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Visions & Reflections (Minireview)

Myostatin as a therapeutic target for musculoskeletal disease

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Myostatin regulates muscle cell homeostasis in adults

Adult skeletal muscle is a dynamic tissue undergoing a continuous round of stem cell activation, proliferation and differentiation. A number of myogenic cell types at various stages of development exist in muscle tissue at any one time: satellite cells (muscle stem cells), myoblasts, myocytes (activated proliferating myoblasts), differentiated myocytes and finally, the muscle syncitia that make up fast and slow twitch muscle fibers (Fig. 1A). Atrophy of muscle cells or of the motor neurons that innervate them results in muscular wasting and loss of function. The regulation of skeletal muscle homeostasis is tightly controlled to maintain a delicate balance of cell types within the tissue at all times. Myostatin (MSTN), also known as GDF-8, is a member of the TGF-β family of growth and differentiation factors [1]. It serves to negatively regulate stem cell activation [2] as well as promote survival of the muscle syncitia. Naturally occurring null mutations in the myostatin gene of cows, sheep, dogs, swine and humans are hyper muscular with myostatin null mice displaying a similar phenotype [3]. In particular, dogs carrying at least one mutant myostatin allele displayed enhanced racing performance, confirming a functional benefit to loss of myostatin [4]. Correspondingly, overexpression of myostatin in adult mice causes profound muscle and fat loss without an alteration in satiety [5] and in humans, myostatin levels are elevated in sarcopenic individuals [6].

Myostatin is produced in increasing amounts as cells progress through the developmental cascade such that the rate of satellite cell activation, proliferation and differentiation is negatively regulated by signals arising from the fully developed muscle cells. The complex signal transduction network through which myostatin regulates myoblast maturation is described in Figure 1A and is nicely reviewed by Joulia-Ekaza and Cabello [3] and references therein. Myostatin binds to and activates a heterodimeric complex of activin receptor 2B (Acvr2b) and ALK4 or ALK5 that is expressed by myogenic stem cells and proliferating myoblasts. Acvr2b receptor activation triggers multiple intracellular signaling cascades including the SMAD and MAPK pathways that activate the AKT and p21/Rb pathways and inhibit expression of the muscle regulatory factors (MRFs). MRFs, including MyoD, positively regulate myostatin gene transcription during myocyte maturation. This results in the inhibition of satellite cell activation, blocking of the cell cycle in proliferating myoblasts and consequently slows further myogenesis. Myostatin signaling also prolongs the survival of fully differentiated muscle syncitia *via* the p53 pathway and stabilizes the acetylcholine receptors that underpin neuromuscular junctions. The myostatin signaling cascade also forms an autoregulatory loop to repress its own transcription

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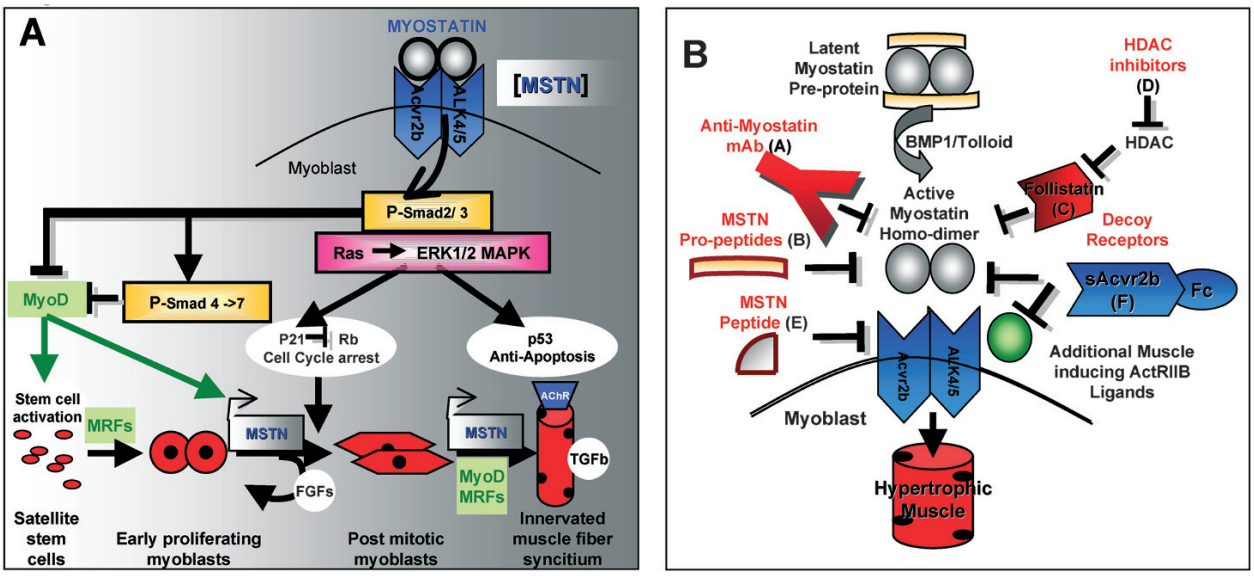

Figure 1. (*A*) Myostatin (MSTN) levels tightly regulate skeletal muscle cell homeostasis in adult tissue. Myostatin is synthesized and secreted by muscle cells in increasing trace amounts as they proceed through the myoblast activation, proliferation and differentiation pathway. Myostatin signals through the activin receptor IIB (Acvr2b) ALK4/5 heterodimer to activate Smad2/3 with the consequent nuclear translocation of Smad4 and blocking of MyoD transactivation in an autoregulatory feedback loop. In addition, Smad3 sequesters MyoD in the cytoplasm to prevent it from entering the nucleus and activating the stem cell population *via* the induction of myogenic regulatory factors (MRFs) [29]. Once in the nucleus, Smad4 not only inhibits myostatin transcription but also activates Smad7 expression [30]. Smad7 is a key component of the autoregulatory loop that not only represses myostatin transcription but also activates Smurf-mediated ubiquitination and subsequent degradation of the myostatin receptor/ligand complex (not shown) [30]. Activated Smad2/3 also serves to activate the Erk 1/2 MAPK pathway (yellow box). In proliferating myoblasts this pathway arrests cell proliferation and differentiation through the p21/Rb cascade. In differentiated cells the Erk 1/2 MAPK pathway activates p53 to promote anti-apoptotic pathways, which, in combination with TGF-β signaling, maintain the muscle syncitia and the expression of acetylcholine receptor alpha (ACR) component of the neuromuscular junction. Arrows, inductive signals; T-bars, inhibitory signals; gray, myostatin levels; yellow, SMAD pathway; green, myogenic regulatory transcription factors. (*B*) Points of therapeutic intervention to promote muscle building. Several components that regulate the availability of the active myostatin homodimer provide possible therapeutic candidates with which to block myostatin activity. Anti-myostatin monoclonal antibodies (A) such as RK35 and JA16 [13, 15, 31, 32] have been used to specifically block myostatin signaling. Exogenous myostatin propeptides (B) have also been used to render the myostatin homodimer inactive [17, 18]. Follistatin (C) acts as an endogenous decoy receptor, binding extracellular myostatin and sequestering it to the external cell membrane. Follistatin expression is induced by treatment with histone deacetylase (HDAC) inhibitors (D) [26]. A C-terminal fragment of the mature myostatin has been postulated as a dominant negative modulator of receptor signaling (E) [33]. A soluble decoy receptor Acvr2b-Fc is used to sequester myostatin ligand binding and so prevent it from binding to the endogenous transmembrane receptor [28]. Acvr2b-Fc also blocks signaling by additional, as yet unidentified, Acvr2b ligands that induce muscle maturation (F). Treatment with any of these anabolic reagents results in hyper-muscularization. Figure adapted from [8].

as well as to degrade the myostatin/Acvr2b signaling complex [3]. This complex mode of action creates a careful balance of differentiated cell types for optimal muscular function as well as for rapid regeneration of damaged muscle tissue.

Regulation of bioavailable myostatin is very tightly controlled *in vivo*

Given its key role in muscle homeostasis, it is not surprising that the availability of bioactive myostatin is very tightly regulated through post-translational modification and protein sequestration. The protein is produced as an inactive dimeric precursor that undergoes catalytic cleavages resulting in a 26-kDa N-terminal propeptide and a 12.5-kDa C-terminal peptide, a homodimer that is the biologically active protein. Following biosynthesis it is retained in the cytoplasm of the producing cell by the addition of a Titin cap [7]. Upon secretion, mature myostatin is held in an inactive conformation by the two propeptides in a tetrameric complex that is disassembled following proteolytic cleavage by a BMP1/Tolloid family enzyme to release the active homodimeric signaling molecule [8]. Recent genetic evidence has shown that mutation of the BMP1/Tolloid cleavage site generates inactive myostatin with increased muscle mass in mice [9]. The availability of the active myostatin homodimer is tightly regulated by multiple factors. Once secreted from the cell, myostatin is chaperoned by a number of additional factors; myostatin is bound by and held on to the extracellular membrane by follistatin in a complex with heparin sulfate proteoglycans [10]. Other complexes, containing GASP or FLRG, which retain myostatin in an inactive form,

have been found in serum [11]. More recently, a novel isoform of promyostatin associated with LTBP3 has been identified in the extracellular matrix of skeletal muscle with proposed activation by a furin-type cleavage [12].

Points of therapeutic intervention

As previously outlined, multiple pathways serve to regulate myostatin levels or to tightly control its activity as a regulator of muscle homeostasis. Its key role in this process presents myostatin as an obvious candidate for therapeutic intervention in promoting muscle regeneration in multiple disease states in which muscle atrophy is a key symptom. An important aspect to consider when designing therapeutics is ease of access to the target molecule. Biological (protein-based) drugs can be engineered to block the activity of secreted or membrane-bound proteins. Consequently, the complex sequence of extracellular processing that regulates myostatin availability present multiple possibilities for blockade. Intervention at this point also allows fine modulation of the myostatin pathway so as not to overly deplete any one cell type. To date, six potential myostatin inhibitory therapeutics have been evaluated in animal models of disease, including muscular dystrophy (MD) and motor neuron disease. A summary of these studies, with the pertinent citations, is presented in Table 1 and discussed below in the order depicted in Figure 1B.

Neutralizing antibodies to myostatin

Following the reports of genetic mutants in myostatin, studies were conducted to confirm that its inhibition affects skeletal muscle in the absence of any genetic gain- or loss-of-function paradigms. Several neutralizing monoclonal antibodies to myostatin have been generated and characterized both *in vitro* and *in vivo*. These studies provided clear evidence that postnatal inhibition of myostatin might provide a therapeutic benefit in muscle-wasting diseases.

Muscular dystrophies. Myopathies, including MD, comprise a group of progressive muscle-wasting disorders that lead to atrophy and eventual disability. To combat this, neutralizing antibodies to myostatin have been evaluated in three mouse MD models to assess the efficacy of muscle building in these indications. While not every dystrophic pathology was ameliorated in these studies, the aggregate of data support that pharmacological inhibition of myostatin could be a valid approach to treating MD. In particular, a dramatic improvement in muscle mass, integrity and function was observed in the *mdx* mouse model of Duchenne MD (DMD) when treated with anti-myostatin antibodies [31]. In additional models of MD such as the δ-sarcoglycan (*sgcd-/-*) model of LGMD2F, anti-myostatin therapy showed benefit when administered to young animals but not to adults [13]. This suggested that the timing of myostatin blockade therapy might be an important consideration. Potentially differential benefits have also been achieved depending upon the disease type or severity. In the γ-sarcoglycan mutant (*sgcg-/-*) model of LGMD2C a discordance between tissue histology and muscle function was observed following anti-myostatin therapy [32]. Such phenotypic differences have also been noted in genetic studies using the *Mstn-/-* mice. When crossed into either the *mdx* [14] or *sgcd-/-* [13] there were significant improvements in the dystrophic state. In contrast, genetic experiments showed that crossing the dy^{w}/dy^{w} (laminin α2-deficient model of MDC1A) mice with the *Mstn* nulls maintained a severe disease state in spite of elevated muscle regeneration. Together, these results begin to present the complexity of the underlying disease states and provide guidance as to when optimal benefit of myostatin inhibition therapy can be achieved.

Motor neuron disease. Anti-myostatin therapy has also been investigated in rodent models of amyotrophic lateral sclerosis (ALS) [15]. ALS is a late onset, fatal neurodegenerative disorder characterized by motor neuron degeneration and muscle atrophy. Anti-myostatin treatment resulted in significant but temporary increases in muscle mass and grip strength through early disease stages. However, the therapy did not delay disease onset or extend survival. While improved neuronal trophic support from the hypertrophic skeletal muscle may not be sufficient to enhance survival, the improved histopathology of the diaphragm observed in treated animals indicated that anti-myostatin therapy may provide an important benefit to respiratory function. Such functional benefits would greatly improve quality of life for ALS patients should these observations translate into humans.

Inhibition with the myostatin propeptide

A proteolytic cleavage-resistant myostatin propeptide mutant has shown efficacy in building muscle in wild-type mice [16]. Consequently, its potential as a therapeutic has been studied in models of MD. In experiments using the wild-type propeptide to treat the *mdx* mouse, results were very similar to if not better than the anti-myostatin antibody therapy with

Table 1. Summary of *in vivo* studies using myostatin blockade strategies[a].

Myostatin regulatory molecule	Mechanism of action	Animal model	Indication	Result summary	Reference
(A) Neutralizing antibodies to myostatin	Binds myostatin and prevents receptor binding.	*mdx*	Duchenne muscular dystrophy (DMD)	Increased body weight, muscle mass (by hypertrophy), and absolute twitch and tetanic force. Improved diaphragm histology.	[31]
		Sgcd-/-	Limb girdle muscular dystrophy 2F (LGMD2F)	Increased muscle mass and reduced fibrosis when administered to young mice.	[13]
		Sgcg-/-	LGMD2C	Increased body weight, muscle mass (by hypertrophy), and absolute twitch and tetanic force. No improvement in histopathology.	[32]
		SOD1 ^{G93A} Transgenic	Amyotrophic lateral sclerosis (ALS)	Increased body weight, muscle mass and grip strength. Improved diaphragm histology. Therapy did not delay disease onset or extend survival.	[15]
(B) Myostatin propeptide	Propeptide binds myostatin preventing the release of mature myostatin.	*mdx*	DMD	Increased body weight, muscle mass (by hypertrophy), absolute twitch and tetanic force and specific tetanic force. Improved diaphragm histology.	[17]
		Capn3-/-	LGMD2A	Increased muscle mass (by hypertrophy) and absolute tetanic force following viral delivery. Specific force measurements trended positively. No changes noted in muscle histology.	[18]
		Sgca-/-	LGMD2D	Viral delivery of the propeptide did not result in any obvious phenotype.	[18]
(C) Follistatin	Binds to myostatin.	SOD1 ^{G93A} Transgenic	ALS	Viral delivery of follistatin resulted in increased muscle mass (by both hypertrophy and hyperplasia) as well as improved grip strength. No benefit to survival.	[23]
(D) HDAC inhibitors	Induces follistatin.	*mdx*	DMD	Increased muscle mass (by hypertrophy), specific force and exercise performance. Improved adductor histopathology.	[27]
		Sgca-/-	LGMD2D	Muscle hypertrophy in the adductor with improved histopathology.	[27]
(E) Myostatin peptide	Likely prevents myostatin binding to Acvr2b.	Aged mice	Sarcopenia	Promoted muscle regeneration following injury. Increased satellite cell activation. Enhanced muscle grip strength.	[33]
(F) Soluble Acvr2b	Soluble, decoy receptor for myostatin.	*mstn-/-*		Increased muscle mass (by hypertrophy) in the absence of myostatin.	[28]
		Caveolin 3 transgenic mice	LGMD1C	Increased muscle mass (by hypertrophy).	[22]

[a] Detailed experimental methods including modality, dose and therapeutic onset and duration can be found in the cited references.

the notable addition of an improvement in specific force not simply absolute force [17]. In addition, virus-mediated expression of the mutant propeptide has shown benefit in one of two limb girdle MD models tested [18]. The improved functional outcome over the neutralizing antibodies might be explained by the observation that the propeptide has dramatically enhanced myostatin binding properties as well as the ability to bind its closely related family member GDF11. Additional genetic studies further support these findings where transgenic expression of the myostatin propeptide has resulted in increased muscle mass [19–21]. Transgenic overexpression of the wild-type myostatin propeptide has also been shown to rescue muscle atrophy and enhance both muscle strength and recovery from impaired functional deficits in the caveolin 3 transgenic model of LGMD1C [22]. Interestingly, two of these studies report that myostatin propeptide treatment alters dietary fat utilization towards muscle and away from adipose tissue, suggestive of a potential role for myostatin blockade therapies in preventing obesity.

Follistatin

Follistatin is an extracellular antagonist of myostatin that can bind to myostatin with a higher affinity than its receptor Acrv2b, and is thus proposed to block myostatin signaling [10, 21]. Transgenic expression of

follistatin in mice results in dramatic increases in muscle mass, both hypertrophy and hyperplasia, even greater than that detected in *Mstn-/-* mice [21]. Although it was not formally shown that the expressed follistatin inhibited myostatin activity, it is likely that other follistatin-interacting proteins may have a role in regulating muscle mass, and indeed may be ligands for Acvr2b. In the $SOD1^{G93A}$ mouse model of ALS, viral delivery of follistatin to skeletal muscle resulted in maintained and enhanced muscle strength. However, as is the case following anti-myostatin treatment in this model, follistatin-treated mice did not show any survival benefit. In contrast, viral delivery of follistatin to the CNS both enhanced muscle function and extended life, reinforcing the notion that cross talk between the CNS and muscle is essential for the maintenance of both tissues [23]. In addition to follistatin, other extracellular myostatin modulators, including FLRG and GASP, are beginning to show potential as *in vivo* modulators of myostatin following transgenic or viral-mediated expression in mice [24, 25].

Histone deacetylase inhibitors

Deacetylase inhibitors have been shown to increase myoblast fusion resulting in increased fiber cross-sectional area [26]. Since muscle hypertrophy had been shown to be beneficial in the *mdx* mouse, these inhibitors were evaluated for disease amelioration in two MD mouse models. Mechanistically, it has been shown that deacetylase inhibitors induce *follistatin* gene expression and thereby inhibit myostatin activity. Importantly, benefits in specific force and endurance were achieved in the *mdx* mouse following histone deacetylase (HDAC) inhibitor therapy [27]. It remains unknown why specific force production is improved upon HDAC inhibitor (or myostatin propeptide) treatment but not, so far, following neutralizing antibody therapy or in the *mstn-/-* mice. One intriguing possibility is that other follistatin or myostatin propeptide interacting proteins may be involved.

Myostatin peptide

A truncated peptide spanning amino acids 266–350 of myostatin has been used as a myostatin signaling antagonist, presumably by blocking Acvr2b binding sites [33]. *In vivo*, this peptide antagonist was shown to promote muscle repair, activate satellite cells and improve muscle function as measured by grip strength in aged mice. Further studies with this and variants of this peptide in various muscle disease models will be interesting.

Soluble activin receptor 2B

Transgenic expression of a dominant negative mutant of the myostatin receptor, Acvr2b, has been shown to result in up to a 125 % increase in skeletal muscle mass [21]. Using a purified recombinant soluble form of the extracellular domain of the receptor fused to an immunoglobulin Fc domain, Acvr2b-Fc has been shown to also increase muscle mass in wild-type mice [28]. This inhibition is not solely due to signaling *via* Acvr2b or the closely related Acvr2 since Acvr2b-Fc treatment functioned as an anabolic muscle agent in both *acvr2-/-* and *acvr2b-/-* mice. Therefore, both receptors may be involved in regulating muscle mass. Strikingly, Acvr2b-Fc was also able to further increase the muscle mass of *mstn-/-* mice, suggesting that at least one other of its ligands also functions as a negative regulator of muscle. GDF11 and activin have already been put forward as putative candidates [28]. To date, Acvr2b-Fc appears the most efficacious myostatin inhibitor reported. In disease models, Acvr2b-Fc has only been evaluated in the LMGD1C mouse. Although no functional studies were performed, Acvr2b-Fc increased muscle mass and cross-sectional fiber area reversing the muscle atrophy in the model [22]. It remains to be determined whether the observed improvement in muscle integrity observed in any Acvr2b-Fc treated animals translates to improved force production.

Conclusions

All of these therapeutics target different portions of the myostatin extracellular pathway and affect myogenesis and function to different degrees. Muscle is a complex tissue and myostatin is a critical component of its homeostasis. The current literature suggests that the further down the extracellular myostatin processing and signaling cascade that the pathway is targeted, the more effective the anabolic treatment (Table 1). More detailed study is needed to support this observation. The varied response to myostatin blockade in the different MD models highlights that the underlying heterogeneity of the disease must be considered. For example, modality of myostatin blockade, time and duration of intervention, underlying genetic defect, and integrity of the sarcolemma must all be assessed. These studies have also furthered our understanding of adult muscle biology. In particular, the unexpected efficacy of Acvr2b-Fc in building hypertrophic muscle in the *acvr2-/-*, *acvr2b-/-* and *mstn-/-* mice indicated that additional factors exist that potentiate the formation of new muscle beyond myostatin blockade alone. This raises exciting possi-

bilities for myostatin inhibitors as therapeutics, either alone or in combination with drugs targeting additional aspects of the pathology, in treating degenerative muscle and neuromuscular diseases.

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