growth that may be comparable to MSTN (Lee, 2007). In a report by Kota et al. (2009), a skeletal muscle-specific alternatively spliced isoform of human FST was expressed through an adeno-associated virus serotype 1 vector and injected into the quadriceps of macaque monkeys. The treated monkeys had significantly larger quadriceps and increased strength compared with the controls, and there were no long-term adverse effects on other organs.

DOMINANT NEGATIVE FORM OF ACTIVIN RECEPTOR IIB

Mature MSTN dimers bind to ActRIIB to induce a signaling cascade, which modulates the expression of various target genes. A dominant negative form of ActRIIB (dnActRIIB) was constructed with a truncated kinase domain and expressed in mice using a skeletal muscle-specific myosin light chain promoter to demonstrate the effects of sequestering MSTN and preventing the signaling cascade (Fig. 2; Lee and McPherron, 2001). The transgenic lines had varying increases in muscle mass depending on the expression of the transgene. When compared with overexpression of FST in the same study, all lines showed similar but less drastic results regarding larger muscle mass. The gastrocnemius and plantaris muscles from the transgenic mice had greater fiber numbers and fiber size than the nontransgenic counterparts (Lee and McPherron, 2001). In addition, Lee et al. (2005) generated a soluble form of ActRIIB and injected it into 6-wk-old mice. Because muscle fiber number was fixed at this age, the observed increases in muscle mass within the treated mice were due to hypertrophy through protein synthesis.

Skeletal muscle regeneration is essential for the success of different therapies for Duchenne muscular dystrophy, but MSTN reduces regenerative activities and hinders the efficiencies of the therapies. One of these treatments is transplantation of myoblasts to introduce the dystrophin gene into affected fibers (Palmieri et al., 2010). Fakhfakh et al. (2011) transplanted myoblasts with an ActRIIB into dystrophic mice. Analysis of the tibialis anterior revealed higher numbers of dystrophin-positive fibers in the mice treated with dnActRIIB myoblasts than in mice treated with normal myoblasts. This treatment increased expression of MRF for proliferation and fusion of the myoblasts.

MSTN PROPEPTIDE

During processing of full-length MSTN, dimerized pro-MSTN is cleaved by furin to generate the N-terminal propeptides and C-terminal receptor-binding domain (McFarlane et al., 2005). The propeptides can noncovalently bind to the C-terminal domain, forming the latent MSTN complex, and prevent further interactions with ActRIIB (Thies et al., 2001). Two propeptides can also inhibit processing of 1 pro-MSTN dimer with or without the presence of the N-linked glycosylation through binding via a critical peptide sequence (Fig. 3; Jiang et al., 2004). Members of the BMP-1/TLD family are metalloproteinases that can cleave the propeptide and release mature MSTN (Wolfman et al., 2003). A mutant form of the propeptide that resisted cleavage increased muscle growth throughout the body when injected into adult mice, and the effects were dose-dependent (Wolfman et al., 2003). In addition, Lee and McPherron (2001) overexpressed the MSTN propeptide in skeletal muscle using the same myosin light chain promoter as for FST and the dominant negative form of ActRIIB. Once again, significant increases in muscle mass were achieved, and the results were comparable to those of the dnActRIIB. As a model for musculoskeletal damage, recombinant MSTN propeptide was injected into mice that were subjected to fibula osteotomy and damage to muscles in the surrounding area. When MSTN was blocked through propeptide treatment, regeneration of bone and skeletal muscle was enhanced, significantly decreasing recovery time (Hamrick et al., 2010).

Transgenic mice overexpressing the porcine MSTN propeptide exhibited enhanced muscle size (Ma et al., 2015). Moreover, a porcine MSTN propeptide with a mutated BMP-1/TLD site has been synthesized in Escherichia coli. The mutant propeptide had the same level of MSTN inhibitory activity as the wild-type propeptide in an in vitro gene reporter assay, which implies that it may have strong agricultural implications for increasing muscle mass in pigs (Haq et al., 2013).

Within the propeptide, there are particular AA residues close to the amino-terminus that are necessary for the inhibitory activity (Fig. 3). Jiang et al. (2004) generated 15 truncated versions of the human MSTN propeptide through the bacterial expression system and identified residues 42 through 115 as the region that was essential for MSTN inhibition. This conserved region of the MSTN propeptide in flatfish had inhibitory activity with the same potency as the full propeptide sequence (Lee et al., 2016a). Smaller peptides (23–24 AA) derived from the murine MSTN propeptide inhibit MSTN activity and Smad2 phosphorylation in vitro. One of the peptides was intramuscularly injected into the tibialis anterior of dystrophin-deficient mdx mice and increased the weight of the muscle (Takayama et al., 2015). This suggests that smaller-sized sequences from the MSTN propeptide can be used for different applications to inhibit MSTN activity, such as for agricultural and biomedical purposes.
Usage of antibodies targeting MSTN activity and signaling may be a promising treatment for sarcopenia and other muscle-wasting diseases as well as promoting muscle growth in poultry and livestock (Fig. 2). Lach-Trifilieff et al. (2014) developed a human anti-ActRIIB antibody that binds to the receptor, acting as a competitive inhibitor to ligands. Proliferation and differentiation of human myoblasts were enhanced after administration of the antibody in vitro, and skeletal muscle size in mice was increased when injected with a murinized version of the antibody. The antibodies REGN1033 and ActRIIB-hFc have a high affinity for MSTN and prevent binding to ActRIIB. Injections of these antibodies into mice increased the weights of the gastrocnemius and tibialis anterior without altering heart weight (Latres et al., 2015). Another anti-MSTN antibody (PF-354) prevented muscle loss due to aging when injected into 18-mo-old mice and resulted in maintenance of soleus, gastrocnemius, and quadriceps sizes and maximum forces (Murphy et al., 2010). Recently, the anti-MSTN antibody ATA 842 was administered to young and old mice and increased muscle mass and grip strength. In addition, whole body insulin sensitivity was improved in old mice following ATA 842 treatment (Camporez et al., 2016). Kim et al. (2006) injected anti-MSTN antibodies (mAb-c134) into the yolk of broiler eggs. Body weight and muscle mass were increased in the treatment broilers at 35 d after hatch when compared with the control with no injection. Injection of pAb-AVM46, a polyclonal anti-chicken MSTN that recognizes the propeptide, into the yolk of broiler eggs resulted in significantly lower combined thigh and leg weight than the control group at 28 d after hatch (Kim et al., 2007). This implies that MSTN activity is increased when the antibody is bound to the propeptide.

**Figure 3.** Myostatin processing is inhibited through several mechanisms. Myostatin-B (MSTN-B) in avians can potentially bind promyostatin at a critical AA sequence to prevent proteolytic processing to the mature form. Two propeptides can also bind promyostatin in the same region to prevent proteolytic processing. Smaller sequences of the propeptide are speculated to be crucial for binding to promyostatin and inhibiting processing.
ALTERNATIVE SPlicing ISOFORMS

Alternative splicing is a highly controlled process that produces multiple transcripts from 1 unprocessed pre-mRNA through inclusion or exclusion of exons and noncoding regions. This explains the phenomenon of how a single gene can generate many transcripts to be translated into several distinct proteins (Matlin et al., 2005). Avian species have multiple alternative splicing isoforms of MSTN that are differentially expressed among various tissues, including adipose tissue, skeletal muscle, heart, liver, lung, and kidney (Shin et al., 2015). A shorter isoform of full-length MSTN that contained part of the sequence for the propeptide but lacked the entire C-terminal region to code mature MSTN was the first to be discovered (Castelhano-Barbosa et al., 2005). Four MSTN splicing variants were revealed in growing skeletal muscle of Peking ducks (Huang et al., 2011). Real-time PCR demonstrated that the classical MSTN transcript, MSTN-A, and MSTN-B, which encodes a portion of the N-terminal propeptide, had significantly higher expression in breast and leg muscles than the other 2 isoforms, MSTN-C and MSTN-D. This suggests that MSTN-A and -B may have an important role in myogenesis and posthatch muscle development (Huang et al., 2011). Furthermore, these alternative splicing isoforms are present in migratory birds, such as white-throated sparrows and European starlings; however, their potential functions were not investigated (Price et al., 2011).

More recently, Shin et al. (2015) confirmed 4 alternative splicing isoforms of MSTN (-A to -D) in domestic avian species and discovered another isoform (MSTN-E). Splicing of 3 isoforms, MSTN-B, -C, and -E, generates a premature stop codon that results in a truncated peptide devoid of the mature MSTN domain. MSTN-D has a deletion of the first 297 bp of exon 2; however, the reading frame is conserved and this isoform still produces the mature MSTN domain. Pectoralis major muscle and thigh muscle of chickens, turkeys, and quail had high expression of MSTN-A and -B that was dynamic through embryonic and posthatch stages, suggesting that these 2 isoforms regulate critical stages of muscle development in avian species. Overexpression of MSTN-A in quail muscle clone 7 (QM7) cells reduced differentiation into myotubes compared with the control. On the contrary, overexpression of MSTN-B in QM7 cells increased both proliferation and differentiation into myotubes. Co-immunoprecipitation analysis revealed that MSTN-B binds to MSTN-A at a critical AA sequence and prevents proteolytic processing of MSTN-A to the mature form (Fig. 3). Because MSTN-B contains this critical binding region, expression of this alternative splicing variant may be a useful marker for improving muscle mass in poultry.

A splicing variant of ovine MSTN was identified that has a deletion of the receptor-binding sequence within the C-terminal domain. When the splicing variant was overexpressed in C2C12 myoblasts, a greater amount of proliferation was observed as well as up-regulation of MyoD and myogenin (Jeanplong et al., 2013). Further investigation of alternative splicing in mammalian MSTN sequences may reveal potential applications for animal agriculture as selection markers to enhance muscle growth characteristics.

SUMMARY AND FUTURE PERSPECTIVES

With the discovery of myostatin as an antimyogenic factor in animals and humans, inactivation of MSTN has been a promising strategy for improving muscle growth of food animals and treating human diseases associated with muscle weakness and dystrophy. Approaches for inhibition of MSTN binding to ActRIIB include FST that antagonizes MSTN by protein–protein interactions and antibodies for mature MSTN and ActRIIB. The MSTN propeptide and truncated versions exhibit inhibitory activity against MSTN. Most recently, an alternative spliced isoform of avian MSTN encoding 129 AA binds to pro-MSTN, inhibits proteolytic processing of pro-MSTN, and thereby reduces the release of mature MSTN in cell culture. Considering the aforementioned promyogenic mechanism of the alternative splicing isoform of avian MSTN, further studies are needed to investigate whether the short peptides within residues 42 through 115 have promyogenic activities with the same mechanism of action as the alternative splicing isoform of avian MSTN. As the in vivo evidence in support of MSTN inhibition through short peptides is mounting, mechanisms of their action and methods of their administration should be determined to practically use them in food animals or human medicine. In addition, the promyogenic potential of the alternative splicing isoform of MSTN should be further validated in vivo by generating transgenic poultry overexpressing this form. With no commercial MSTN inhibitors currently available, production and administration of short peptides from the MSTN propeptide or selection of superior livestock and poultry breeders through expression of MSTN alternative splicing isoforms may be the safer and less intensive alternatives for practical applications.

LITERATURE CITED
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