Targeting myostatin for therapies against muscle-wasting disorders
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In addition to gene correction therapy and cell transplantation techniques, multidisciplinary approaches to drug discovery and development offer promising therapeutic strategies for intractable genetic muscular disorders including muscular dystrophy. Inhibition of the production and activity of myostatin, a potent growth factor that determines skeletal muscle size, is a novel strategy for the treatment of muscle-wasting disorders such as muscular dystrophy, cachexia and sarcopenia. Myostatin blockers include myostatin-blocking antibodies, myostatin propeptide, follistatin and follistatin-related proteins, soluble myostatin receptors, small interfering RNA and small chemical inhibitors. This review describes the discovery and development of myostatin inhibitors.

Abbreviations
ActR activin receptor, **ALK** activin receptor-like kinase, **BMP** bone morphogenetic protein, **DMD** Duchenne muscular dystrophy, **FLRG** follistatin-related gene, **GDF** growth and differentiation factor, **LGMD** limb-girdle muscular dystrophy, **RNAi** RNA interference, **TGF-β** transforming growth factor-β

Introduction
The transforming growth factor-β (TGF-β) superfamily is the largest growth factor subfamily and includes TGF-βs, activin, myostatin and bone morphogenetic proteins (BMPs) [1]. Among these growth factors, myostatin plays an essential role in the negative regulation of muscle growth and determines the mass and size of skeletal muscle [1-3]. The inhibition of myostatin activity is a promising therapeutic strategy for restoring muscle mass and strength in muscle-wasting disorders such as muscular dystrophy, cachexia and sarcopenia [1-4]. As with other growth factors in the TGF-β superfamily, the actions of myostatin are tightly regulated by multiple molecular mechanisms [2]. The production of myostatin is controlled by the processing of precursor proteins. In addition, extracellular binding proteins limit the actions of active myostatin. Signal transduction of myostatin occurs via cell surface transmembrane serine/threonine kinase receptors and intracellular Smad proteins [5,6]. Thus, the activity of myostatin is also tightly controlled within the cell. Potential myostatin inhibitors such as myostatin-blocking antibodies, myostatin propeptide, follistatin domain-containing proteins, soluble myostatin receptors, antisense and small interfering RNA, and chemical TGF-β-inhibiting compounds are being developed. These inhibitors and their derivatives could provide new drugs for the treatment of muscle-wasting disorders. This review describes the development of a clinically useful strategy for targeting myostatin.

Structure and activation of myostatin
Myostatin, also known as growth and differentiation factor-8 (GDF-8), was identified through screening of a novel member of the TGF-β superfamily (Figure 1) [7••]. Myostatin is almost exclusively expressed in skeletal muscle, but it is also found, to a lesser extent, in adipose tissues. Myostatin gene-deleted mice were demonstrated to have hypermuscular phenotypes [7••]. Both an increased number of muscle fibers and an increased fiber size were responsible for the increased muscle mass in these myostatin-null mice. Intriguingly, inactivating mutations in the myostatin gene have been identified in double-muscle cattle breeds, sheep and dogs [8-11,12•,13]. Recently, increased skeletal muscle mass as a result of myostatin mutation has even been reported in humans [14••]. These findings indicated that myostatin works as a negative regulator of skeletal muscle growth and development. In adult mice, the inhibition of myostatin resulted in an increase in skeletal muscle by hypertrophy [15•,16]. Therefore, myostatin inhibition is a promising therapeutic approach toward restoring muscle mass and strength in muscle-wasting conditions.

The synthesis and processing of myostatin in the cell is prototypic of members of the TGF-β superfamily [2]. Myostatin is first synthesized as a precursor protein consisting of a signal peptide, an N-terminal propeptide domain and a C-terminal domain (Figure 1). Sequential proteolytic cleavages play a role in myostatin activation. The first cleavage removes the signal peptide, then in the second cleavage, a furin-like protease recognizes the RXRR motif and generates the N-terminal myostatin propeptide and the C-terminal myostatin fragment (Figure 1). It is currently believed that the myostatin precursor forms a homodimer through a disulfide bond before cleavage. The C-terminal dimeric 26-kDa protein acts as the biologically active myostatin, which is referred to simply as myostatin [2].

**Figure 1. Sequence alignment of myostatin from various organisms.**
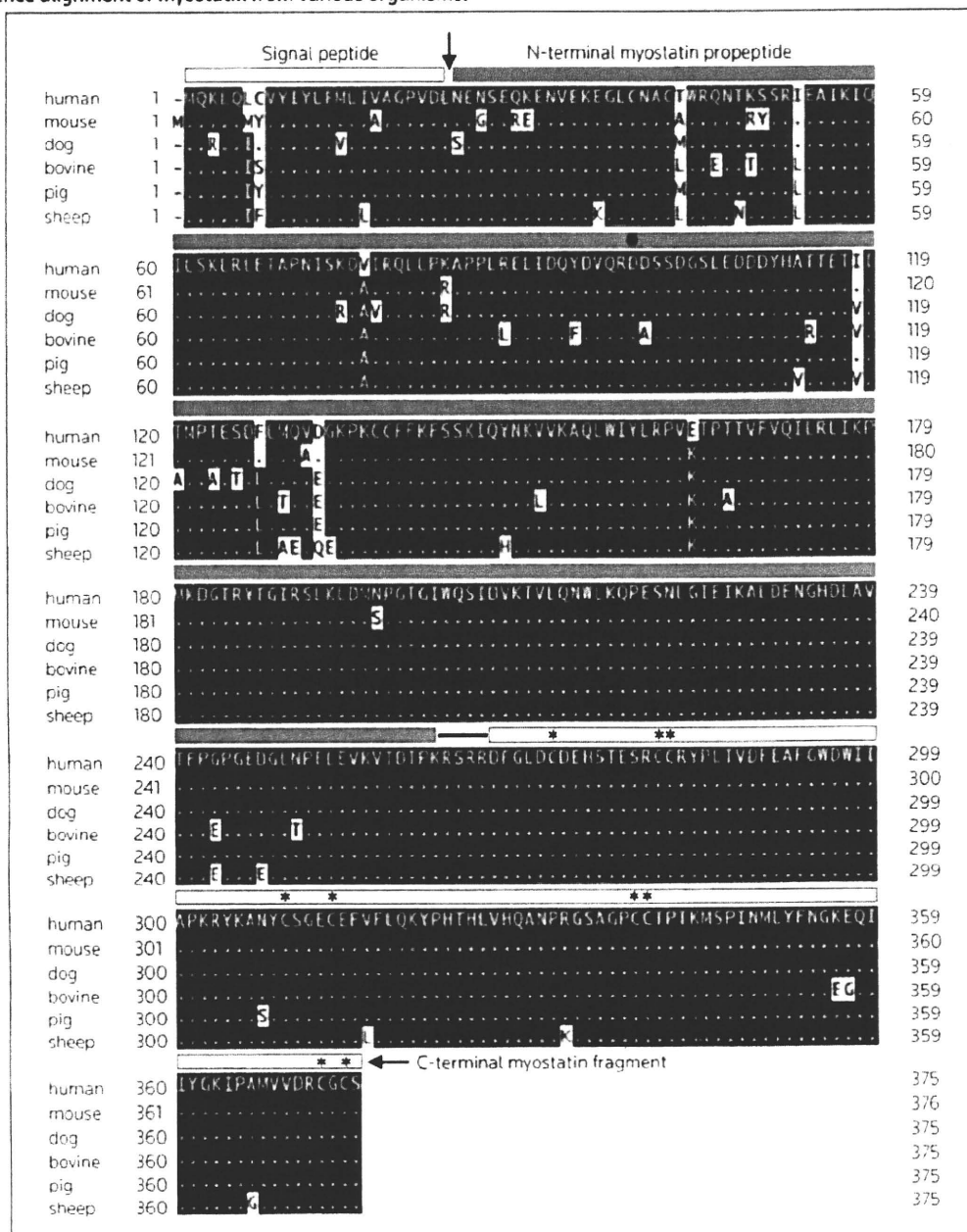
Shaded residues – amino acids matching with the human sequence; Arrow – cleavage site by signal peptidase to remove the signal peptide; Dot – cleavage site by metalloproteinase to activate the myostatin latent complex; Line – RXRR cleavage site by furin-like protease to generate the N-terminal myostatin propeptide and the C-terminal myostatin fragment; White box – signal peptide; Gray box – N-terminal myostatin propeptide; White box with asterisks – C-terminal myostatin fragment; Asterisks – conserved cysteine residues.

Myostatin circulates in serum in a latent form that is complexed with several myostatin-binding proteins (Figure 2) [17•]. Intriguingly, the N-terminal myostatin propeptide remains non-covalently bound to the active myostatin and is a major myostatin-binding protein in serum. The myostatin latent complex can be activated by additional cleavage of the myostatin propeptide by the bone morphogenetic protein-1/tolloid (BMP-1/TLD) family of metalloproteinases [18]. Cleavage occurs between residues Arg^{75} and Asp^{76} of the propeptide (numbered from the N-terminus after signal peptide cleavage) [18]. The mutated form of the propeptide in which Asp^{76} is converted to Ala^{76} (D76A) is resistant to proteolysis and performs as a better myostatin inhibitor than the native myostatin propeptide [19].

Figure 2. Myostatin inhibitors.
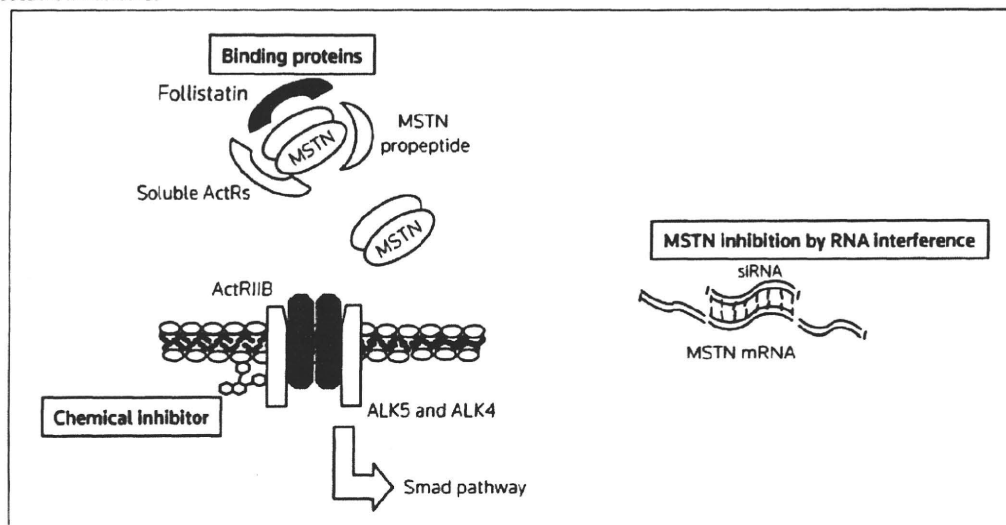
ActR activin receptor, **ALK** activin receptor-like kinase, **MSTN** myostatin, **siRNA** small interfering RNA.

In addition to myostatin propeptide, follistatin-related gene (FLRG) protein and GDF-associated serum protein-1, both of which are follistatin domain-containing proteins, associate with myostatin [17•,20]. Follistatin associates with myostatin both *in vitro* and *in vivo*. Interestingly, these three myostatin-binding proteins are efficient myostatin inhibitors and prevent myostatin from binding to its receptor. In addition, decorin, a small leucine-rich proteoglycan of the extracellular matrix, binds myostatin and regulates its activity in myogenic cells [21]. Recently, it was reported that, unlike in serum, myostatin is present extracellularly as uncleaved pro-myostatin in skeletal muscle, and that an extracellular pro-myostatin constitutes the major pool of latent myostatin in muscle [22]. Thus, the processing of myostatin is regulated in a tissue-specific manner.

Myostatin signaling pathways

Myostatin signals through two types of transmembrane serine/threonine kinase receptors, called type II activin receptors (ActRIIB and ActRIIA) and type I activin receptors (activin receptor-like kinases 5 and 4 [ALK5 and 4]; Figure 2) [5,23•]. The myostatin signaling pathway is similar to that of activin and TGF-β, and is mediated by Smad2 and Smad3 [1,2]. Smad proteins enter cell nuclei upon activation of myostatin and associate with a transcriptional coactivator for gene expression. Increased numbers of satellite cells, which are mononuclear stem cells found between the basal lamina and sarcolemma that are involved in muscle growth, are present in myostatin-deficient mice [24]. One report suggested that myostatin regulates the self-renewal and proliferation of satellite cells by controlling Pax7 expression via the Erk1/2 pathway [24]; however, another recent report demonstrated that myostatin acted *in vivo* to regulate the balance between proliferation and differentiation of embryonic muscle progenitors by promoting their terminal differentiation through the activation of p21 and MyoD [25].

Thus, the effect of myostatin on muscle progenitors is more complex than previously realized and is likely to be context-dependent. Further research is required to elucidate the precise functions of myostatin in muscle.

Myostatin inhibition and therapeutic strategy for muscle-wasting disorders

Myostatin inhibition is effective in increasing skeletal muscle mass and strength, both in the postnatal period and in adults [15•,16]. This suggests that targeting myostatin would be a suitable therapy for muscle-wasting diseases such as muscular dystrophy, cachexia and sarcopenia (Table 1). In particular, a therapy for muscular dystrophy by myostatin blockade will attract clinical attention, as no effective therapy for the disease is available yet. Myostatin inhibition has been reported to be effective in several forms of muscular dystrophies in mouse models, and myostatin antibodies have now been evaluated in human clinical trials [1-4] (see below).

Table 1. Applications of targeting myostatin.

Area of use	Applications
Medical	• Muscle-wasting diseases (eg, muscular dystrophy, cachexia, sarcopenia) • Increasing muscle strength • Diabetes/obesity
Agricultural	• Meat production (bovine, sheep, pig, chicken, fish) • Enhancing racing performance in animals

Cachexia is the severe wasting condition observed in patients with advanced stages of diseases such as cancer and infection. Loss of weight, muscle atrophy and fatigue are evident in cachexic patients. Cachexia is mainly thought to be caused by inadequate food intake, increased metabolic rate and tissue protein breakdown; however, the causes of

cachexia are not fully understood. Systemically administered myostatin induced cachexia with profound muscle and fat loss in mice [26•]. Antagonism of myostatin by follistatin or myostatin propeptide was effective in slowing such myostatin-induced weight loss [26•].

Sarcopenia is derived from the Greek word meaning poverty of flesh, and is the degenerative loss of skeletal muscle mass and strength with aging. The most atrophy is observed in the fast twitch type II myofibers. Multiple factors, including physical inactivity, motor-unit remodeling, decreased hormone levels and decreased protein synthesis, may contribute to sarcopenia. Elevated levels of circulating tumor necrosis factor-α (TNF-α) and adaptations in TNF-α signaling in aged skeletal muscle may be contributing factors in the activation of apoptosis. Short-term blockade of myostatin enhanced muscle regeneration in aged mice after injury and during sarcopenia [27•]. Myostatin antagonism led to satellite cell activation, and resulted in enhanced muscle regeneration in injured aged mice [27•].

Thus, targeting myostatin has significant therapeutic potential in muscle-wasting disorders. In agricultural applications, meat production is the focus of attention (Table 1). It is also of note that racing performance is enhanced in heterozygous myostatin-deficient whippet dogs [12•].

Development of myostatin inhibitors
Myostatin-blocking antibodies

Several myostatin-blocking antibodies have been developed by using phage display technology and protein/antibody engineering [28••,101,102]. Antibody-mediated myostatin blockade in *mdx* mice, a model of Duchenne muscular dystrophy (DMD), was found to ameliorate the pathophysiology and muscle weakness associated with the disease [28••]. This finding indicated that, although normal levels of dystrophin were not regained, myostatin inhibition offers a novel therapy for DMD. The biosafety and effectiveness of the humanized myostatin antibody stamulumab (MYO-029, Wyeth), have been evaluated in clinical studies in the US in patients with muscular dystrophy; however, in February 2008, Wyeth reported it had discontinued development of stamulumab after analysis of clinical data.

Myostatin propeptide

Myostatin propeptide associates with myostatin in serum and works as one of the myostatin inhibitors. Transgenic myostatin propeptide expression prevented muscular atrophy in P104L-mutant caveolin-3 mice [29•]. The myostatin propeptide stabilized by fusion with the immunoglobulin G – fragment crystallizable (IgG-Fc) region was effective in ameliorating the symptoms of *mdx* mice [30]. Myostatin propeptide D76A, in which the Asp[76] residue was converted to an Ala[76] residue, was resistant to proteolysis, worked as a better myostatin inhibitor than the native myostatin propeptide [19] and was effective in limb-girdle muscular dystrophy (LGMD) 2A model mice with calpain-3 gene mutations [31].

Soluble myostatin receptors

A soluble form of ActRIIB, also known as ACVR2B, had potent myostatin-inhibitory activity and caused dramatic increases in muscle mass [23•]. Only 2 weeks were required for the soluble form of ACVR2B to increase the muscle mass in mice by up to 60% [23•,103,104]. Because the soluble form of ACVR2B augmented muscle mass even in myostatin-knockout mice, it has been suggested that it also inhibits other ligands, including activins and GDF11, that regulate skeletal muscle growth in addition to inhibiting myostatin [32].

Follistatin and follistatin-related proteins

Follistatin was originally identified as a single-chain polypeptide with weak inhibitory activity toward follicle-stimulating hormone secretion by anterior pituitary cells and was later demonstrated to be an activin-binding protein [33]. Follistatin and FLRG were shown to bind to myostatin and inhibit its activity [34,105] and also induced dramatic increases in muscle mass when overexpressed as transgenes in mice [32]. Follistatin and FLRG are likely to inhibit other regulators of muscle mass with similar activity to myostatin, as overexpression of follistatin or FLRG still caused substantial muscle growth in mice lacking myostatin [32]. Because the inhibition of activin by follistatin was very efficient, follistatin may have effects on skeletal muscles by regulating both myostatin and activin. It was reported that single gene administration of myostatin inhibitors, including follistatin, was enough to enhance skeletal muscle mass for long periods [35].

Recently, the authors developed a myostatin inhibitor derived from follistatin, designated FS I-I, and characterized its effects on muscle mass and strength in *mdx* mice [36••]. Follistatin is composed of an N-terminal domain and three cysteine-rich follistatin domains (FS I, FS II and FS III) [33,37-39]. X-ray crystallographic analyses revealed that the minimal activin-inhibiting fragment of follistatin was comprised of the FS I and FS II domains, and that the individual FS domains may have different activities (Figure 3) [37-39]. A follistatin mutant containing two FS I domains was synthesized, and its binding activities toward myostatin and activin were characterized [36••]. Interestingly, FS I-I retained myostatin binding, but demonstrated significantly weaker activin-binding activity; the dissociation constants of follistatin for activin and myostatin were 1.72 and 12.3 nM, respectively, while, in contrast, the dissociation constants of FS I-I for activin and myostatin were 64.3 μM and 46.8 nM, respectively. Transgenic mice expressing FS I-I under the control of a skeletal muscle-specific promoter showed increased skeletal muscle mass and muscle strength, and hyperplasia and hypertrophy were both observed. FS I-I transgenic mice were crossed with *mdx* mice and characterized. The skeletal muscles in the *mdx*/FS I-I mice were enlarged and showed reduced cell infiltration [36••]. Muscle strength was also recovered in *mdx*/FS I-I mice. These results indicated that myostatin blockade by FS I-I has therapeutic potential for muscular dystrophy [36••]. As myostatin blockade by myostatin propeptide, follistatin and follistatin-derived peptide caused neither an

Figure 3. Crystallography of follistatin bound to activin.
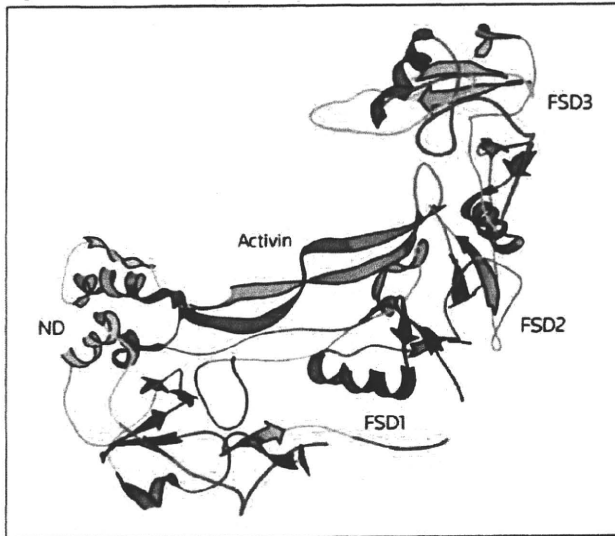
Because the X-ray crystal structure of myostatin is not currently available, structurally related activin is shown. The image is displayed using KiNG software. (*Protein Data Bank DOI: 10.2210.pdb2p6a/pdb*). **FSD** follistatin domain, **ND** N-terminal domain.

anti-idiotypic response, nor an antibody-dependent toxic response, they may be superior to myostatin antibody in terms of *in vivo* administration [30]. In addition, follistatin domain-containing proteins associated not only with mature myostatin, but also with myostatin propeptide [20]. Thus, unlike myostatin antibody, follistatin and related proteins may have regulatory functions other than inhibiting mature myostatin activity. It should also be noted that deacetylase inhibitors that are useful for functional and morphological recovery of dystrophic muscle increased the rate of myoblast fusion, leading to enlarged myotubes by the induction of follistatin in satellite cells [40].
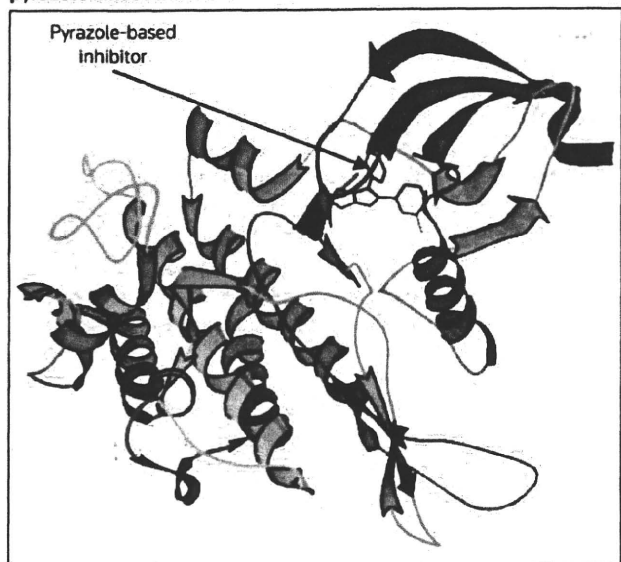
Myostatin inhibition by RNAi
RNA interference (RNAi) is a form of post-transcriptional gene silencing (Figure 2). Double-stranded RNA induces degradation of the homologous endogenous transcripts, which results in the reduction or loss of gene activity. A plasmid expressing a short hairpin interfering RNA (shRNA) was designed and electroporated in rat tibialis anterior muscle [41]. RNAi for myostatin was capable of reducing myostatin mRNA and protein, and increasing muscle weight and fiber size *in vivo* [41,42]. Satellite cell number was also increased by more than 2-fold [41]. The RNA oligonucleotide suppressed myostatin expression through upregulation of the MyoD pathway [43]. Importantly, RNA oligonucleotide-dependent myostatin suppression led to the increase in muscle growth both in dystrophic and cachectic mice, as well as in normal mice, indicating a therapeutic potential [42,43]. Thus, myostatin inhibition by RNAi provides an additional opportunity that needs to be investigated further.

Chemical TGF-β inhibitors
ATP-competitive inhibitors of the kinase domain of the TGF-β type I receptor (TβRI, also known as ALK5) have been developed [4,44]. These inhibitors were based on a pyrazole core, and included pyridinylimidazoles and their derivatives (Figure 4). SB-431542 (GlaxoSmithKline plc; Figure 5) was the first selective ALK5 inhibitor to be developed, and it was used as a pharmacological research tool to investigate the role of ALK5 in cellular mechanisms [4]. Treatment with SB-431542 increased lean tissue content and decreased fat content, possibly by inhibiting myostatin *in vivo* [106]. Various small-molecule kinase inhibitors that inhibit the structurally related ALK4, ALK5 and ALK7 at low concentrations have been reported in the patent literature [106,107]. Therefore, chemical TGF-β inhibitors are not specific to myostatin, and inhibit activins and TGF-βs that signal through ALK4, ALK5 and ALK7 [4].

Conclusions
Myostatin is an important regulator that controls skeletal muscle mass. Targeting myostatin has attracted clinical

Figure 4. Crystallography of TGF-β type I receptor kinase with a pyrazole kinase inhibitor.

The image is displayed using KiNG software. (*Protein Data Bank DOI: 10.2210.pdb1rw8/pdb*).
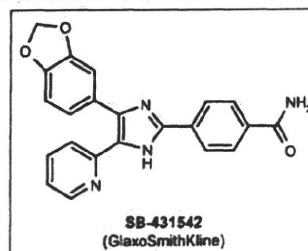**TGF-β** transforming growth factor-β.

Figure 5. The structure of SB-431542.

SB-431542
(GlaxoSmithKline)

Table 2. Muscular dystrophies and myostatin inhibition.

Disease	Gene locus	Gene products	Myostatin blockage in model animal	Reference
DMD	Xp21	Dystrophin	Effective	[28••,30,36••]
LGMD1C	3p25	Caveolin-3	Effective	[29•]
LGMD2A	15q15	Calpain-3	Effective by gene therapy	[31]
LGMD2F	5q33-34	δ-sarcoglycan	Early therapy is effective	[46]
LGMD2C	13q12	γ-sarcoglycan	Muscle function improved, histopathology not improved	[48]
MDC1A	6q22	Laminin-α2	Not effective	[47]

DMD Duchenne muscular dystrophy, **LGMD** limb-girdle muscular dystrophy, **MDC** muscular dystrophy congenital.

attention for therapy against muscle-wasting disorders such as muscular dystrophy, cachexia and sarcopenia. Myostatin inhibition also has potential in the production of livestock for meat production (Table 1). The processing and production of active myostatin is controlled by multiple processes. Mature myostatin forms a non-covalent, inactive complex with myostatin propeptide, which serves as an inhibitor of myostatin signaling. Physiologically, the BMP-1/TLD family of metalloproteinases cleave myostatin propeptide and this cleavage activates latent myostatin. Myostatin also associates with a number of binding proteins including follistatin and FLRG (Figure 2). Myostatin signals through a combination of activin type II receptors (ActRIIB and ActRIIA) and type I receptors (ALK5 and ALK4; Figure 2). When activated, these receptors phosphorylate Smad2/3, associate with the common Smad4, and then translocate into the nucleus to activate gene transcription [1-6]. In addition to the Smad pathway, multiple non-Smad pathways, including the Erk1/2 mitogen-activated protein kinase pathway and the phosphatidylinositol 3-kinase/Akt pathway, are regulated by myostatin in a context-dependent manner [45].

There are multiple strategies for inhibiting myostatin activity. Myostatin inhibitors, such as monoclonal myostatin antibodies, myostatin propeptide, follistatin, and soluble myostatin receptors, could be lead compounds in drug development for muscle-wasting disorders. Pyrazole-based selective inhibitors of TGF-β type I receptor kinase that are also rational myostatin inhibitors have been developed (Figures 2 and 4). RNAi-based transcriptional degradation of myostatin mRNA is a recently developed strategy for myostatin inhibition (Figure 2).

There are various types of muscular dystrophy, including DMD, LGMDs, and congenital muscular dystrophies (Table 2). Myostatin blockade could be effective in treating these various types of muscular dystrophy and is considered to be most effective when combined with gene correction. In addition to *mdx* mice, two models of LGMDs, caveolin-3 mutation and calpain-3 deficiency, showed pathophysiologies that were ameliorated by myostatin blockade (Table 2) [29•,31]. In the δ-sarcoglycan-deficient LGMD2F model, an age-dependent effect of myostatin inhibition was reported [46]. Myostatin inhibition was beneficial when delivered early, when the disease was relatively mild, whereas it was not effective in the advanced stages of the disease. It was also demonstrated that myostatin blockade was highly variable in its effects on individual muscles [46]. This may reflect the finding that the effect of myostatin was context-dependent in order to regulate the balance between proliferation and differentiation [25]. It should also be noted that myostatin elimination did not combat laminin-α2 deficiency in model mice, but rather increased their postnatal mortality as a result of fat loss [47]. In the case of γ-sarcoglycan-deficient LGMD2C dystrophic mice, myostatin inhibition led to increased fiber size, muscle mass and absolute force; however, no clear improvement in muscle histopathology was evident [48]. One report demonstrated that a lack of myostatin resulted in excessive muscle growth, but impaired force generation [49]. Although the targeting of myostatin had great merit in increasing muscle mass and force, these data disclosed the disease-specific limitations to therapeutic strategies of myostatin blockade in the more severe models of various muscular dystrophies [48].

In summary, recent studies into the development of myostatin inhibitors have been presented and their application and possible limitations as therapies for muscle-wasting disorders have been discussed.

Acknowledgments

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Myostatin inhibition by a follistatin-derived peptide ameliorates the pathophysiology of muscular dystrophy model mice

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Gene-targeted therapies, such as adeno-associated viral vector (AAV)-mediated gene therapy and cell-mediated therapy using myogenic stem cells, are hopeful molecular strategies for muscular dystrophy. In addition, drug therapies based on the pathophysiology of muscular dystrophy patients are desirable. Multidisciplinary approaches to drug design would offer promising therapeutic strategies. Myostatin, a member of the transforming growth factor-β superfamily, is predominantly produced by skeletal muscle and negatively regulates the growth and differentiation of cells of the skeletal muscle lineage. Myostatin inhibition would increase the skeletal muscle mass and prevent muscle degeneration, regardless of the type of muscular dystrophy. Myostatin inhibitors include myostatin antibodies, myostatin propeptide, follistatin and follistatin-related protein. Although follistatin possesses potent myostatin-inhibiting activity, it works as an efficient inhibitor of activins. Unlike myostatin, activins regulate the growth and differentiation of nearly all cell types, including cells of the gonads, pituitary gland and skeletal muscle. We have developed a myostatin-specific inhibitor derived from follistatin, designated FS I-I. Transgenic mice expressing this myostatin-inhibiting peptide under the control of a skeletal muscle-specific promoter showed increased skeletal muscle mass and strength. *mdx* mice were crossed with FS I-I transgenic mice and any improvement of the pathological signs was investigated. The resulting *mdx*/FS I-I mice exhibited increased skeletal muscle mass and reduced cell infiltration in muscles. Muscle strength was also recovered in *mdx*/FS I-I mice. Our data indicate that myostatin inhibition by this follistatin-derived peptide has therapeutic potential for muscular dystrophy.

Key words: Myostatin, follistatin, muscular dystrophy

Actions of Myostatin

Skeletal myogenesis is under tight regulation by growth factor signaling. Myostatin is an endogenous negative regulator of muscle growth and plays a major role in determining skeletal muscle mass. Myostatin, also known as growth and differentiation factor-8 (GDF8), belongs to the transforming growth factor (TGF)-β superfamily (1, 2). Similar to other TGF-β superfamily members, myostatin is synthesized as a precursor protein that is biologically inactive. Production of mature myostatin occurs through dimerization of the precursor and subsequent proteolytic processing. Cleavage by furin-like protease is responsible of separating the N-terminal propeptide from the C-terminal mature myostatin, while cleavage of the latent propeptide by the bone morphogenetic protein-1/tolloid (BMP1/TLD) family of metalloproteinases is responsible for activation of latent myostatin (3). The C-terminal dimeric 26-kDa protein acts as mature myostatin. Mice with targeted deletion of the myostatin gene show dramatic and widespread increases in skeletal muscle mass (2). Both muscle fiber hypertrophy and muscle cell hyperplasia are observed.

Myostatin signals through two types of transmembrane serine/threonine kinase receptors, namely activin type II receptors (ACVR2B and ACVR2A) and activin receptor-like kinases 4 and 5 (ALK4 and 5). Its intracellular signaling pathway is similar to those of activin and TGF-β, and mediated by the Smad proteins Smad2 and Smad3 (1, 2, 4). Myostatin negatively regulates G1-to-S progression in the cell cycle and maintains the quiescent status of satellite cells (5). As a result, increased numbers of satellite cells are present in myostatin-deficient mice (5). Involvement of the MAP kinase pathway as well as the Smad pathway is a characteristic of the myostatin-regulated skeletal muscle differentiation program (6). However, the precise mechanism of action and the skeletal-muscle specific signaling of myostatin have not yet been fully elucidated.

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Myostatin Inhibition as a Therapeutic Strategy for Muscular Dystrophy

Interestingly, inhibition of myostatin activity is capable of increasing muscle mass and strength in the postnatal period and even in adults. These observations suggest that targeting of myostatin would be a suitable therapy for degenerative muscle diseases, such as muscular dystrophy and cachexia, and may be able to prevent muscle wasting due to aging (1, 2, 7). In fact, antibody-mediated myostatin blockade in *mdx* mice, a model for Duchenne muscular dystrophy, was found to ameliorate the pathophysiology and muscle weakness (8). Myostatin propeptide-mediated amelioration of the symptoms in *mdx* mice, limb-girdle muscular dystrophy (LGMD) 1C model mice with caveolin-3 gene mutations and LGMD2A model mice with calpain 3 gene mutations has also been reported (9-11). However, elimination of myostatin did not recover the pathology in laminin-α2-deficient model mice and rather increased their mortality (12). Thus, the effectiveness of myostatin inhibition depends on the disease state (Table 1). In addition to myostatin propeptide and myostatin antibodies, follistatin and follistatin domain-containing proteins can bind to myostatin *in vivo* and act as effective myostatin inhibitors (1, 13, 14). Small chemical compounds that block the kinase activity of myostatin type I receptor would also serve as myostatin inhibitors (13).

Development of Myostatin Inhibitors for Therapies against Muscular Dystrophy

Phage display technology and antibody engineering have been used to develop myostatin-blocking antibodies. The biosafety and effectiveness of humanized myostatin antibodies, designated MYO-029, are being evaluated in phase I/II studies in the United States in 108 patients suffering from muscular dystrophy (3).

Multiple myostatin-binding proteins, such as myostatin propeptide, follistatin and follistatin-related protein, have been characterized. After cleavage of myostatin precursors, myostatin propeptide associates with mature myostatin in sera (14). Proteolytic cleavage of the propeptide at aspartate-76 by the BMP-1/TLD family of metalloproteinases is an important step for activa-

Table 1. Muscular dystrophies and myostatin inhibition.

Disease	Mode of inheritance	Gene locus	Gene products	Myostatin blockage	Ref [Method of myostatin inhibition]
Duchenne	XR	Xp21	Dystrophin	Effective in *mdx* mouse	Bogdanovich et al., (8) [1] Wagner et al., (21) [2] Bogdanovich et al., (9) [3] Nakatani et al., (17) [4]
LGMD1C (CAV3)	AD	3p25	Caveolin-3	Effective in model mouse	Ohsawa et al., (10) [5]
LGMD2A (CAPN3)	AR	15q15	Calpain-3	Gene therapy is effective	Bartoli et al., (11) [6]
LGMD2D (SGCA)	AR	17q12-21	α-sarcoglycan	Gene therapy is not effective	Bartoli et al., (11) [6]
LGMD2F (SGCD)	AR	5q33-34	δ-sarcoglycan	Early therapy is effective Treat early	Parsons et al., (22) [1, 2]
MDC1A (LAMA2)	AR	6q22	Laminin α-2	Not effective in *dy* mouse Severe fat loss	Li et al., (12) [2]

The effects of myostatin blockade on various types of muscular dystrophy are summarized. Myostatin inhibition is applicable as a therapy for multiple types of muscular dystrophy. Transgenic approaches, systemic injection and gene therapy have been tried. Myostatin blockade by myostatin antibodies, modified myostatin propeptide or follistatin-derived peptides is effective for ameliorating the pathophysiology in *mdx* mice. Myostatin inhibition is also effective for ameliorating several types of limb-girdle-type muscular dystrophy caused by mutations of caveolin-3 or calpain-3. Effective therapy would be possible by early treatment. It is noteworthy that elimination of myostatin does not improve the phenotypes of laminin-α2-deficient model mice. Method of myostatin inhibition is shown as brackets. [1], myostatin antibody treatment; [2], crossing with myostatin K/O mice; [3], myostatin propeptide treatment; [4], crossing with mutated follistatin Tg mice; [5], crossing with myostatin propeptide Tg mice; [6], AAV-mediated mutated myostatin propeptide expression. References are shown with parentheses.

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tion of the mature disulfide-bonded C-terminal myostatin dimer (2, 3). Mutation of the myostatin propeptide at the BMP-1/TLD cleavage site by replacing aspartate-76 with alanine (D76A) produces a better myostatin inhibitor than the wild-type propeptide *in vitro* and *in vivo* (9, 11).

Although the activin type IIB receptor, ACVR2B, is characterized as a receptor for activins and nodal, it is the primary ligand-binding myostatin receptor that transmits myostatin signaling. A soluble form of ACVR2B has potent myostatin-inhibitory activity and causes dramatic increases in muscle mass (15). Only 2 weeks are required for the soluble form of ACVR2B to increase the muscle mass in mice by up to 60% (15). Since the soluble form of ACVR2B even augments muscle mass in myostatin-knockout mice, it has been suggested that it also inhibits other ligands including activins and GDF11 that regulate skeletal muscle growth in addition to myostatin (15).

Myostatin Inhibitor Derived from Follistatin

Follistatin was originally identified as a single-chain polypeptide with a weak inhibitory activity toward follicle-stimulating hormone secretion by anterior pituitary cells. Later, follistatin was found to be an activin-binding protein (1). Gene knockout analyses revealed that follistatin gene ablation causes multiple effects, including skeletal and skin abnormalities, suggesting that follistatin may have additional functions other than activin inhibition (1). Follistatin and follistatin-related gene, FLRG, were shown to bind to myostatin and inhibit its activity (1, 2, 15, 16). Similar to myostatin, activins belong to the TGF-β superfamily and have pleiotrophic effects on numerous tissues. Since activins have a variety of functions in tissues other than skeletal muscles and their inhibition by follistatin is very efficient, follistatin has multiple effects on not only skeletal muscles but also other tissues. In fact, transgenic expression of the follistatin gene has profound effects on reproductive performance and fertility (1).

Recently, we developed a myostatin inhibitor derived from follistatin, designated FS I-I, and characterized its effects on muscle mass and strength in *mdx* mice (17). Since myostatin blockade is one of the most promising therapies for muscular dystrophy, the results of our study should provide an additional rational therapeutic strategy for intractable muscular diseases, including muscular dystrophy (17).

Follistatin is composed of an N-terminal domain and three cysteine-rich follistatin domains (FS I, FS II and FS III) (1). Recent crystallographic analyses have revealed that the minimal activin-inhibiting fragment of follistatin is comprised of the FS I and FS II domains, and that the individual FS domains may have different activities (18, 19). We created a follistatin mutant containing two FS I domains, and characterized its binding activities toward myostatin and activin A. Interestingly, FS I-I retained its myostatin binding, but showed significantly weaker activin-binding activity. The dissociation constants of follistatin for activin and myostatin are 1.72 and 12.3 pM, respectively. In contrast, the dissociation constants of FS I-I for activin and myostatin are 64.3 nM and 46.8 pM, respectively. FS I-I was capable of inhibiting the actions of myostatin in multiple assays, but hardly affected the activin activity (17). Transgenic mice expressing FS I-I under the control of a skeletal muscle-specific promoter showed increased skeletal muscle mass, especially in the pectoralis major, triceps brachii, gluteus and quadriceps femoris muscles. Muscle strength was also increased. Hyperplasia and hypertrophy were both observed. FS I-I transgenic mice did not show any behavioral abnormalities and reproduced normally. We crossed FS I-I transgenic mice with *mdx* mice, a model for Duchenne muscular dystrophy. Notably, the skeletal muscles in the resulting *mdx*/FS I-I mice were enlarged and showed reduced cell infiltration (17). The numbers of infiltrated macrophages in skeletal muscles were dramatically decreased in *mdx*/FS I-I mice compared with *mdx* mice (17). Muscle strength was also recovered in *mdx*/FS I-I mice. These results indicate that myostatin blockade by FS I-I has therapeutic potential for muscular dystrophy and should provide a rational therapeutic strategy for intractable muscular diseases. The possibility that injections of this myostatin inhibitor derived from follistatin may affect the pathophysiology of muscular dystrophy model mice or human patients remains to be determined.

Conclusions

The ability to control the actions of myostatin has great potential for a number of research fields and offers medical applications. Myostatin activity determines the skeletal muscle mass. Myostatin blockade is effective for increasing muscle mass, even in adults (1, 2). Thus, myostatin is considered to be one of the rational drug targets for muscle-wasting diseases, such as muscular dystrophy. There are multiple strategies for inhibiting myostatin activity. Myostatin inhibitors, such as monoclonal myostatin antibodies, myostatin propeptide and follistatin, could be promising lead compounds in drug development for muscular dystrophy and related disorders (1, 2, 17).

There are various types of muscular dystrophy, including Duchenne/Becker muscular dystrophies, congenital muscular dystrophies and limb-girdle muscular dystrophies (20). Myostatin blockade could increase the skeletal muscle mass, regardless of the type of muscu-

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lar dystrophy. Antibody-mediated or myostatin propeptide-mediated myostatin blockade in *mdx* mice, a model for Duchenne type muscular dystrophy, ameliorates the pathophysiology and increases muscle strength (8, 9, 18) (Table 1). Crossing of myostatin knockout mice with *mdx* mice also attenuates severity of muscular dystrophy (21). The pathophysiologies of three models of limb-girdle muscular dystrophy, including δ-sarcoglycan-deficiency, caveolin-3 mutations and calpain-3-deficiency, are also ameliorated by myostatin blockade (10, 11, 22). However, myostatin elimination did not combat laminin-α2-deficiency in mice, but rather increased their postnatal mortality due to fat loss (12). Similarly, myostatin inhibition was not effective for prolonging the survival of LGMD2D model mice with mutations of α-sarcoglycan (11). However, since the expression by AAV-myostatin propeptide used in the study was extremely low, it is still possible that different mode of action, such as the use of neutralizing myostatin antibody could be beneficial for α-sarcoglycan deficiency (11).

Myostatin inhibition would increase the relative ratio of fast myofibers to slow myofibers. Exercise in myostatin-deficient cattle led to early exhaustion, which may have been caused by a decrease in the number of mitochondria (23). However, a decreased number of mitochondria associated with myostatin absence was specific for myostatin-knockout mice and not observed in myostatin-inhibitor-expressing transgenic mice (our un-

published observations). Thus, regulation of the number of mitochondria seems to depend on the way in which myostatin is inhibited. This observation suggests that myostatin inhibition by our follistatin-derived peptide would not decrease the number of mitochondria, although this aspect needs to be clarified in future studies.

Follistatin and FLRG are efficient myostatin blockers, and inhibit not only myostatin but also activins. We have developed a myostatin inhibitor derived from follistatin, designated FS I-I, that does not affect activin activity (17). FS I-I is capable of ameliorating the pathophysiology of *mdx* mice. It must be determined whether FS I-I affects other TGF-β-like ligands that regulate muscle fiber growth. Since transgenic expression of FS I-I is effective for treatment of *mdx* mice, FS I-I and related follistatin-derived myostatin inhibitors would join the list of potential therapeutic myostatin inhibitors.

Myostatin inhibitor peptides could be directly infused into muscular dystrophy patients. In addition, a delivery system using myogenic cells is also possible. Furthermore, myostatin inhibition could be combined with other therapeutic approaches. Myostatin inhibition is considered to be most effective when combined with gene correction or other ways of delivering dystrophin (24). In this sense, one advantage of myostatin inhibitor peptides is their application to combined therapy for muscular dystrophy. If cDNAs for myostatin inhibitor peptides can be expressed in myogenic stem cells, cell-mediated
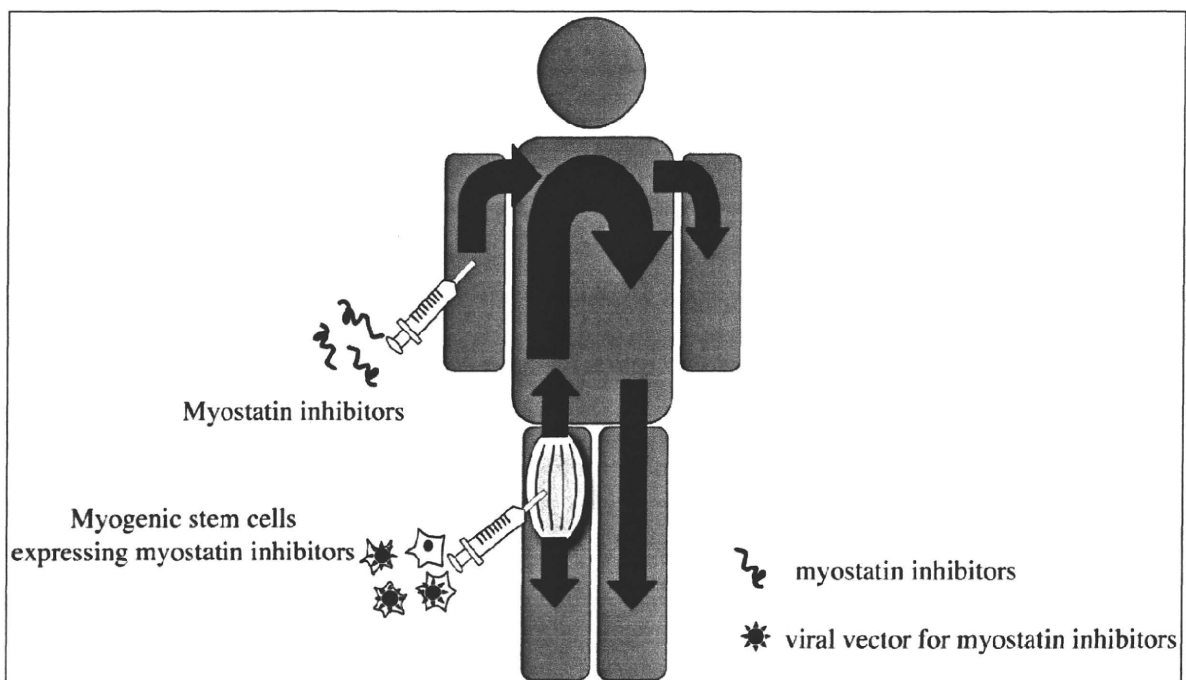

Figure 1. Potential delivery systems for myostatin inhibitors *in vivo.*

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therapy with myostatin inhibition would become possible (Fig. 1). By using this method, defective genes such as dystrophin would be amended by myogenic stem cells. Alternatively, viral vectors containing myostatin inhibitor peptides could be combined with other possible therapies for muscular dystrophy, such as exon-skipping reagents or genes (24).

Studying the role of myostatin in tissues other than skeletal muscle is important to avoid the possible adverse effects of myostatin inhibition. In this respect, it is important to determine whether or not myostatin acts solely on skeletal muscles. Adipose tissues are affected by myostatin signaling. Reduction of adipose tissue mass is observed in myostatin-null mice. Whether myostatin directly acts on adipocytes or factors from hypertrophied skeletal muscle secrete factors affecting adipocyte remains to be determined.

Finally, ethical issues must be considered for use of myostatin inhibition. Athletes are already interested in myostatin for increasement of their muscle strength. There is a discussion that myostatin inhibition would be non-steroidal doping methods that are difficult to identify.

In summary, I have presented an outline of myostatin inhibition therapy for muscular dystrophy with emphasis on a myostatin inhibitor derived from follistatin. I hope that this novel therapeutic strategy will prove useful toward establishing realistic therapies for intractable diseases, such as muscular dystrophy.

Acknowledgements

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SHORT COMMUNICATION

Atelocollagen-mediated local and systemic applications of myostatin-targeting siRNA increase skeletal muscle mass

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RNA interference (RNAi) offers a novel therapeutic strategy based on the highly specific and efficient silencing of a target gene. Since it relies on small interfering RNAs (siRNAs), a major issue is the delivery of therapeutically active siRNAs into the target tissue/target cells in vivo. For safety reasons, strategies based on vector delivery may be of only limited clinical use. The more desirable approach is to directly apply active siRNAs in vivo. Here, we report the effectiveness of in vivo siRNA delivery into skeletal muscles of normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen (ATCOL). ATCOL-mediated local application of siRNA targeting myostatin, a negative regulator of skeletal muscle growth, in mouse skeletal muscles or intravenously, caused a marked increase in the muscle mass within a few weeks after application. These results imply that ATCOL-mediated application of siRNAs is a powerful tool for future therapeutic use for diseases including muscular atrophy.
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RNA interference (RNAi) is the process of sequence-specific, posttranscriptional gene silencing in plants and animals from flatworms to human,[1] which is mediated by ~22-nucleotide small interfering RNAs (siRNAs) generated from longer double-stranded RNA. Since it was demonstrated that siRNAs can intervene gene silencing in mammalian cells without induction of interferon synthesis or nonspecific gene suppression,[2] an increasing number of remedies utilizing highly specific siRNAs targeted against disease-causing or disease-promoting genes have been developed.[3] Effective delivery of active siRNAs to target organs or tissues is therefore the key to the development of RNAi as a broad therapeutic platform. For this purpose, different strategies have been used to deliver and achieve RNAi-mediated gene silencing in vivo;[3] for example, polymers represent a class of materials that meet the needs of a particular siRNA delivery system, condensing siRNAs

into nano-sized particles taken up by cells.[4] However, some of the synthetic polymers, which have been used for delivery of nucleic acids, may trigger cell death in a variety of cell lines and thus suffer from limitations for its application in siRNA delivery in vivo.[4] On the other hand, atelocollagen (ATCOL), a pepsin-treated type I collagen lacking in telopeptides in N and C terminals that confer its antigenicity, has been shown to elicit an efficient delivery of chemically unmodified siRNAs to metastatic tumors in vivo.[5–7] In this study, we sought to examine the effectiveness of siRNA-ATCOL therapy for a nontumorous systemic disease, targeted against myostatin (growth/differentiation factor 8, GDF8), a negative regulator of skeletal muscle growth.[8]

Skeletal muscles are the crucial morphofunctional organs, and their atrophy causes severe conditions for life such as muscular dystrophies. Duchenne muscular dystrophy (DMD), for instance, is a severe muscle wasting disorder affecting 1 out of 3500 male birth.[9] There is currently no effective treatment, but gene therapy approaches are offering viable avenues for treatment development.[10] As one of therapeutic approaches, inhibition of myostatin by using anti-myostatin-blocking antibodies has been employed to increase muscle mass.[11] However, generating antibodies against recombinant target proteins is time consuming and requires a lot of efforts. Recently, we demonstrated that inhibition of myostatin by overexpression of the myostatin prodomain[12] prevented muscular atrophy and
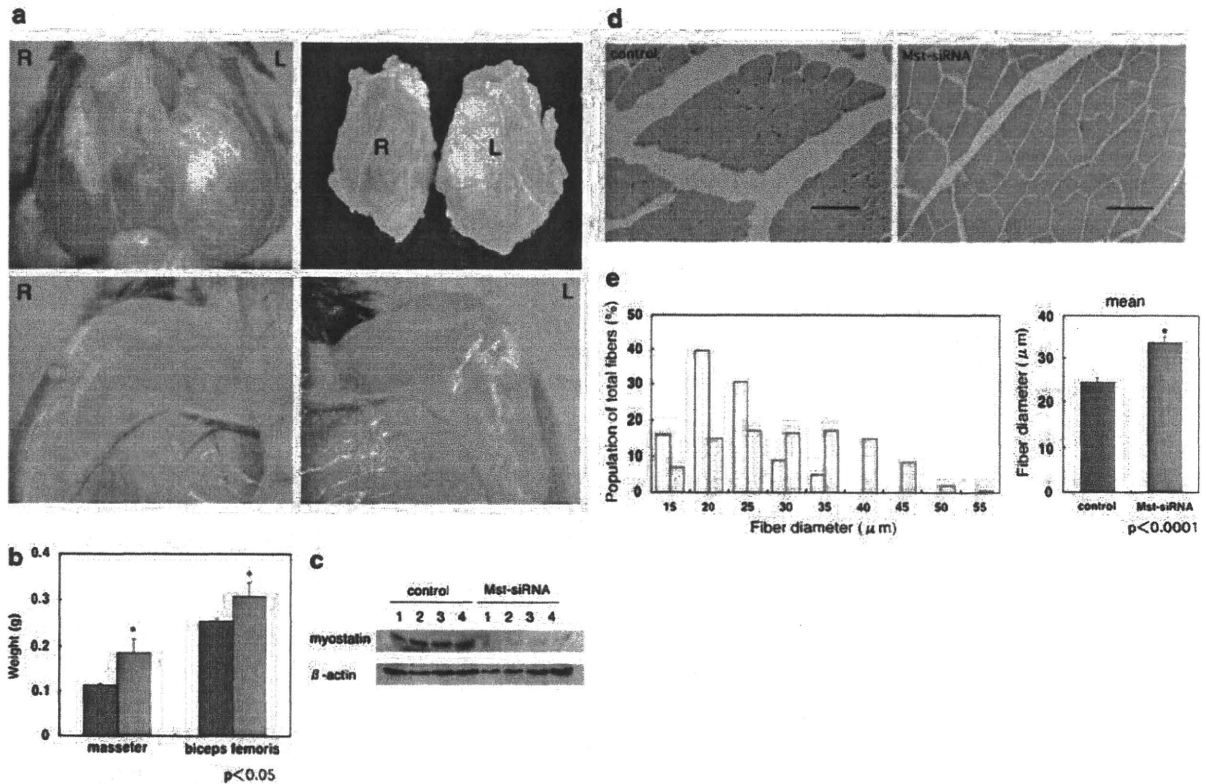
Figure 1 Local administration of the Mst-siRNA/atelocollagen (ATCOL) complex increases skeletal muscle mass and fiber size in wild-type mice through inhibition of myostatin expression. For the experiments depicted in (a–e) Mst-siRNAs (final concentration, 10 μM) were mixed with ATCOL (final concentration for local administration, 0.5%.[5]) (AteloGene, Kohken, Tokyo, Japan) according to the manufacturer's instructions. After anesthesia of mice (20-week-old male C57BL/6) by Nembutal (25 mg/kg, i.p.), the Mst-siRNA/ATCOL complex was injected into the masseter and biceps femoris muscles on the left side. As a control, scrambled siRNA/ATCOL complex was injected into the contralateral (right) muscles. After 2 weeks, the muscles on both sides were harvested and processed for analysis. (a) Photographs of muscles. Increased muscle mass were observed in the Mst-siRNA/ATCOL-treated (L) masseter (upper panels) and biceps femoris (lower panels), but not in the contralateral muscles (R). (b) Muscle weight. Mst-siRNA/ATCOL-treated muscles had an increased weight significantly compared to those with control siRNA/ATCOL (masseter, 0.185 ± 0.041 versus 0.115 ± 0.019 g; biceps, 0.307 ± 0.040 versus 0.232 ± 0.039 g; $n = 4$; $P < 0.05$). Student's t-test was used for determining statistical significance. Graphical representation of data uses the following convention: mean ± s.d.; treated muscles or mice in red; control muscles or mice in blue. (c) Western blot analysis of myostatin (52 kDa) in the control and Mst-siRNA/ATCOL-treated masseter muscles, assessed at 2 weeks after single injection. Total 80 μg of masseter muscle homogenates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes for immunoblotting. After a blocking reaction (5% nonfat milk/1% bovine serum albumin in phosphate-buffered saline (PBS) and 0.05% Triton X-100), the blots were incubated for 1 h at room temperature with mouse monoclonal anti-myostatin antibody (1:500; R&D Systems, Minneapolis, MN, USA) or anti-β-actin. After incubation with a secondary antibody (1:10000; horseradish peroxidase-conjugated anti-rat antibody; Biosource International, Camarillo, CA, USA), the blots were developed using the ECL Plus kit (Amersham, Buckinghamshire, UK). We used a purified myostatin protein and proteins extracted from cells transformed with a myostatin cDNA to confirm that the bands are due to 52 kDa myostatin. (d) Hematoxylin and eosin staining of the control and Mst-siRNA/ATCOL-treated masseter muscle. Muscles were fixed in 4% paraformaldehyde/PBS at 4 °C overnight, dehydrated and paraffin-embedded. Serial sections (5 μm thickness) were cut at mid-belly of muscle and stained. Scale bar, 50 μm. (e) Distribution of myofibril sizes of the control (blue bars) and Mst-siRNA/ATCOL-treated (red bars) muscles. The right panel shows the average myofibril size (33.6 ± 1.5 versus 24.4 ± 1.1 μm; $n = 200$; $P < 0.0001$). NIH Image (NIH, USA) software was used for morphometric measurements.

normalized intracellular myostatin signaling in the model mice for limb-girdle muscular dystrophy 1C.[13] On the other hand, Magee et al.[14] demonstrated that downregulation of myostatin expression by transduction of a plasmid expressing a short-hairpin interfering RNA (shRNA) against myostatin using electroporation can increase local skeletal muscle mass. For safety reasons, however, strategies based on vector delivery may be of only limited clinical use. The more desirable approach is to directly apply active siRNAs in vivo. As one of the practical platforms for siRNA delivery, we sought to employ an ATCOL-mediated oligonucleotide delivery system to apply myostatin-targeting siRNA into muscles.

We utilized the siRNA sequences reported previously[14] (GDF8 siRNA26, 5'-AAGATGACGATTAT CACGCTA-3', position 426–446). It has been noted that this sequence can target myostatin mRNA not only of mouse but also human, rat, rabbit, cow, macaque and baboon, based on Blast search (National Center for Biotechnology Information).[14] To confirm the silencing effect of this siRNA, we constructed a plasmid of pSilencer 2.1-U6 neo containing the target sequence and transfected the plasmid into a mouse myoblast cell line, C2C12 cells, which had been made forced to stably express myostatin. We confirmed that the RNAi construct could effectively downregulate the expression
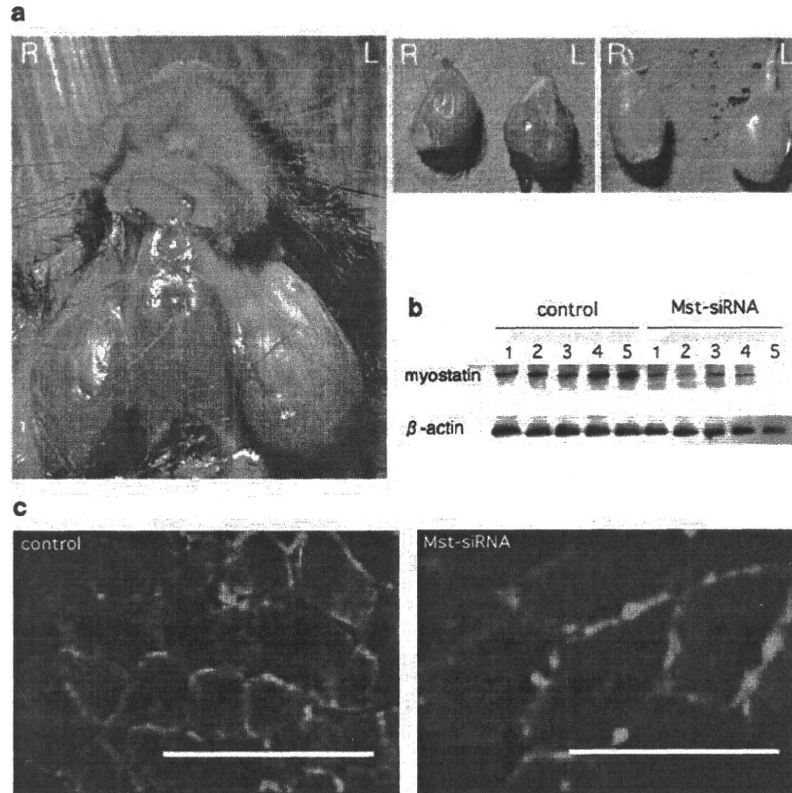
Figure 2 Mst-siRNA/atelocollagen (ATCOL) treatment improves myofibril size in *mdx* mice. (**a**) Photographs of muscles. The leftward masseter (left and middle panels) and tibial (right panel) muscles injected with the Mst-siRNA/ATCOL complex intramuscularly show a marked increased muscle mass in 20-week-old *mdx* male mice. (**b**) Western blot analysis of the control and Mst-siRNA/ATCOL-treated masseter muscles, assessed at 2 weeks after single injection. Myostatin protein levels in the muscles injected with the Mst-siRNA/ATCOL complex are markedly decreased, but not in the contralateral muscles injected with the control-siRNA/ATCOL. (**c**) Immunohistochemical analysis of the cross-sectional myofiber area of the masseter muscle, with the anti-laminin α2 antibody (4H8-2, Sigma, St Louis, MO, USA), showing increased fiber size in the Mst-siRNA/ATCOL-treated (right panel) muscle, compared to that of control (left panel). Alexafluor 594-conjugated anti-rat immunoglobulin G antibodies (A-11007, Invitrogen, Carlsbad, CA, USA) were used for immunohistochemistry. Scale bar, 100 μm.

of myostatin in the C2C12 cells[15] (Supplementary Figure S1).

We prepared the nanoparticle complex containing the GDF8 siRNA26 (10 μM) and ATCOL. Then, we injected the GDF8 siRNA26-ATCOL (Mst-siRNA/ATCOL) complex into the masseter and biceps femoris muscles of 20-week-old C57BL/6 mice. As a control, we injected control-scrambled siRNAs/ATCOL complex in the contralateral muscles. We observed gross morphology of the muscles and dissected the muscle tissues 2 weeks after injection. After injection of the Mst-siRNA/ATCOL complex, both muscles (on the left side) were enlarged, while no significant change was observed on the contralateral side (Figure 1a). We also measured the muscle weight, finding that the Mst-siRNA/ATCOL-treated muscles weighed significantly more than those on the control side (Figure 1b). The Mst-siRNA/ATCOL-treated muscles were further examined by a western blot analysis for myostatin (52 kDa), showing the decreased expression of myostatin on the treated side (Figure 1c). We quantified each result as a ratio to the internal control and statistically analyzed a difference between control (average ratio 0.90 ± 0.07) and treated (average ratio 0.44 ± 0.22) muscles. This difference is significant ($P < 0.01$, Student's *t*-test, $n = 4$). Histological analysis

showed that the myofibril sizes of the masseter muscles treated with the Mst-siRNA/ATCOL complex were larger than those of the control (Figure 1d). Examining the sizes of 200 myofibers per group, the population of myofibril sizes indicated a shift from smaller to larger fibers in the Mst-siRNA/ATCOL-treated muscle (Figure 1e). The average myofibril size of the muscle treated with Mst-siRNA/ATCOL gained approximately 1.3 times more than that of control (Figure 1e). No obvious morphological change was observed in other tissues than the treated masseter muscles. In the meanwhile, we did not observe any general sign of ill health and deaths during the period of experiment. These results indicate that the increase of the Mst-siRNA/ATCOL-treated muscle mass is caused by their hypertrophy and that the siRNA complex gives no obvious adverse effects.

We next questioned whether this effect of hypertrophy after local injection of the Mst-siRNA/ATCOL complex observed in normal mice was relevant to dystrophin-deficient *mdx* mouse, an animal model for DMD.[16] We intramuscularly injected the same Mst-siRNA/ATCOL complex into the masseter and tibial muscles on the left side of 20-week-old *mdx* male mice. Within 2 weeks after the single injection, a dramatically increased muscle
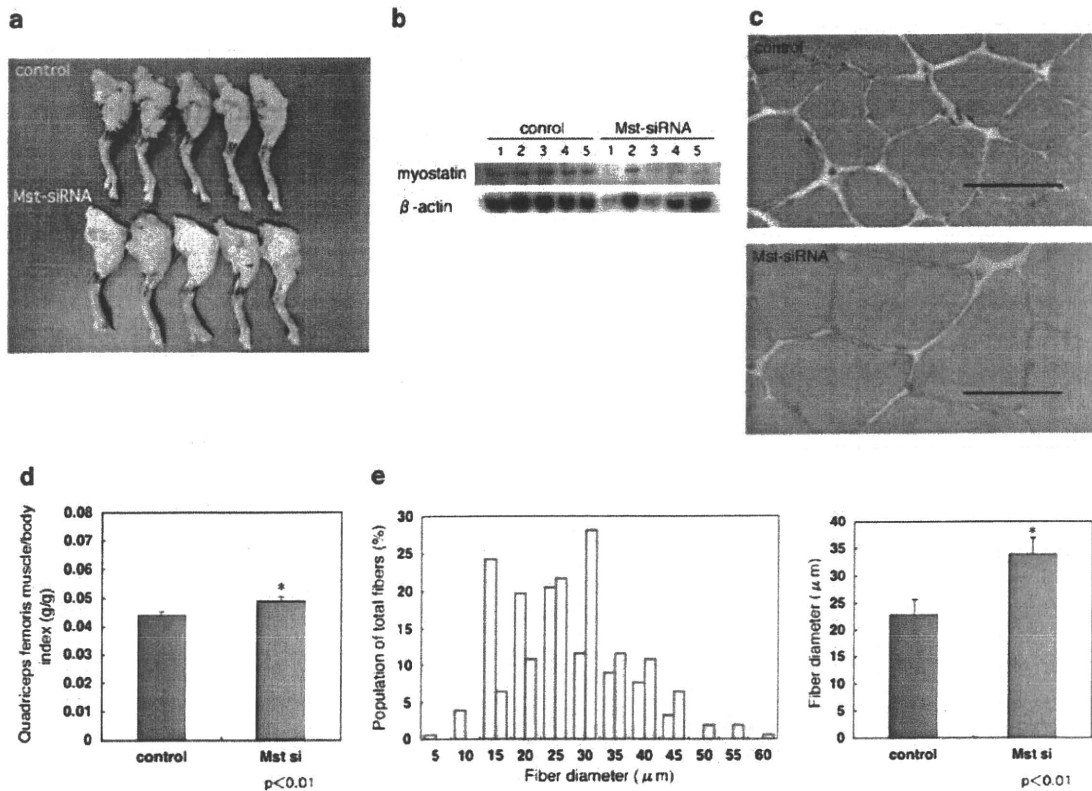
Figure 3 Systemic administration of the Mst-siRNA/atelocollagen (ATCOL) complex induces muscle enlargement in the mouse through inhibition of myostatin expression. For systemic administration, the siRNA (final concentration, 40 µM)/ATCOL (final concentration, 0.05%[6] complex, 200 µl) was introduced intravenously via orbital veins at 4, 7 and 14 days after the first application (*n*=5). As a control, control-scrambled siRNAs were injected into wild-type male mice (20 weeks, *n*=5). After 3 weeks, the quadriceps muscles on both sides were harvested and processed for analysis. (a) Photographs of lower limbs from control (upper panel) and Mst-siRNA/ATCOL-treated (lower panel) mice. (b) Western blot analysis of the control and Mst-siRNA/ATCOL-treated muscles (quadriceps femoris), assessed at 3 weeks after triple injection. (c) Hematoxylin and eosin staining of the control (upper panel) and Mst-siRNA/ATCOL-treated quadriceps muscle (lower panel). Scale bar, 50 µm. (d) Comparison of muscle weight/body weight index between the Mst-siRNA/ATCOL and control-siRNA/ATCOL-treated mice (0.048 ± 0.002 versus 0.043 ± 0.001 $n=5$; $P<0.01$). (e) Distribution of myofibril sizes of the control and Mst-siRNA/ATCOL-treated quadriceps muscles. The right panel shows the average myofibril size (33.92 ± 2.91 versus 22.95 ± 1.54 µm, $n=156$; $P<0.01$).

mass was observed in the Mst-siRNA/ATCOL-treated muscle (Figure 2a). Western blot analysis showed that the protein levels of myostatin in the muscles treated with the Mst-siRNA/ATCOL complex were significantly decreased (average ratio 0.55 ± 0.03), but not in the contralateral muscles treated with control siRNAs/ATCOL complex (average ratio 0.83 ± 0.01) (Figure 2b; $P<0.05$, $n=5$). Furthermore, immunohistochemical analysis on the masseter using an anti-laminin α2 antibody showed increase in the mean myofiber size of the Mst-siRNA/ATCOL-treated muscle (Figure 2c), as is the case for the wild-type (not shown). On the basis of these results, it seems that myostatin maintains satellite cells or muscle stem cells in a quiescent state. Reduced myostatin activity would lead to activation of these cells and fusion into existing fibers (Supplementary Figure S1e and f), resulting in fiber hypertrophy as proposed previously.[14]

We further examined whether systemic administration of the Mst-siRNA/ATCOL complex would have an effect on silencing the myostatin expression and lead to muscle enlargement. The Mst- or control siRNA/ATCOL complex was applied intravenously into normal mice four times in 3 weeks. Strikingly, we observed an obvious enlargement of skeletal muscles of lower limbs (Figure

3a), masseters and other muscles. Since change in the muscles of lower limbs is much larger than others, we used them for further analyses. We confirmed reduction of myostatin proteins in the muscles treated with the Mst-siRNA/ATCOL complex (average ratio 0.67 ± 0.11) (Figure 3b; $P<0.01$, $n=5$; average ratio for control 0.87 ± 0.03). We observed that the treated lower limbs are much larger than the controls, although the average body weights were 26.7 ± 0.7 and 25.8 ± 0.4 g for controls and treated mice, respectively. No increase in the body weight of the treated mouse was observed, probably because increase in the muscle weight compensated for reduction of fat accumulation.[17] To show increase in muscle weights, we used the muscle weight/body weight ratio (Figure 3d), in case the body weight exhibited variation. Significant increase in muscle fiber size (Figures 3c and e) was also observed after 3 weeks. These results indicate that siRNAs targeting against myostatin, intravenously administered with ATCOL, can specifically repress the expression of myostatin, inducing muscle hypertrophy in normal mice.

We present evidence that local and systemic applications of siRNA against myostatin coupled with ATCOL markedly stimulate muscle growth *in vivo* within a few

weeks. Local application of siRNA/ATCOL complex was shown to be effective to target the vascular endothelial growth factor gene in a xenografted tumor,[18] while ATCOL was used for systemic siRNA delivery into tumor-bearing mouse models and proved to be effective for silencing exogenous genes as luciferase and metastasis-associated genes as EZH2.[6] However, it has not been elucidated until this study whether the siRNA complex could have an effect of muscle growth on normal tissues by repression of muscle-specific genes. It has been thought that the enhanced permeability and retention (EPR) effect in tumor tissues could facilitate selective targeting of siRNA/polymer complex.[6] In spite of the significance of the EPR effect in tumor therapies, it is noticeable that normal and nontumor diseased tissues can be targets for siRNA-based drugs applied systemically. It was reported that nuclease activity to siRNA could be prevented[18] and cellular uptake of siRNAs was elevated by ATCOL.[5] Although the precise mechanisms by which ATCOL achieves these effects have not been elucidated to date, ATCOL complexed with DNA molecules was demonstrated to be efficiently transduced into mammalian cells.[19] Thus, similarly siRNA/ATCOL complexes may be transduced into cells probably by the same mechanisms as observed for DNA molecules. As a simple administration of myostatin-siRNA/ATCOL complex has a muscle growth effect, this novel method for fighting against muscle atrophy would be of considerable value for clinical applications. In tumor-bearing mice, it was reported that ATCOL could distribute siRNAs against luciferase to normal liver, lung, spleen and kidney tissues as well as bone-metastatic lesions.[6] ATCOL was also reported to display low toxicity and low immunogenicity when it is transplanted in vivo.[20,21] Taken together with our results, application of siRNAs with ATCOL would be promising for a therapeutic remedy against various diseases not only of muscles, but also of these organs.

Acknowledgements

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Cell Communication and Signaling

Review

Activin signaling as an emerging target for therapeutic interventions

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Abstract

After the initial discovery of activins as important regulators of reproduction, novel and diverse roles have been unraveled for them. Activins are expressed in various tissues and have a broad range of activities including the regulation of gonadal function, hormonal homeostasis, growth and differentiation of musculoskeletal tissues, regulation of growth and metastasis of cancer cells, proliferation and differentiation of embryonic stem cells, and even higher brain functions. Activins signal through a combination of type I and II transmembrane serine/threonine kinase receptors. Activin receptors are shared by multiple transforming growth factor-β (TGF-β) ligands such as myostatin, growth and differentiation factor-11 and nodal. Thus, although the activity of each ligand is distinct, they are also redundant, both physiologically and pathologically in vivo. Activin receptors activated by ligands phosphorylate the receptor-regulated Smads for TGF-β, Smad2 and 3. The Smad proteins then undergo multimerization with the co-mediator Smad4, and translocate into the nucleus to regulate the transcription of target genes in cooperation with nuclear cofactors. Signaling through receptors and Smads is controlled by multiple mechanisms including phosphorylation and other posttranslational modifications such as sumoylation, which affect potein localization, stability and transcriptional activity. Non-Smad signaling also plays an important role in activin signaling. Extracellularly, follistatin and related proteins bind to activins and related TGF-β ligands, and control the signaling and availability of ligands.

The functions of activins through activin receptors are pleiotrophic, cell type-specific and contextual, and they are involved in the etiology and pathogenesis of a variety of diseases. Accordingly, activin signaling may be a target for therapeutic interventions. In this review, we summarize the current knowledge on activin signaling and discuss the potential roles of this pathway as a molecular target of therapy for metabolic diseases, musculoskeletal disorders, cancers and neural damages.

Signaling of activins and related growth factors through activin receptors

Biosynthesis of activin and related growth factors

Activins belong to the transforming growth factor-β (TGF-β) family of growth and differentiation factors [1,2]. They form dimers composed of two inhibin β subunits. Four β subunits have been identified in mammals (βA, βB, βC and βE), whereas only a single inhibin α-subunit has been discovered so far. The βA and βB transcripts are found in nearly all tissues, whereas βC and βE subunits are expressedpredominantly in the liver. Both β and α subunits are synthesized as precursor polypeptides. After dimerization of the precursors, prodomains are cleaved by furin and/or related proprotein convertases in the endoplasmic reticulum and a mature dimeric polypeptide is released. Homodimers of inhibin βA or βB subunits, activin A and activin B, respectively, or heterodimeric activin AB exist in various tissues. Inhibins, heterodimeric proteins composed of an α-subunit linked to β-subunits by disulfide bonds, act as activin antagonists. In the case of myostatin, another TGF-β family protein related to activins, cleavage and maturation of the ligand may occur extracellularly in a tissue-specific manner [3].

Activin receptors

Activin signals are transmitted through two types of transmembrane serine/threonine kinase receptors, type I and type II activin receptors in target cells [1,4]. Activin receptors are prototypes of single-pass transmembrane serine/threonine kinases. Intriguingly, activin receptors are shared by other TGF-β family proteins, such as myostatin, growth and differentiation factor 11 (GDF11) and nodal. Therefore, several activities of these ligands are redundant with those of activins. Myostatin has been characterized as a skeletal muscle-specific cytokine regulating skeletal muscle mass [5]. GDF11 is structurally similar to myostatin, and is involved in neurogenesis in the spinal cord and olfactory bulb [6]. GDF11 also regulates kidney development and endocrine pancreas development [7,8]. Nodal is a central player in patterning the early embryo during the induction of mesoderm and endoderm [9], and acts as an authentic mesoderm inducer in mammalian species. Some of these activities are shared with activins.

Activin type II receptor, ACVR2 or ActRIIA, has been identified and characterized as a transmembrane serine/threonine kinase for activin A [10]. A second activin type II receptor, ACVR2B or ActRIIB, has also been identified [4]. In addition, TGF-β type II receptor, BMP type II receptor and Müllerian duct inhibiting substance type II receptor specific to each ligand have been characterized [2]. To date, seven type I receptors, activin receptor-like kinases 1 to 7 (ALK1-7), have been characterized for the TGF-β family [11]. Like type II receptors, type I receptors possess a serine/threonine kinase domain. However, different from type II receptors, type I receptors have a unique GS domain near the intracellular juxtamembrane regions preceding the kinase domain. The amino acid sequences of L45 loops of type I receptors located between the kinase subdomains IV and V are responsible for the preference of Smad proteins and determine the specificity between the activin/TGF-β subgroup (ALK4, 5, 7) and BMP subgroup (ALK1, 2, 3, 6) [2,11]. ALK4 is known as activin type IB receptor, ACVR1B or ActRIB, whereas ALK7 is known as activin type IC receptor, ACVR1C. ALK4 and ALK7 are type I receptors for activins and nodal, and ALK4 and ALK5 are receptors for myostatin and GDF11 (Table S1; additional file 1) [1,2]. Once activins bind to ActRIIA or ActRIIB, type I receptors are recruited to the ligand/ActRII complex, and the GS domains of type I receptors become phosphorylated by ActRII kinases. Activin/TGF-β-specific Smad, Smad2 and Smad 3, are phosphorylated by activated type I receptors (Figure 1). In the case of nodal, the co-receptor Cripto and related factors are required for the complete activation [9]. Cripto facilitates nodal signaling by binding to both nodal and activin receptors. Interestingly, Cripto may also act as an inhibitory factor for activin signaling when overexpressed [12](Table S1; additional file 1).

A pseudo-receptor BMP and activin membrane-bound inhibitor, BAMBI, has been identified [13]. BAMBI interacts with multiple type I receptors for TGF-β family ligands and inhibits the formation of the active receptor signaling complex. Thus, BAMBI serves as an endogenous dominant negative receptor [13]. BAMBI is characterized as a β-catenin target in colorectal tumors [14].

Regulation of activin receptors

Regulatory proteins for activin receptors control the signaling activity of activins and related growth factors. A FYVE domain-containing protein, the Smad anchor for receptor activation (SARA), interacts with both the type I receptor and Smads.

Complex formation of activin receptors with SARA and Smad in EEA-1 positive early endosomes may be an essential step for efficient activin/TGF-β signaling [15,16]. Activin type II receptors (ActRIIA and ActRIIB) have consensus amino acids for PSD-95/Discs-large/ZO-1 (PDZ) protein interaction at their COOH-terminus [1]. This characteristic is unique among receptors of the TGF-β family [17]. Activin-receptor interacting proteins (ARIPs), which have PDZ domains, associate with the COOH-terminus of ActRIIs and regulate activin signaling. ARIP1 has multiple WW and PDZ domains for protein-protein interactions, and regulates the localization of activin receptors and negatively controls signaling [17]. Intriguingly, ARIP-1 acts as a scaffold for N-methyl-D-aspartate (NMDA) receptor activation in hippocampal neurons, and is also