However, elimination of myostatin did not improve the phenotype in a laminin-α2-deficient dy^w mouse (13). Thus, myostatin inhibition may show disease-specific effects. It is also of note that one report showed that lack of myostatin results in excessive muscle growth but impaired force generation (14). In addition to myostatin propeptide and myostatin antibodies, follistatin (FS) and FS domain-containing proteins can bind to myostatin *in vivo* and act as effective myostatin inhibitors (7, 15, 16). FS was originally identified as a single-chain polypeptide with a weak inhibitory activity toward follicle-stimulating hormone secretion from anterior pituitary cells. Later, FS was found to be an activin-binding protein (16–18). Gene knockout analyses revealed that FS gene ablation causes multiple effects, including skeletal and cutaneous abnormalities, suggesting that FS may have additional functions other than activin inhibition (19). Recently, FS was shown to bind to myostatin and inhibit its activity (20). Similar to myostatin, activins belong to the TGF-β superfamily and have pleiotrophic effects on numerous tissues. Because activins have a variety of functions in tissues other than skeletal muscles, and their inhibition by FS is very efficient, FS has multiple effects on not only skeletal muscles but also other tissues. In fact, transgenic expression of the FS gene under the control of the metallothionein promoter has profound effects on reproductive performance and fertility (21). Recent crystallographic analyses have revealed that the minimal activin-inhibiting fragment of FS comprises the FS I and FS II domains, and each FS domain may have different ligand binding activity (22, 23).

In the present study, we developed and characterized a myostatin inhibitor derived from FS, designated FS I-I, and further investigated its effects on muscle mass and strength in *mdx* mice. Because 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.

MATERIALS AND METHODS

DNA constructs

A human FS cDNA was used as a template for polymerase chain reaction (PCR) amplification (24). A cDNA fragment containing the N-terminal region and FS I domain (FS-A) was amplified using the sense primer 5′-CGGAATTCATGGTCCGCGCGAGGCACCAC-3′ and antisense primer 5′-CGAAGCTTTTTACATCTGCCTTGG-3′. A cDNA fragment containing only the FS I domain (FS-B) was amplified using the sense primer 5′-GCAAGCTTTGTGAGAACGTGGACTGTG-3′ and antisense primer 5′-GCCTCGAGATATCTTCACAAGTCTTTTTACATCTGCC-3′. The FS-A and FS-B cDNA fragments were subcloned into the pGEM-T Easy cloning vector (Promega, Tokyo, Japan), and the *Eco*RI-*Hind*III fragment of FS-A and *Hind*III-*Sal*I fragment of FS-B were then inserted into the pBluescript cloning vector. The resultant cDNA contained the N-terminal region and the two FS I domains in tandem (FS I-I in pBluescript). The *Eco*RI-*Spe*I fragment of FS I-I in pBluescript was subcloned into the pcDNA3 expression vector (Invitrogen, Tokyo, Japan). To create an FS I-I-Fc cDNA, a *Bam*HI-*Xba*I fragment of human Fc was subcloned into pcDNA3 (25), and digested with *Kpn*I and *Bam*HI. Next, a *Kpn*I-*Bam*HI fragment covering the whole FS I-I sequence was subcloned into the *Kpn*I-*Bam*HI-digested human Fc in pcDNA3 (FS I-I-Fc in pcDNA3). Coding sequences of FS I-I-Fc consist of nucleotide 1–501 and 283–501 of GenBank accession number CR541813, fused to *Bam*HI-*Xba*I fragment of human IgG Fc portion (25).

GST-FS I-I enzyme-linked immunosorbent assay analysis

The *Pst*I-*Eco*RV fragment of FS I-I in pcDNA3 was subcloned into pBluescript, and the *Sma*I-*Xho*I fragment was then subcloned into the bacterial expression vector pGEX6P-1 (GE Healthcare Bioscience, Tokyo, Japan). pGEX6P-1-FS I-I cDNA or empty pGEX6P-1 vector cDNA was transformed in Origami competent cells (Takara Bio, Tokyo, Japan). A GST-FS I-I fusion protein and naive GST protein were expressed and purified using glutathione-Sepharose beads, as described previously (26). Enzyme-linked immunosorbent assay (ELISA) plates (Sumilon Bakelite Co., Ltd., Tokyo, Japan) were coated with either 100 μl of 1 μg/ml bovine activin A (WAKO Chemicals, Osaka, Japan) or human myostatin in carbonate buffer (pH 9.6) at 4°C overnight, and then washed twice with washing buffer (0.05% Tween-20 in 25 mM Tris-buffered saline, pH 7.5). After blocking with Blocking One solution (Nakalai Tesque, Kyoto, Japan) for 45 min at room temperature, the wells were washed three times with washing buffer, and incubated with various amounts of GST or GST-FS I-I proteins at 37°C for 60 min in PBS containing Blocking One solution and 0.01% Tween-20, according to the manufacturer's protocol. After three washes with washing buffer, the wells were incubated with a rabbit polyclonal anti-GST antibody at a dilution of 1:750 in Blocking One solution at 37°C for 50 min, washed, incubated with a horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1000, and then incubated with o-phenylenediamine dihydrochloride for color development. The optical densities at 450 nm were measured.

Purification of human FS I-I-Fc protein

Chinese hamster ovary (CHO-K1) cells were transfected with the human FS I-I-Fc cDNA in pcDNA3 by electroporation, and clonal cells were established by limiting dilution (27). CHO-K1 cells stably expressing the FS I-I-Fc cDNA were grown in α-modified essential medium (MEM)-10% fetal calf serum (FCS) until they reached confluency, and then conditioned by EX-CELL301 medium (Nichirei Biosciences, Tokyo, Japan). The conditioned medium was passed through a heparin-Sepharose 6 Fast Flow column, followed by protein A-Sepharose Fast Flow chromatography (GE Healthcare Biosciences, Bucks, UK). The FS I-I-Fc protein was eluted with 5 ml of 100 mM glycine-HCl (pH 3.0), and immediately neutralized with 500 μl of 1 M Tris-HCl (pH 8.0). A total of 850 μg of FS I-I-Fc protein was obtained from 4 liters of conditioned medium.

Surface plasmon resonance biosensor analysis

All surface plasmon resonance (SPR) measurements were taken using a BIAcore X system (Biacore AB, Tokyo, Japan), as described previously (28). For immobilization of samples, proteins were dissolved in 20 mM sodium acetate (pH 4.5) at a concentration of 10 μg/ml and immobilized on a CM5 sensor chip at a flow rate of 5 μl/min at 25°C using an

amine-coupling method. Kinetic measurements were performed by injection of each analyte for 240 s followed by dissociation in buffer flow for 240 s at a flow rate of 20 μl/min at 25°C. The immobilized ligands were regenerated after each cycle using a 40 μl injection of 10 mM HEPES (pH 7.4)/2 M guanidine-HCl. The kinetic parameters, association rate constant (k_{on}) and dissociation rate constant (k_{off}) were determined using BIAevaluation software version 3.0 (Biacore AB). Activin A was purified from bovine follicular fluid, as described previously (29). Human FS and mouse myostatin were obtained from R&D Systems (Minneapolis, MN, USA).

Biological assay for myostatin signaling

A204 rhabdomyosarcoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% FCS. Cells were plated at a density of 8×10^4 cells/well in 24-well dishes and were transfected with 0.5 ng of CAGA-lux and 0.1 ng of a cytomegalovirus promoter-driven β-galactosidase (CMV-β-gal) DNA using the GeneJammer transfection reagent (Stratagene, La Jolla, CA, USA), according to the manufacturer's protocol. At 24 h after transfection, the cells were stimulated with either myostatin or activin A in DMEM containing 1% FCS for 24 h. To examine whether FS or FS I-I-Fc had inhibitory effects on myostatin or activin, FS or FS I-I-Fc was simultaneously added with either myostatin or activin. The luciferase activities were measured and normalized by the corresponding β-galactosidase activities, as described previously (30).

CHO-K1 cells were transfected with 0.2 ng each of ActRIIB and wild-type ALK5 or ALK4 expression plasmids, 0.3 ng of a CAGA-lux reporter plasmid and 0.1 ng of the CMV-β-gal plasmid per well of 12-well dishes using the Transfast liposome reagent (Promega K.K. Japan, Tokyo, Japan) (30). 10T1/2 mouse fibroblastic cells were transfected with 0.2 ng each of a MyoD plasmid, Smad3 plasmid, and wild-type ALK5 or ALK4 expression plasmid, 0.3 ng of a MyoD-responsive 6R-lux reporter plasmid and 0.1 ng of the CMV-β-gal plasmid per well of 12-well dishes using the Effectene reagent (Qiagen in Japan, Tokyo, Japan) (31). At 24 h after transfection, the cells were stimulated with 20 ng/ml of myostatin either alone or in the presence of increasing amounts of FS I-I-Fc.

Generation and characterization of FS I-I transgenic mice

An *Eco*RI-*Xho*I fragment containing the whole FS I-I cDNA was subcloned into the CAG-GS vector (CAGGS-FS I-I) (32). Next, an *Eco*RI-*Sma*I fragment covering the whole coding sequence of FS I-I from CAGGS-FS I-I was subcloned into the MDAF2 vector containing the myosin light chain promoter, SV40 processing sites and MLC1/3 enhancer (33). The FS I-I transgene with the skeletal muscle-specific promoter consisting of 3.9 kb was recovered by *Cla*I digestion, and transgenic mice were generated by standard pronuclear microinjection techniques (21). The presence of the transgene was determined by PCR amplification of tail genomic DNA. The PCR was performed using the sense primer 5′-CACCACTGCTCTTCCAAGTGTC-3′ (MDAF2S) and antisense primer 5′-GTCACACCACAGAAGTAAGGTC-3′ (MDAF2AS) with a program of 35 cycles of denaturation for 30 s at 94°C, annealing for 15 s and extension for 30 s at 72°C. The annealing temperature was altered from 74°C to 54°C in five steps of 4°C intervals at 3 cycles per step, followed by 20 additional cycles at 54°C. The final extension reaction was carried out for 10 min at 72°C. The PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining.

Transgenic founders were mated to wild-type C57BL/6 mice, and the offspring were analyzed. Individual muscles were manually dissected from five mice and weighed. To obtain transgenic *mdx* mice expressing FS I-I, female *mdx* mice were crossed with male FS I-I transgenic mice, and the male F1 generation was screened for the presence of the FS I-I transgene as described above and used for analyses.

Immunoblot analysis

Sections of the quadriceps femoris muscles from FS I-I transgenic mice and age-matched control mice were homogenized in a buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM NaF, 5 mM β-glycerophosphate, 1 mM Na vanadate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, and 1 μg/ml aprotinin) and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants were recovered as cell lysates. Aliquots of the lysates containing 250 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (27). The membranes were probed with antiphosphorylated Smad2, anti-Smad2, antiphosphorylated Erk1/2 and anti-Erk1/2 antibodies (all obtained from Cell Signaling Technology, Beverly, MA, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence reactions, images of the developed immunoblots were captured using a cooled CCD camera system (Light-Capture; ATTO, Tokyo, Japan).

Immunohistochemical analysis and determination of muscle fiber size and number

Mice were subjected to histological analysis at 5, 9, and 13 wk of age. The quadriceps femoris and diaphragm muscles were snap-frozen in liquid nitrogen-cooled isopentane and sectioned using a cryostat (Leica Microsystems Japan, Tokyo, Japan). The sections were stained with hematoxylin and eosin. The myofiber size and total myofiber number in individual skeletal muscles were measured from fluorescence images of antilaminin-α2-stained sections using WinROOF software (Mitani Corporation, Fukui, Japan). Infiltration of macrophages into the skeletal muscles was detected by staining with an anti-Mac1 (CD11b/18) antibody (COSMO BIO, Tokyo, Japan).

Rotarod analysis and grip strength test

A rotarod RRAC-3002 (O'Hara & Co. Ltd., Tokyo, Japan) was used. Mice were subjected to the rotarod analysis in the acceleration mode (3–30 rpm/min) over 5 min. In total, 21 wild-type male mice and 20 FS I-I male mice were analyzed.

The peak grip force was measured using an MK-380S grip strength meter (Muromachi Kikai Co. Ltd., Tokyo, Japan), according to the manufacturer's protocol. Male mice (9–13 wk of age) were allowed to grasp a wire mesh with their forelimbs and hindlimbs, and then pulled steadily by their tails horizontally until they lost their grip. The maximal force values were recorded. Measurements were performed 8 times using wild-type mice, FS I-I transgenic male mice, *mdx* mice and *mdx*/FS I-I mice (n=6–8).

RESULTS

Characterization of a myostatin inhibitor derived from FS

FS is composed of an N-terminal domain and three FS domains (FS I, FS II, and FS III). We created FS

mutants containing two FS I domains, designated FS I-I and FS I-I-III, and characterized their binding activities toward myostatin and activin A (**Fig. 1*A***). Since the two mutants were found to have similar activities and FS I-I is shorter than FS I-I-III, we characterized FS I-I in the subsequent experiments. Interestingly, FS I-I retained its myostatin binding but showed a significantly weaker activin-binding activity. The results of solid-phase binding assays of GST-FS I-I with myostatin and activin A are shown in Fig. 1*B*. GST alone did not show any binding to either myostatin or activin. In contrast, GST-FS I-I lost its binding activity toward activin but bound to myostatin in a dose-dependent manner (Fig. 1*B*). To determine the affinities of FS I-I for myostatin and activin, we prepared a highly purified FS I-I recombinant protein. Since the naive FS I-I protein showed weak heparin-binding activity and was difficult to purify, we used an FS I-I-Fc fusion protein and characterized its binding activities toward myostatin and activin by SPR biosensor analysis. First, we analyzed the affinities of FS for myostatin and activin A. The association constant (k_{on}) of FS for activin A was slightly faster than that for myostatin, while the dissociation constants (k_{off}) of FS for myostatin and activin A were comparable. We further calculated the affinities (K_d) of FS for myostatin and activin A to be 12.3 and 1.72 nM, respectively (**Table 1**). Next, we analyzed the binding activities of FS I-I-Fc to myostatin and activin. Myostatin and activin A were separately immobilized on sensor chips, and various concentrations of FS I-I-Fc were passed over these sensor chips as analytes. As shown in Table 1, the association constant (k_{on}) of FS I-I-Fc for myostatin was much faster than that for activin A, while the dissociation constants (k_{off}) of FS I-I-Fc for myostatin and activin A were comparable. On the basis of the association and dissociation rate constants obtained through the SPR analyses, we calculated the affinities (K_d) of FS I-I-Fc for myostatin and activin A to be 46.8 nM and 64.3 μM, respectively (Table 1). Thus, FS showed high-affinity binding activities toward both myostatin and activin, whereas FS I-I retained the high-affinity myostatin-binding activity but lost the high-affinity activin-binding activity.

Next, we studied the actions of FS I-I on the myostatin- and activin-signaling pathways. The intracellular signaling pathway of myostatin is similar to those of activin and TGF-β. Myostatin signals through a combination of activin type II receptors (ActRIIB and ActRIIA) and activin receptor-like kinases 5 and 4 (ALK5 and ALK4) (34). The activated receptors phosphorylate Smad2/3, which then associate with the common Smad, Smad4, and translocate into the nucleus to activate gene transcription (1, 2, 16). Myostatin signaling can be monitored by a conventional reporter assay, called the CAGA-lux assay, in human A204 rhabdomyo-

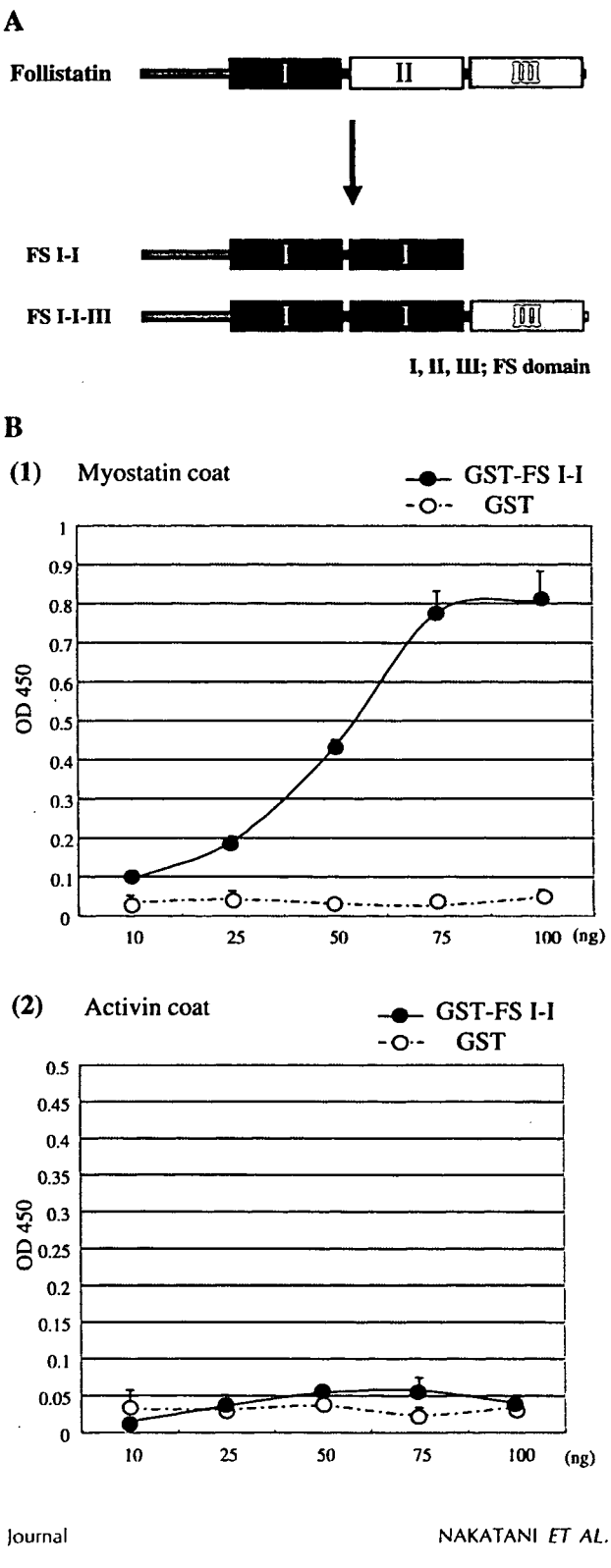

Figure 1. Structure and binding activities of follistatin (FS) and two FS mutants. *A)* FS has three cysteine-rich domains, called FS domains, which are shown as boxes. The FS mutants have two consecutive FS I domains. *B)* Binding activities of GST-FS I-I (●) and GST (○) toward myostatin and activin A. ELISA plates coated with myostatin (*1*) or activin (*2*) were incubated with various amounts of GST fusion proteins. The relative binding activities were quantified by a colorimetric assay. GST does not bind to either myostatin or activin, whereas GST-FS I-I shows myostatin-binding activity in a dose-dependent manner.

TABLE 1. *Kinetic rate constants for binding of follistatin and FS I-I-Fc to activin A and myostatin evaluated by surface plasmon resonance biosensor analysis*

Ligand	Analyte	k_{on}	k_{off}	K_d
Follistatin	Activin A	8.31×10^{5}	1.43×10^{-3}	1.72×10^{-9}
	Myostatin	5.29×10^{4}	6.51×10^{-4}	1.23×10^{-8}
FS I-I	Activin A	4.12	2.65×10^{-4}	6.43×10^{-5}
	Myostatin	1.06×10^{4}	4.98×10^{-4}	4.68×10^{-8}

sarcoma cells, which are responsive to both myostatin and activin. The reporter activity stimulated by myostatin was repressed by coincubation with FS I-I-Fc in a dose-dependent manner (**Fig. 2*A***, left panel). The IC_{50} (median inhibitory dose) of FS I-I-Fc for inhibition of 20 ng/ml of myostatin was estimated to be 60 ng/ml. In contrast, the reporter activity stimulated by activin was not repressed by coincubation with FS I-I-Fc, even at 1 μg/ml (Fig. 2*A*, right panel). As expected, FS efficiently inhibited both the myostatin and activin activities. The myostatin-inhibiting activity of FS I-I was further studied in another cell line, CHO-K1 cells (Fig. 2*B*). In this experiment, the myostatin signaling was augmented by exogenous transfection of ActRIIB expression plasmid either with wild-type ALK5 or ALK4 expression plasmids. Similar to the A204 cells, the myostatin activity was efficiently blocked by FS I-I. We also studied the actions of FS I-I using a different reporter construct.

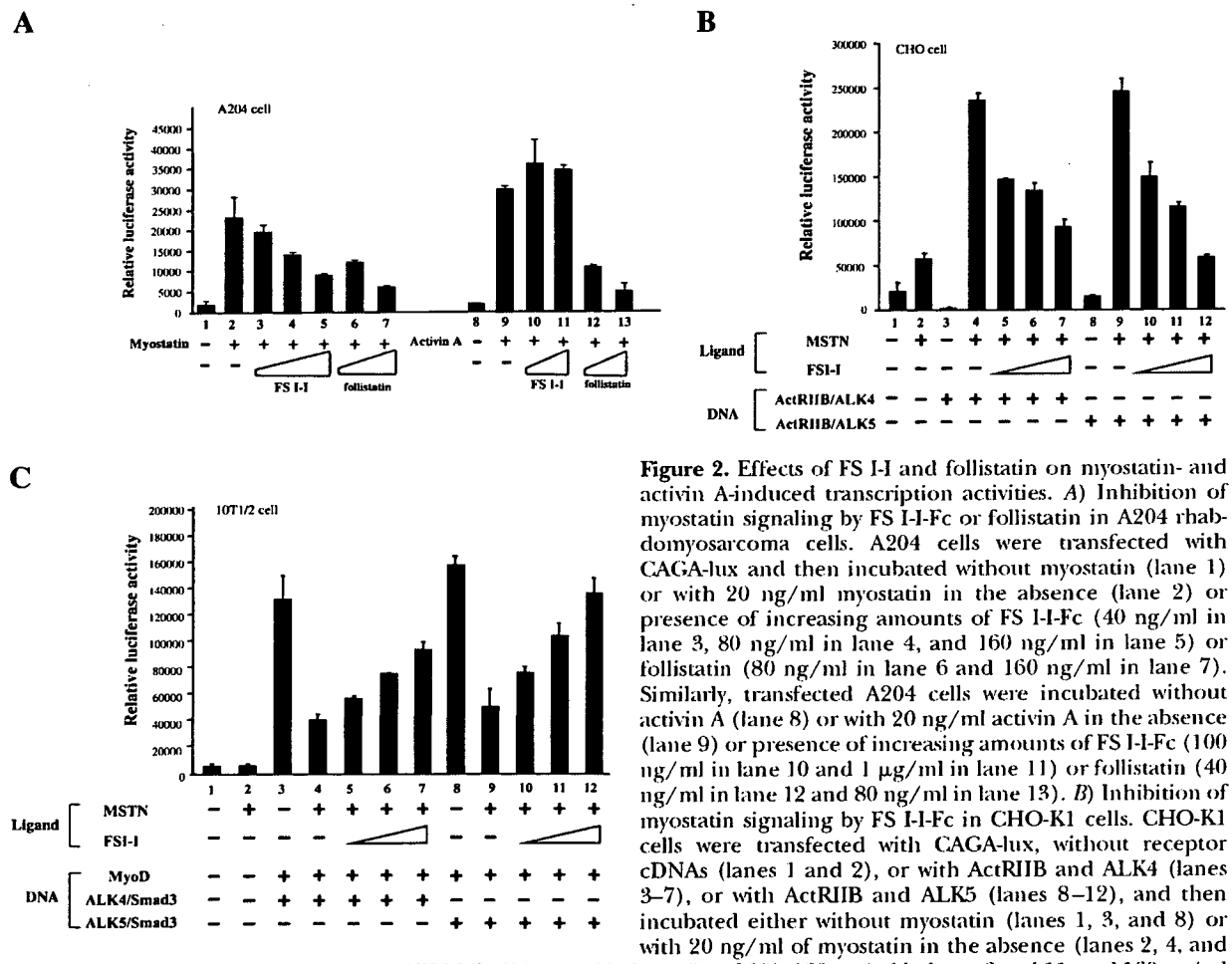

Figure 2. Effects of FS I-I and follistatin on myostatin- and activin A-induced transcription activities. *A*) Inhibition of myostatin signaling by FS I-I-Fc or follistatin in A204 rhabdomyosarcoma cells. A204 cells were transfected with CAGA-lux and then incubated without myostatin (lane 1) or with 20 ng/ml myostatin in the absence (lane 2) or presence of increasing amounts of FS I-I-Fc (40 ng/ml in lane 3, 80 ng/ml in lane 4, and 160 ng/ml in lane 5) or follistatin (80 ng/ml in lane 6 and 160 ng/ml in lane 7). Similarly, transfected A204 cells were incubated without activin A (lane 8) or with 20 ng/ml activin A in the absence (lane 9) or presence of increasing amounts of FS I-I-Fc (100 ng/ml in lane 10 and 1 μg/ml in lane 11) or follistatin (40 ng/ml in lane 12 and 80 ng/ml in lane 13). *B*) Inhibition of myostatin signaling by FS I-I-Fc in CHO-K1 cells. CHO-K1 cells were transfected with CAGA-lux, without receptor cDNAs (lanes 1 and 2), or with ActRIIB and ALK4 (lanes 3–7), or with ActRIIB and ALK5 (lanes 8–12), and then incubated either without myostatin (lanes 1, 3, and 8) or with 20 ng/ml of myostatin in the absence (lanes 2, 4, and 9) or presence of increasing amounts of FS I-I-Fc (80 ng/ml in lanes 5 and 10), 120 ng/ml in lanes 6 and 11, and 160 ng/ml in lanes 7 and 12). *C*) Suppression of MyoD-induced transcription by myostatin is inhibited by FS I-I-Fc. 10T1/2 cells were transfected with MyoD-dependent 6R-lux, MyoD, ALK4, and Smad3 (lanes 3–7), or with 6R-lux, MyoD, ALK5, and Smad3 (lanes 8–12), and incubated either without myostatin (lanes 1, 3, and 8) or with 20 ng/ml myostatin (lanes 2, 4–7, and 9–12) in the absence (lanes 2, 4, and 9) or presence of increasing amounts of FS I-I-Fc (80 ng/ml in lanes 5 and 10, 120 ng/ml in lanes 6 and 11, and 160 ng/ml in lanes 7 and 12). In lanes 1 and 2, the cells were transfected with 6R-lux plasmid, without MyoD/Smad3/ALK plasmids. The luciferase activity of each lysate was measured. The values represent the means ± SE of triplicate determinations.

10T1/2 cells differentiate into myoblastic cells on forced expression of a MyoD cDNA, and myostatin is capable of repressing the activity of a MyoD-dependent reporter, 6R-lux. In this experiment, Smad3 and wild-type ALK5 or ALK4 plasmids were cotransfected to evaluate the actions of myostatin. As shown in Fig. 2*C*, the inhibition of the MyoD-dependent reporter activity by myostatin was recovered by coincubation with FS I-I-Fc. In summary, FS I-I was capable of inhibiting the actions of myostatin in multiple assays but hardly affected the activin activity. These observations are consistent with the low binding activity of FS I-I toward activin (Fig. 1*B* and Table 1).

Analysis of transgenic mice overexpressing FS I-I in skeletal muscles

To evaluate the actions of FS I-I *in vivo*, we overexpressed FS I-I in mice. FS I-I was placed downstream of a skeletal muscle-specific myosin light chain promoter (**Fig. 3*A***) (33). Four founders positive for the transgene by PCR genotyping were obtained (Fig. 3*B*). Protein expression of FS I-I was also verified by Western blot analysis (Fig. 3*C*). We selected one high-expressing line that showed significant increases in skeletal muscle mass for further characterization. Lysates from the quadriceps femoris muscles of male FS I-I transgenic mice and matched wild-type littermates were prepared and subjected to immunoblot analysis for phosphorylated and total Smad2 (**Fig. 4*A***). Although the total Smad2 levels were comparable between wild-type and transgenic mice, phosphorylated Smad2 was reduced in the transgenic mice compared with the level in wild-type mice. Since FS I-I inhibits myostatin *in vitro*, and myostatin signals through phosphorylation of Smad2 and Smad3, it is reasonable that the FS I-I expressed in skeletal muscles suppressed myostatin signaling in FS I-I transgenic mice. Interestingly, we found that phosphorylation of Erk1/2, a MAP kinase family member, was also reduced in FS I-I transgenic mice. Next, changes in the body weight and skeletal muscle mass were studied (Fig. 4*B*). No difference in the weights was detected until 5 wk of age. From 6 to 14 wk of age, the weight of the transgenic male mice was greater than that of wild-type littermates. However, after 15 wk, the weight of the wild-type mice became larger than that of FS I-I transgenic mice. This is likely to be due to an increase in the adipose tissue mass in wild-type mice. Transgenic mice exhibited pronounced muscles, especially the pectoralis major, triceps brachii, gluteus, and quadriceps femoris muscles (Fig. 4*C*). Representative skeletal muscles were dissected out and weighed. The weight of the tibialis anterior muscle of transgenic mice showed 1.33-fold increase compared with that of wild-type mice (Fig. 4*D* and **Table 2**). Other individual muscles also showed increases (Table 2). To investigate whether the increases in skeletal muscle mass and weight arose from hypertrophy and/or hyperplasia, we measured the myofiber size and number (Table 2). As shown in Table 2, FS I-I transgenic mice and their wild-type littermates

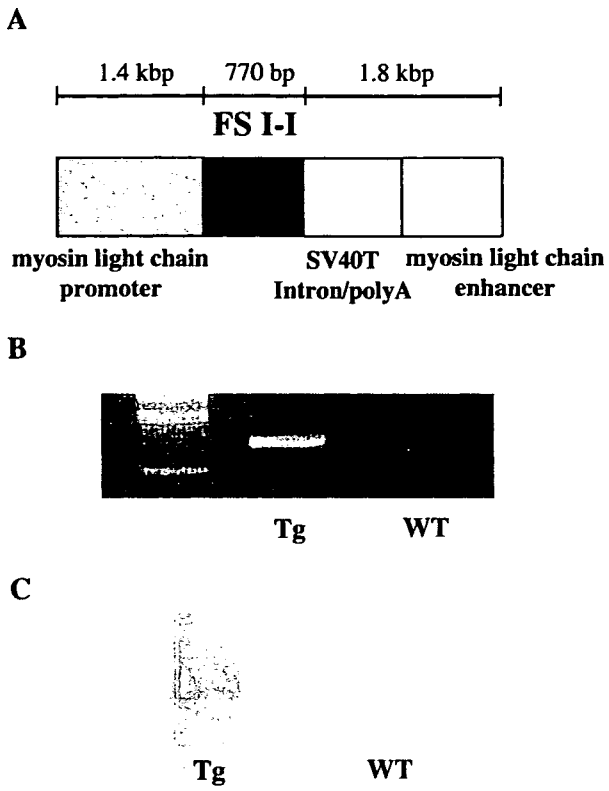

Figure 3. Transgenic expression of the myostatin inhibitor FS I-I. *A*) The FS I-I transgene consists of the myosin light chain promoter, FS I-I cDNA, SV40T antigen intron/poly(A) and myosin light chain enhancer. bp, base pairs; kbp, kilobase pairs. *B*, *C*) Total RNA samples (*B*) and protein lysates (*C*) from the quadriceps femoris muscles of transgenic offspring (Tg) and nontransgenic wild-type (WT) mice were analyzed by PCR (*B*) or immunoblotting (*C*).

had 3,372 ± 467 and 2,707 ± 284 fibers in their tibialis anterior muscles, respectively, indicating that the fiber number in the transgenic mice showed 1.25-fold increase. In other skeletal muscles such as the extensor digitorum longus, the fiber numbers were increased by 1.2-fold. Next, we quantified the myofiber size by staining each fiber of the quadriceps femoris muscle with an antilaminin-α2 antibody (Fig. 4*E*). Enlargement of the myofibers in FS I-I transgenic mice was noted. Quantification of the fiber areas of individual myofibers is shown in Fig. 4*F*. The mean fiber areas were increased in FS I-I transgenic mice. The estimated mean fiber sizes in wild-type littermates and FS I-I transgenic mice were 1924 and 2504 μm^2, respectively. These results indicate that both hypertrophy and hyperplasia were responsible for the increase in muscle mass in FS I-I transgenic mice.

FS I-I transgenic mice did not show any behavioral abnormalities and reproduced normally. To assess the functionality of the increased muscle mass, we used two independent methods, namely the rotarod test and grip strength test. In the rotarod test, FS I-I transgenic mice performed better than control mice (**Fig. 5*A***). The endurance times for wild-type and FS I-I transgenic

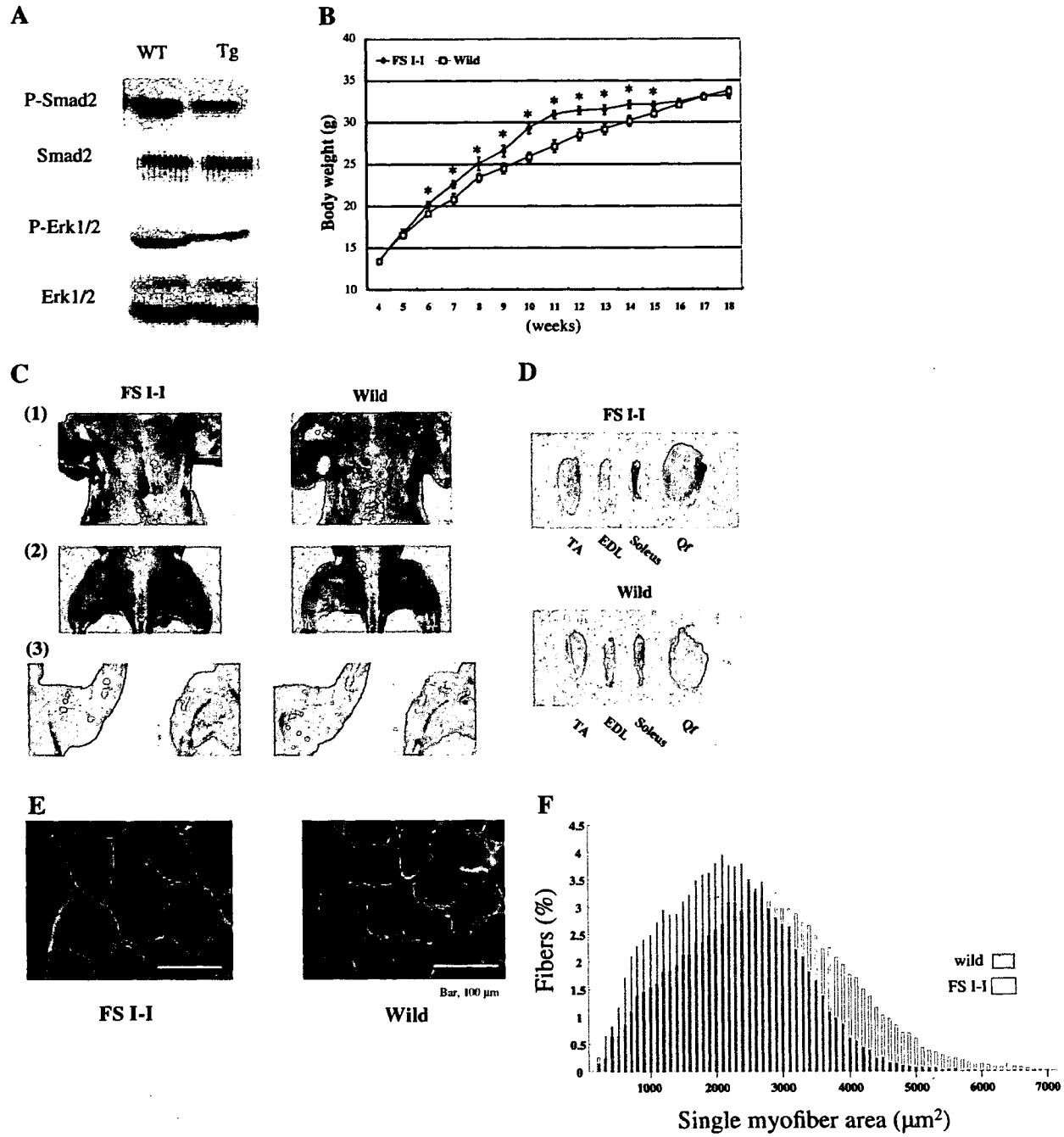

Figure 4. Analyses of FS I-I expressing transgenic mice. *A*) Immunoblot analyses for Smad and Erk1/2. Aliquots (250 μg protein) of lysates of the quadriceps femoris muscles of wild-type (WT) littermates, and transgenic (Tg) mice were analyzed by immunoblotting using antibodies against phosphorylated Smad2 (P-Smad2), Smad2, phosphorylated Erk1/2 (P-ERK1/2), and Erk1/2. *B*) Growth curves of wild-type littermates (WT, □) and FS I-I transgenic mice (FS I-I, ♦). Mice were weighed at the indicated weeks (n=5). *$P < 0.001$, Student's t test. *C*) Phenotypes of FS I-I transgenic mice (left) and wild-type littermates (right). Pictures of the pectoral regions (top panels, *1*), quadriceps femoris muscles (middle panels, *2*) and upper and lower limbs (bottom panels, *3*) of skinned transgenic mice and wild-type littermates are shown. *D*) Pictures of dissected skeletal muscles from FS I-I transgenic and wild-type littermates. TA, tibialis anterior muscle; EDL, extensor digitorum longus muscle; Qf, quadriceps femoris muscle. *E*) Immunohistochemical analysis of the quadriceps femoris muscles from FS I-I transgenic mice and wild-type littermates using an antilaminin-α2 antibody. *F*) Distributions of single myofiber areas in the quadriceps femoris muscles of FS I-I transgenic mice and wild-type littermates at 13 wk of age. From five transgenic mice and five wild-type mice, 500 myofibers per mice (total 2500 myofibers for each genotype) were counted. Percentage of myofibers with indicated areas per total fibers were plotted. Mean areas are 1924 ± 921 and 2504 ± 1603 μm^2 for wild-type and transgenic mice, respectively. $P < 0.0001$, Student's t test.

TABLE 2. *Muscle weights and fiber numbers in FS I-I transgenic mice and wild-type littermates*

Characteristic	TA	Soleus	EDL	Quadriceps
Weight (mg)				
FS I-I	68.0 ± 5.1*	10.2 ± 1.2*	13.4 ± 2.1*	245.1 ± 16.0*
Wild	51.1 ± 3.9	8.4 ± 1.0	11.0 ± 1.2	201.0 ± 20.0
Fiber number				
FS I-I	3372 ± 467**	953 ± 121**	1101 ± 99**	8137 ± 523**
Wild	2707 ± 284	783 ± 52	912 ± 99	7213 ± 492

Ten male mice were analyzed for their muscle weights and the fiber number counts. TA, tibialis anterior muscle; EDL, extensor digitorum longus muscle. *$P < 0.005$; **$P < 0.001$, Student's *t* test

mice were 160.8 ± 48.7 and 211.8 ± 76.6 s, respectively. We further studied the muscle function by the grip strength test (Fig. 5*B*). In the grip test, the mice grasped a mesh with both their forelimbs and hindlimbs, and the horizontal force required to dislodge their grip was measured. The mean values for wild-type and FS I-I transgenic mice were 218.4 ± 17.0 and 297.9 ± 32.7 g, respectively. Thus, these two independent tests for assessing muscle strength both indicated that the skeletal muscles of FS I-I transgenic mice were functional and had increased strength.

Amelioration of the pathophysiology of *mdx* mice by crossing with FS I-I transgenic mice

Myostatin inhibition is one of the most promising therapeutic approaches for muscular dystrophy. We analyzed the effects of FS I-I on the *mdx* mouse model of Duchenne muscular dystrophy. *mdx* mice are deficient in the dystrophin gene carried on the X chromosome. Male FS I-I mice were crossed with female *mdx* mice to generate *mdx*/FS I-I mice. The skeletal muscles of *mdx*/FS I-I mice were larger than those of *mdx* mice (**Fig. 6*A***). One of the characteristics of *mdx* mice was irregularity of myofiber size. The diameters of the myofibers in the quadriceps femoris of *mdx*/FS I-I mice became larger than those of *mdx* mice (Fig. 6*A*). More importantly, the sizes of the myofibers in *mdx*/FS I-I mice were homogeneous (Fig. 6*A*). Massive cell infiltration and tissue damage were evident in the skeletal muscles of *mdx* mice (Fig. 6*B*). Interestingly, at both early (5 wk, Fig. 6*B*; see also ref. 1) and late (9 wk, Fig. 6*B*; see also ref. 2) phases, the cell infiltration and fibrous changes observed in the diaphragm of *mdx* mice were significantly reduced in *mdx*/FS I-I mice. At 9 wk of age, several sections without cell infiltration were observed histologically (Fig. 6*B*; see also ref. 2). Similarly, recovery from cell infiltration was observed in the quadriceps femoris muscle in *mdx*/FS I-I mice (Fig. 6*C*). We quantified the cell infiltration in the skeletal muscles by counting Mac1-positive macrophages in tissue sections of the diaphragm. In *mdx* mice, 8.45% of the total surface area of the diaphragm was positive for Mac1, compared to only 4.03% in *mdx*/FS I-I mice, indicating that macrophage infiltration was significantly reduced in *mdx*/FS I-I mice. In addition, a grip strength test showed that the muscle strength in *mdx*/FS I-I mice was increased compared with that in *mdx* mice and became comparable with that of wild-type mice (Fig. 6*E*).

DISCUSSION

Duchenne muscular dystrophy is an X-linked degenerative disorder caused by mutations of dystrophin. In addition to gene therapy and cell therapy, myostatin blockade is one of the promising therapeutic strategies for muscular diseases, such as muscular dystrophy (35). Inhibition of myostatin is capable of increasing skeletal muscle mass, even in adults, and myostatin blockade could increase skeletal muscle mass regardless of the type of muscular dystrophy. Experimentally, the pathology of the *mdx* mouse model of Duchenne muscular

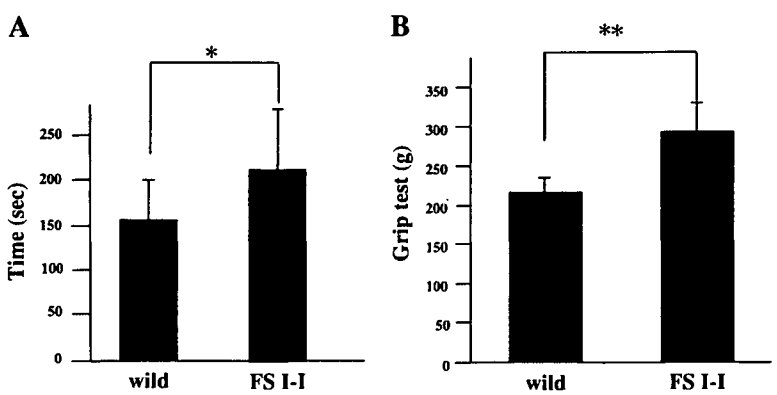

Figure 5. Analyses of muscle strength of FS I-I transgenic mice. *A*) FS I-I transgenic male mice and wild-type littermates were subjected to rotarod tests. The endurance times (s) on the rotarod apparatus are plotted. Data are expressed as means ± SD. *$P < 0.01$, Student's *t* test. *B*) Peak force measurements (*g*) of grip strength for FS I-I transgenic mice and wild-type littermates at 10–12 wk of age. Data are expressed as means ± SD (n=6). **$P < 0.001$, Student's *t* test.

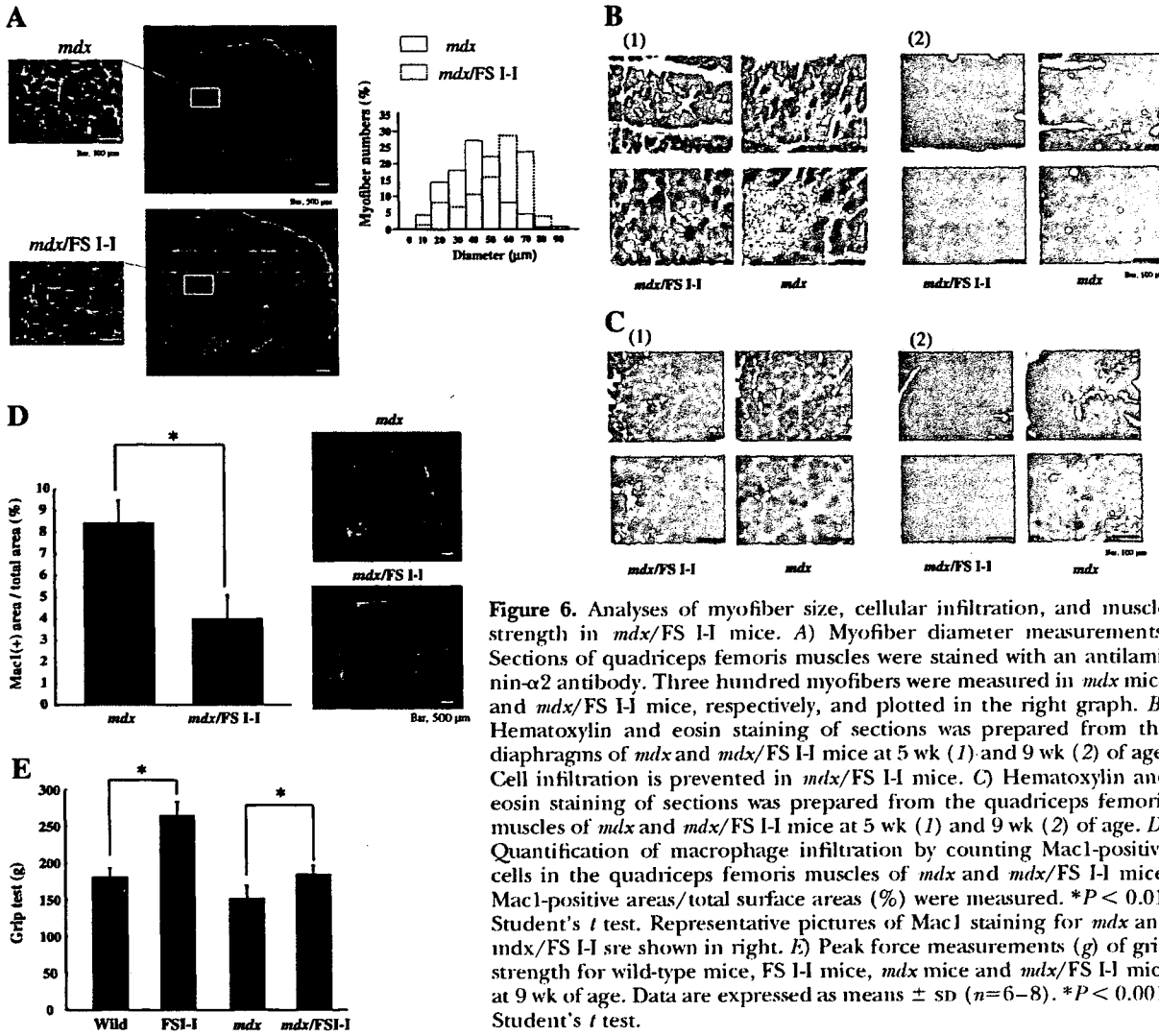

Figure 6. Analyses of myofiber size, cellular infiltration, and muscle strength in *mdx*/FS I-I mice. *A*) Myofiber diameter measurements. Sections of quadriceps femoris muscles were stained with an antilaminin-α2 antibody. Three hundred myofibers were measured in *mdx* mice and *mdx*/FS I-I mice, respectively, and plotted in the right graph. *B*) Hematoxylin and eosin staining of sections was prepared from the diaphragms of *mdx* and *mdx*/FS I-I mice at 5 wk (*1*) and 9 wk (*2*) of age. Cell infiltration is prevented in *mdx*/FS I-I mice. *C*) Hematoxylin and eosin staining of sections was prepared from the quadriceps femoris muscles of *mdx* and *mdx*/FS I-I mice at 5 wk (*1*) and 9 wk (*2*) of age. *D*) Quantification of macrophage infiltration by counting Mac1-positive cells in the quadriceps femoris muscles of *mdx* and *mdx*/FS I-I mice. Mac1-positive areas/total surface areas (%) were measured. $*P < 0.01$, Student's *t* test. Representative pictures of Mac1 staining for *mdx* and mdx/FS I-I sre shown in right. *E*) Peak force measurements (*g*) of grip strength for wild-type mice, FS I-I mice, *mdx* mice and *mdx*/FS I-I mice at 9 wk of age. Data are expressed as means ± SD (n=6–8). $*P < 0.001$, Student's *t* test.

dystrophy can be recovered by myostatin inhibition (11, 12, 36). In addition, three models of limb-girdle muscular dystrophy, including δ-sarcoglycan deficiency, caveolin-3 mutation, and calpain-3 deficiency, are also ameliorated by myostatin blockade (37–39). Even the muscle atrophy associated with amyotrophic lateral sclerosis and aging can be ameliorated by myostatin inhibition (40). It is also noteworthy that elimination of myostatin did not combat a laminin-α2-deficient dy^W mouse model but did increase postnatal lethality due to fat loss in one study (13).

There are multiple ways to inhibit myostatin (1, 2). Myostatin propeptide, FS, FLRG, and antimyostatin antibodies bind to myostatin and inhibit its activities. FS is a naturally occurring hormone that shows potent myostatin inhibition (2). It is also an activin-binding protein that causes activin inhibition (2, 16, 20). Myostatin is primarily produced in skeletal muscles and acts specifically on skeletal muscles. In contrast, activins are produced by various organs, including the gonads, pituitary, brain, liver, and gastrointestinal tract, and have pleiotrophic effects on various cell types.

In the present study, we developed a myostatin-specific inhibitor derived from FS, which did not affect the activin bioactivity. This molecule, designated FS I-I, consists of the FS N-terminal domain and two consecutive FS I domains. The precise mechanisms for how FS I-I discriminates between myostatin and activins remain to be determined. Recent crystallographic analyses have revealed that the minimal activin-inhibiting fragment of FS comprises the FS I and FS II domains. Two FS I-II molecules encircle and wrap around the back of the 'wings' of activin, thereby blocking its type II receptor-binding site. In particular, arginine residue 192 in the FS II domain is a key player in this interaction and inserts itself into the activin finger (22, 23). It is also worthwhile to note that an analogous arginine residue is conserved in the COOH-terminal activin-binding region of FLRG (22, 26). Although myostatin and activins are structurally similar among the TGF-β superfamily members, several key residues are different (1). Our findings that FS I-I binds and inhibits myostatin much more effectively than its effects on activin argue that the FS II domain, which is important for

binding to activin, could be dispensable for the interaction with myostatin, although the precise mechanism remains to be determined.

We established transgenic mouse lines stably expressing FS I-I under the control of a skeletal muscle-specific promoter. The FS I-I overexpressing mice showed increased skeletal muscle mass. The mice were fertile and did not show any abnormalities except for increased muscle mass. A lack of reproductive phenotype in FS I-I overexpressor could be due to low-affinity binding of FS I-I to activin *in vivo*. However, since a skeletal muscle-specific promoter was used, it is likely that FS I-I did not express at high levels in tissues other than skeletal muscle where activins were expressed. The individual weights of the tibialis anterior, quadriceps femoris, and extensor digitorum longus muscles were apparently increased. The masses of other skeletal muscles were also increased, whereas the sizes of other internal organs, including the heart, did not differ from those of control mice (data not shown). Both hypertrophy and hyperplasia of the myofibers were found to be responsible for the increased muscle weight.

Similar to our FS I-I transgenic mice, both hypertrophy and hyperplasia are observed in skeletal muscles in myostatin knockout mice (3). The myosin light chain promoter used in the present study is activated weakly during the prenatal period and becomes active in the postnatal period (41). Thus, myostatin is inhibited after the postnatal period in FS I-I transgenic mice, whereas myostatin is missing throughout embryonic development and the postnatal period in myostatin knockout mice. Our findings argue that inhibition of myostatin, even in the postnatal period, could regulate both the number and size of the myofibers. Interestingly, the increased skeletal muscle mass induced by myostatin inhibition can be caused by hyperplasia without hypertrophy (42, 43), hypertrophy without hyperplasia (44, 45) or both hyperplasia and hypertrophy (3, 20, present study). The question of whether these differences are caused by species differences and/or the extents of myostatin inhibition remains to be determined.

The functionality of the skeletal muscles of FS I-I transgenic mice was studied by two independent approaches, namely the rotarod test and grip strength test. Both of these analyses revealed that the skeletal muscles of FS I-I transgenic mice were functional and had increased strength. The possibility that FS I-I could ameliorate the pathophysiology of *mdx* Duchenne muscular dystrophy model mice was studied by crossing FS I-I transgenic mice with *mdx* mice. The myofibers of the skeletal muscles in *mdx*/FS I-I mice became homogeneous and were larger than those in *mdx* mice. Furthermore, the fibrosis and cell infiltration normally observed in the diaphragm and quadriceps femoris muscle of *mdx* mice were significantly ameliorated. Muscle strength was also recovered in *mdx*/FS I-I mice.

In summary, we have developed and characterized a myostatin inhibitor, FS I-I, derived from FS. The myostatin inhibition by FS I-I is capable of promoting muscle regeneration and preventing muscle fibrosis in *mdx* mice without any adverse effects. An antimyostatin antibody is currently under investigation in clinical trials for the treatment of several forms of muscular dystrophy. Thus, similar to other myostatin inhibitors, FS I-I could be a candidate for a lead compound for the development of drugs for muscular dystrophy. Although myostatin inhibition by FS I-I did not restore the dystrophin expression in *mdx* mice, it could offer effective amelioration of the symptoms of muscular dystrophy caused by the primary loss of dystrophin. FJ

We thank Dr. Y.-M. Suzuki for the *mdx* mice, Dr. K. Inokuchi for advice on the rotarod analysis, Dr. S. Yoshida for the 6R-lux plasmid, Dr. Y. Yokota for the MyoD plasmid, and Dr. S-J. Lee for the skeletal muscle-specific promoter construct. This research was supported by a grant-in-aid for Scientific Research on Priority Areas (18052019) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a research grant (17A-10) for nervous and mental disorders; and a research grant (17231401) for research on psychiatric and neurological diseases and mental health from the Ministry of Health, Labour, and Welfare.

REFERENCES

1. Lee, S. J. (2004) Regulation of muscle mass by myostatin. *Annu. Rev. Cell Dev. Biol.* **20,** 61–86
2. Tsuchida, K. (2006) The role of myostatin and bone morphogenetic proteins in muscular disorders. *Expert. Opin. Biol. Ther.* **6,** 147–152
3. McPherron, A. C., Lawler, A. M., and Lee, S. J. (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387,** 83–90
4. McPherron, A. C., and Lee, S. J. (1997) Double muscling in cattle due to mutations in the myostatin gene. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12457–12461
5. Schuelke, M., Wagner, K. R., and Stolz, L. E. (2004) Myostatin mutation associated with gross muscle hypertrophy in a child. *N. Engl. J. Med.* **350,** 2682–2688
6. Clop, A., Marcq, F., Takeda, H., Pirottin, D., Tordoir, X., Bibe, B., Bouix, J., Caiment, F., Elsen, J. M., Eychenne, F., Larzul, C., Laville, E., Meish, F., Milenkovic, D., Tobin, J., Charlier, C., Georges, M. (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat. Genet.* **38,** 813–818
7. Hill, J. J., Davies, M. V., Pearson, A. A., Wang, J. H., Hewick, R. M., Wolfman, N. M., and Qiu, Y. (2003) The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *J. Biol. Chem.* **277,** 40735–40741
8. Whittemore, L. A., Song, K., Li, X., Aghajanian, J., Davies, M., Girgenrath, S., Hill, J. J., Jalenak, M., Kelley, P., Knight, A., Maylor, R., O'Hara, D., Pearson, A., Quazi, A., Ryerson, S., Tan, X. Y., Tomkinson, K. N., Veldman, G. M., Widom, A., Wright, J. F., Wudyka, S., Zhao, L., and Wolfman, N. M. (2003) Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochem. Biophys. Res. Commun.* **300,** 965–971
9. Grobet, L., Pirottin, D., Farnir, F., Poncelet, D., Royo, L. J., Brouwers, B., Christians, E., Desmecht, D., Coignoul, F., Kahn, R., and Georges, M. (2003) Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene. *Genesis* **35,** 227–238
10. Khurana, T. S., and Davies, K. E. (2003) Pharmacological strategies for muscular dystrophy. *Nat. Rev. Drug Discov.* **2,** 379–390
11. Bogdanovich, S., Krag, T. O. B., Barton, E. R., Morris, L. D., Whittemore, L.-A., Ahima, R. S., and Khurana, T. S. (2002)

Functional improvement of dystrophic muscle by myostatin blockade. *Nature* **420,** 418–421
12. Bogdanovich, S., Perkins, K. J., Krag, T. O. B., Whittemore, L.-A., and Khurana, T. S. (2005) Myostatin propeptide-mediated amelioration of dystrophic pathophysiology. *FASEB J.* **19,** 543–549
13. Li, Z. F., Shelton, G. D., and Engvall, E. (2005) Elimination of myostatin does not combat muscular dystrophy in *dy* mice but increases postnatal lethality. *Am. J. Pathol.* **166,** 491–497
14. Amthor, H., Macharia, R., Navarrete, R., Schuelke, M., Brown, S. C., Otto, A., Voit, T., Muntoni, F., Vrbova, G., Partridge, T., Zammit, P., Bunger, L., and Patel, K. (2007) Lack of myostatin results in excessive muscle growth but impaired force generation. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 1835–1840
15. Hill, J. J., Qiu, Y., Hewick, R. M., and Wolfman, N. M. (2003) Regulation of myostatin in vivo by growth and differentiation factor-associated serum protein-1: a novel protein with protease inhibitor and follistatin domains. *Mol. Endocrinol.* **17,** 1144–1154
16. Tsuchida, K. (2004) Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. *Curr. Drug Targets-Immune Endocr. Metabol. Disord.* **4,** 157–166
17. Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugino, H. (1990) Activin-binding protein from rat ovary is follistatin. *Science* **247,** 836–838
18. Sugino, H., and Tsuchida, K. (2000) Activin and follistatin. In *Skeletal Growth Factor,* Lippincott Williams & Wilkins, Philadelphia, p. 251–263
19. Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R., and Bradley, A. (1995) Multiple defects and perinatal death in mice deficient in follistatin. *Nature* **374,** 360–363
20. Lee, S. J., and McPherron, A. C. (2001) Regulation of myostatin activity and muscle growth. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9306–9311
21. Guo, Q., Kumar, T. R., Woodruff, T., Hadsell, L. A., DeMayo, F. J., and Matzuk, M. M. (1998) Overexpression of mouse follistatin causes reproductive defects in transgenic mice. *Mol. Endocrinol.* **12,** 96–106
22. Harrington, A. E., Morris-Triggs, S. A., Ruotolo, B. T., Robinson, C. V., Ohnuma, S., and Hyvonen, M. (2006) Structural basis for the inhibition of activin signalling by follistatin. *EMBO J.* **25,** 1035–1045
23. Thompson, T. B., Lerch, T. F., Cook, R. W., Woodruff, T. K., and Jardetzky, T. S. (2005) The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding. *Dev. Cell* **9,** 535–543
24. Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S. Y., Ling, N., and Guillemin, R. (1988) Primary structure of the human follistatin precursor and its genomic organization. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4218–4222
25. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* **61,** 1303–1313
26. Tsuchida, K., Arai, K. Y., Kuramoto, Y., Yamakawa, N., Hasegawa, Y., and Sugino, H. (2000) Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-beta family. *J. Biol. Chem.* **275,** 40788–40796
27. Yamakawa, N., Tsuchida, K., and Sugino, H. (2002) The rasGAP-binding protein, Dok-1, mediates activin signaling *via* serine/threonine kinase receptors. *EMBO J.* **21,** 1684–1694
28. Hashimoto, O., Kawasaki, N., Tsuchida, K., Shimasaki, S., Hayakawa, T., and Sugino, H. (2000) Difference between follistatin isoforms in the inhibition of activin signalling: activin neutralizing activity of follistatin isoforms is dependent on their affinity for activin. *Cell. Signal.* **12,** 565–571
29. Nakamura, T., Asashima, M., Eto, Y., Takio, K., Uchiyama, H., Moriya, N., Ariizumi, T., Yashiro, T., Sugino, K., Titani, K., and Sugino, H. (1992) Isolation and characterization of native activin B. *J. Biol. Chem.* **267,** 16385–16389
30. Liu, Z. H., Tsuchida, K., Matsuzaki, T., Bao, Y. L., Kurisaki, A., and Sugino, H. (2006) Characterization of isoforms of activin receptor-interacting protein 2 that augment activin signaling. *J. Endocrinol.* **189,** 409–421
31. Liu, D., Black, B. L., and Derynck, R. (2001) TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. *Genes Dev.* **15,** 2950–2966
32. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108,** 193–200
33. Rosenthal, N., Kornhauser, J. M., Donoghue, M., Rosen, K. M., and Merlie, J. P. (1989) Myosin light chain enhancer activates muscle-specific, developmentally regulated gene expression in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7780–7784
34. Rebbapragada, A., Benchabane, H., Wrana, J. L., Celeste, A. J., and Attisano, L. (2003) Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol. Cell. Biol.* **23,** 7230–7242
35. Engvall, E., and Wewer, U. M. (2003) The new frontier in muscular dystrophy research: booster genes. *FASEB J.* **17,** 1579–1584
36. Wagner, K. R., McPherron, A. C., Winik, N., and Allen, R. E. (2002) Loss of myostatin attenuates severity of muscular dystrophy in *mdx* mice. *Ann. Neurol.* **52,** 832–836
37. Parsons, S. A., Millay, D. P., Sargent, M. A., McNally, E. M., and Molkentin, J. D. (2006) Age-dependent effect of myostatin blockade on disease severity in a murine model of limb-girdle muscular dystrophy. *Am. J. Pathol.* **168,** 1975–1985
38. Ohsawa, Y., Hagiwara, H., Nakatani, M., Yasue, A., Moriyama, K., Murakami, T., Tsuchida, K., Noji, S., and Sunada, Y. (2006) Caveolin-3 regulates myostatin signaling: muscular atrophy of caveolin-3-deficient mice is rescued by myostatin inhibition. *J. Clin. Invest.* **116,** 2924–2934
39. Bartoli, M., Poupiot, J., Vulin, A., Fougerousse, F., Arandel, L., Daniele, N., Roudaut, C., Noulet, F., Garcia, L., Danos, O., and Richard, I. (2007) AAV-mediated delivery of a mutated myostatin propeptide ameliorates calpain 3 but not alpha-sarcoglycan deficiency. *Gene Ther.* **14,** 733–740
40. Holzbaur, E. L., Howland, D. S., Weber, N., Wallace, K., She, Y., Kwak, S., Tchistiakova, L. A., Murphy, E., Hinson, J., Karim, R., Tan, X. Y., Kelley, P., McGill, K. C., Williams, G., Hobbs, C., Doherty, P., Zaleska, M. M., Pangalos, M. N., and Walsh, F. S. (2006) Myostatin inhibition slows muscle atrophy in rodent models of amyotrophic lateral sclerosis. *Neurobiol. Dis.* **23,** 697–707
41. Donoghue, M. J., Merlie, J. P., Rosenthal, N., and Sanes, J. R. (1991) Rostrocaudal gradient of transgene expression in adult skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5847–5851
42. Kambadur, R., Sharma, M., Smith, T. P., and Bass, J. J. (1997) Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. *Genome Res.* **7,** 910–916
43. Nishi, M., Yasue, A., Nishimatu, S., Nohno, T., Yamaoka, T., Itakura, M., Moriyama, K., Ohuchi, H., and Noji, S. (2002) A missense mutant myostatin causes hyperplasia without hypertrophy in the mouse muscle. *Biochem. Biophys. Res. Commun.* **293,** 247–251
44. Zhu, X., Hadhazy, M., Wehling, M., Tidball, J. G., and McNally, E. M. (2000) Dominant negative myostatin produces hypertrophy without hyperplasia in muscle. *FEBS Lett.* **474,** 71–75
45. Yang, J., Ratovitski, T., Brady, J. P., Solomon, M. B., Wells, K. D., and Wall, R. J. (2001) Expression of myostatin pro domain results in muscular transgenic mice. *Mol. Reprod. Dev.* **60,** 351–361

Received for publication April 1, 2007.
Accepted for publication August 16, 2007.

Endocrine Journal 2008, 55 (1), 11–21

REVIEW

Signal Transduction Pathway through Activin Receptors as a Therapeutic Target of Musculoskeletal Diseases and Cancer

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Abstract. Activin, myostatin and other members of the TGF-β superfamily signal through a combination of type II and type I receptors, both of which are transmembrane serine/threonine kinases. Activin type II receptors, ActRIIA and ActRIIB, are primary ligand binding receptors for activins, nodal, myostatin and GDF11. ActRIIs also bind a subset of bone morphogenetic proteins (BMPs). Type I receptors that form complexes with ActRIIs are dependent on ligands. In the case of activins and nodal, activin receptor-like kinases 4 and 7 (ALK4 and ALK7) are the authentic type I receptors. Myostatin and GDF11 utilize ALK5, although ALK4 could also be activated by these growth factors. ALK4, 5 and 7 are structurally and functionally similar and activate receptor-regulated Smads for TGF-β, Smad2 and 3. BMPs signal through a combination of three type II receptors, BMPRII, ActRIIA, and ActRIIB and four type I receptors, ALK1, 2, 3, and 6. BMPs activate BMP-specific Smads, Smad1, 5 and 8. Smad proteins undergo multimerization with co-mediator Smad, Smad4, and translocated into the nucleus to regulate the transcription of target genes in cooperation with nuclear cofactors. The signal transduction pathway through activin type II receptors, ActRIIA and ActRIIB, with type I receptors is involved in various human diseases. In this review, we discuss the role of signaling through activin receptors as therapeutic targets of intractable neuromuscular diseases, endocrine disorders and cancers.

Key words: Activin, Myostatin, Activin receptor, Muscular dystrophy, Bone formation, Cancer, Targeted therapy

(*Endocrine Journal* **55**: 11–21, 2008)

I. An overview of activins, myostatin and BMPs that signal through activin receptors

Activins were first discovered as regulators of secretion of follicle-stimulating hormone from the anterior pituitary [1–3]. Various reproductive and non-reproductive roles of activins have been characterized [3, 4]. Activins have potent mesoderm inducing activity in *Xenopus laevis* embryos [5], although nodal is an authentic mesoderm inducer in many species including mammals [6]. Since nodal shares activin receptors with activins, exogenous administration of activins mimics the actions of an endogenous role of nodal (Table 1). Myostatin, like activins, belongs to the transforming growth factor-β (TGF-β) superfamily and has a distinct role in myogenesis [7]. Myostatin is an endogenous negative regulator of muscle growth and plays a major role in determining skeletal muscle mass. Mice with a targeted deletion of the myostatin gene were shown to have hypermuscular phenotypes [8]. Interestingly, inactivating mutations in the myostatin gene have been identified in double-muscling cattle breeds, sheep, dogs and even humans [9–11]. These findings indicate that myostatin works as a negative regulator of skeletal muscle growth and development. Myostatin is also known as growth and differentiation factor 8 (GDF8) and is structurally closest to GDF11, which is involved in development of spinal cord and kidney organogenesis [12, 13].

The TGF-β superfamily is subdivided into several subfamilies; TGF-β subfamily, activin/inhibin subfamily, myostatin/GDF11 subfamily and BMP subfamily. TGF-β, activin, myostatin, nodal activate TGF-β pathway-restricted Smads, Smad2 and 3 in the cytoplasm,

Received: July 6, 2007
Accepted: July 12, 2007
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Table 1. Type II and type I receptors for activins, myostatin and the related TGF-β family

Ligand	Type II Receptors	Type I Receptors
Activins nodal	ActRIIA (ACVR2) & ActRIIB (ACVR2B)	ALK4 (ACVR1B) & ALK7 (ACVR1C)
Myostatin GDF11	ActRIIB (ACVR2B) & ActRIIA (ACVR2)	ALK5 (TGFBR1) & ALK4 (ACVR1B)
BMPs	BMPRII, ActRIIA, ActRIIB	ALK2 (ACVR1) & ALK1, ALK3, ALK6
TGF-βs	TGF-βRII (TGFBR2)	ALK5 (TGFBR1)

Various combinations of type II and type I receptors elicit signaling of activins and related ligands. Activins and nodal signal through a combination of type II receptors, ActRIIA/ActRIIB and type I receptors, ALK4/7. Myostatin and GDF11 signal through combination of type II receptors, ActRIIA/ActRIIB and type I receptors, ALK5/4. BMPs signal through a combination of type II receptors, BMPRII, ActRIIA and ActRIIB and type I receptors, ALK1/2/3/6.

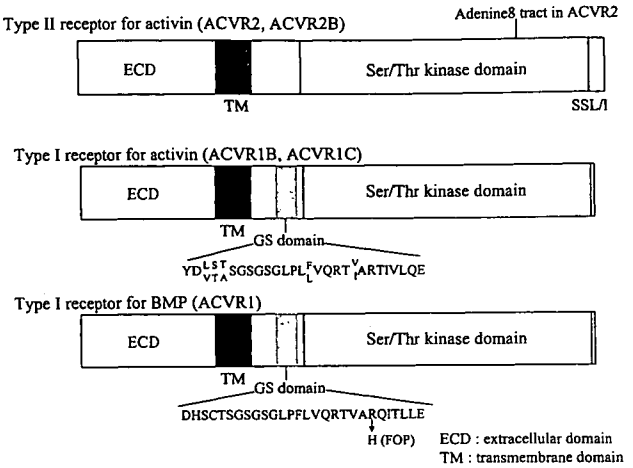

Fig. 1. Schematic structures of activin receptors.
Type II receptors for activin (ACVR2 and ACVR2B) have an extracellular domain, a single transmembrane domain and an intracellular serine/threonine kinase domain. COOH-terminal sequences SSL/I are binding sites for PDZ proteins and are unique to activin type II receptors. In gastrointestinal cancers, polyadenine tracts in ACVR2 were identified as targets for frameshift mutations. Type I receptors have also a similar domain organization. In contrast with the type II receptor, the type I receptor has a GS domain that is located between trasnmembrane domain and kinase domain. The amino acid sequences of the GS domains of type I receptors for activins and BMPs show differences, reflecting the preference of Smad proteins.

whereas BMPs phosphorylate and activate BMP-specific Smad, Smad1, 5 and 8 [14, 15]. Although the intracellular signaling pathway of BMPs is different from that of activins, activin type II receptors (ActRIIA and ActRIIB) are shared and can be activated by BMP6 and BMP7 [16, 17]. This difference is attributed to different type I receptors, which form complexes with ActRIIs by ligand stimulation (Table 1). ALK4/5/7 are structurally similar and activate Smad 2 and 3, receptor-regulated Smads specific for the TGF-β pathway [3, 4, 18, 19], whereas ALK1/2/3/6 activate receptor-regulated Smad specific for the BMP pathway [15] (see next section).

II. Activin Receptors and their Unique Signal Transduction Pathway

Mathews and Vale performed expression cloning studies to identify activin receptors, and successfully isolated a transmembrane serine/threonine kinase receptor in 1991 [20] (Fig. 1). The cloned receptor from AtT20 cells was named as an activin type II receptor, ActRII [20]. ActRII is also known as ActRIIA or ACVR2 [20]. A second type of activin receptor, ActRIIB, also known as ACVR2B, was also identified [21]. ActRIIs are activin receptors; however, they also work as receptors for a subset of BMPs, notably BMP6 and BMP7 [16, 17]. The distinct TGF-β type II receptor (TGF-βRII) and BMP type II receptor (BMPRII) bind and transmit TGF-β and BMP signaling, respectively [14, 15]. Using degenerate DNA primers based on the conserved sequences of ActRII, TGF-βRII and C. elegans Daf-1 gene, a number of receptor serine/threonine kinases have been identified [15, 22, 23] and termed activin receptor-like kinases 1 to 7 (ALK1~7). Subsequent characterization of ALKs revealed that ALK4 (ACVR1B) and ALK7 (ACVR1C) act as type I receptors for activins and nodal [3, 14, 18, 19] (Fig. 1). ALK2 (ACVR1) was termed an activin type I receptor since it could bind activins; however, it is highly likely that ALK2 acts as a type I receptor for BMPs [15]. Myostatin and GDF11 bind ActRIIB and ActRIIA and utilize ALK5 and ALK4 as type I receptors [24–26] (Table 1).

The activin type II receptor has an extracellular ligand binding domain, a single transmembrane domain and an intracellular serine/threonine kinase domain (Fig. 1). Type I receptors also have a similar domain organization. In contrast with the type II receptor, the type I receptor has a GS domain that is

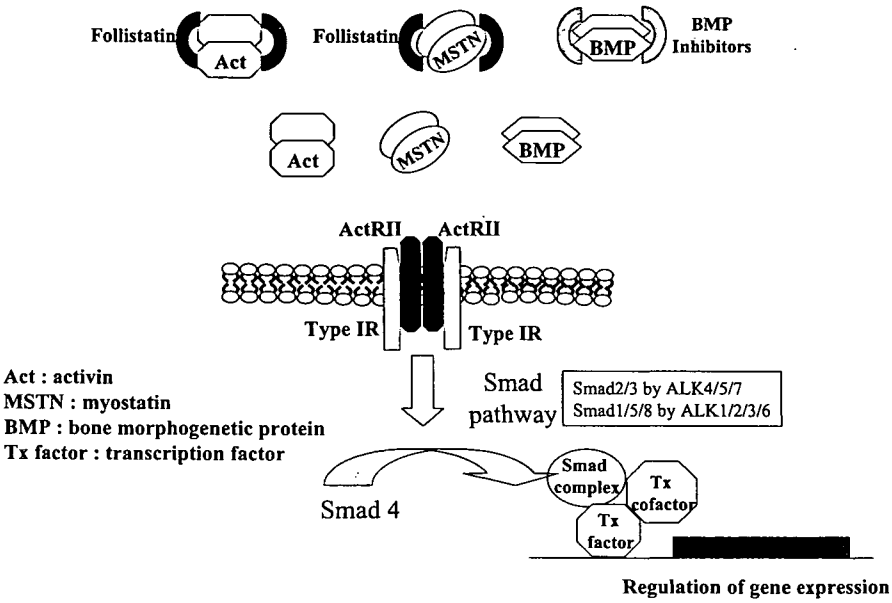

Fig. 2. An overview of signal transduction through activin receptors.
Activin (Act), myostatin (MSTN) and BMP are dimeric ligands. Follistatin binds and neutralizes Act and MSTN, whereas BMP inhibitors, like noggin, bind to and inhibit BMPs. Once Act, MSTN and BMP bind to activin type II receptors, type I receptors are recruited to the complex and GS domains of type I receptors are phosphorylated by ActRIIs. Then, receptor-specific Smads are phosphorylated by activated type I receptors. When ALK4/5/7 is activated by ligand/ActRIIs, TGF-β specific Smads, Smad2/3, are phosphorylated. Whereas, when ALK1/2/3/6 is activated by BMP/ActRIIs, BMP specific Smads, Smad1/5/8 are activated. Activated Smads form a complex with common-Smad4, and are transported to the nucleus to regulate expression of specific genes. Transcription factors and cofactors form complex with Smad and are responsible for the gene expression.

located between transmembrane domain and kinase domain (Fig. 1). The amino acid sequences of GS domains of type I receptors for activins and BMP show distinct differences, reflecting the preference of Smad proteins (Fig. 1). Once ligands bind to activin type II receptors, type I receptors are recruited to the complex and GS domains of type I receptors are phosphorylated by ActRIIs. Then, receptor-specific Smads are phosphorylated by the activated type I receptors. When ALK4/5/7 are activated by ligand/ActRIIs, TGF-β specific Smads, Smad2/3, are phosphorylated (Fig. 2). By contrast, when ALK1/2/3/6 are activated by BMP/ActRIIs, BMP specific Smads, Smad1/5/8 are activated. Activated Smads form a complex with common-Smad4, and are transported to the nucleus to regulate expression of specific genes. Transcription factors and cofactors form complex with Smad and are responsible for the gene expression (Fig. 2).

III. Crystal Structures of Activin Receptors

The crystal structure of the extracellular domain (ECD) of activin type II receptor (ActRII) in complex with ligand, BMP7, has been reported [27]. The ECD of ActRII has a fold similar to that of three finger toxins. Intriguingly, the ligand mediates cooperative receptor assembly without receptor contact [27]. The structure of the activin complex with ActRIIB was also revealed [28, 29]. The activin dimer exhibits an unexpected flexibility dependent on whether or not it contacts with receptors, and the affinity of activin to the two ActRIIBs is greatly affected by spatial localization of ActRIIB in the membrane [28, 29]. The dimeric nature of activin and TGF-β ligands suggests that the ligand/type II receptor/type I receptor form complexes with 1 : 2 : 2 stoichiometry. The knuckle region of activin or BMP-7 dimer has a type II receptor binding site, and the concave-shaped region of the ligands serves as type I receptor binding site [27, 28].

IV. Activin Receptors as Therapeutic Targets of Neuromuscular Diseases, Endocrine Disorders and Cancers

A. Muscular dystrophy and related diseases

Muscular dystrophy is a severe muscle-wasting disorder. Although the genes responsible for various types of muscular dystrophies have been identified, no effective therapies are not available yet. The most common type is Duchenne muscular dystrophy (DMD) in which the dystrophin gene is mutated and defective. The milder Becker muscular dystrophy (BMD), Fukuyama congenital muscular dystrophy [30], myotoninc dystrophy, and multiple types of limb girdle muscular dystrophy (LGMD) are other important muscular dystrophies [31]. Skeletal muscle in dystrophy patients becomes susceptible to tissue inflammation and atrophy. Progressive muscle weakness is also a characteristic feature of the disease. Most of the deficient molecules in muscular dystrophies are components of dystrophin-glycoprotein complexes [32]. Although gene therapy and cell transplantation therapy have greatly advanced, drug therapies to increase muscle size and strength and to reduce inflammation are also needed for patients. Insulin-like growth factor-1 (IGF-1) is a potent growth factor for myoblast growth and proliferation, whereas myostatin negatively controls myoblast growth. Myostatin inhibition is considered to be one of the most hopeful and realistic molecular therapies for muscular dystrophy. Monoclonal antibody-mediated myostatin blockade improved function of dystrophic muscle of *mdx* mice, an animal model of DMD [33]. Studies where myostatin null mice were crossed with *mdx* mice indicated that *mdx* mice lacking myostatin were stronger and more muscular. Furthermore, muscles in *mdx* mice without myostatin showed less fibrosis and fatty remodeling, suggesting improved regeneration process [34]. A number of endogenous peptides including myostatin propeptide and follistatin bind and inhibit myostatin [32, 35]. Myostatin propeptide, stabilized by fusion to IgG-Fc, was injected into *mdx* mice to pharmacologically block myostatin [36]. This strategy is also effective to ameliorate dystrophic pathophysiology [36]. A mouse model of LGMD1C caused by a mutation of caveolin-3 was established and crossed with myostatin propeptide transgenic mice [37, 38]. Muscle atrophy caused by mutant caveolin-3 transgenic mice was dramatically recovered [37]. Myostatin propeptide was used for gene therapy using adeno-associated virus (AAV) vector in LGMD2A caused by mutation in the calpain 3 and LGMD2D caused by mutation in the α-sarcoglycan gene (SGCA) [39]. Interestingly, in calpain 3-deficient mice, muscle mass and force were recovered by myostatin inhibition, whereas in the highly regenerative Sgca-null mice, survival was not improved [39]. In addition, myostatin blockage in early stage in a murine model of δ-sarcoglycan-deficient muscular dystrophy was effective to improve muscle loss, regeneration and reduce fibrosis [40]. Importantly, elimination of myostatin did not combat a laminin-α2-deficient dy^w mouse model but did increase postnatal lethality due to fat loss [41]. Taken together, these studies suggest that although myostatin inhibition can ameliorate several different types of muscular dystrophy, it is important to choose the proper type of dystrophy and disease stages. One report showed that neurogenic muscle atrophy by amyotrophic lateral sclerosis could be slowed by myostatin inhibition [42].

In addition to myostatin antibody and myostatin propeptide, soluble forms of ActRIIB and follistatin-related peptide would effectively block myostatin *in vivo* [24, 43]. The soluble form of ActRIIB has a strong muscle enhancing activity. Only 2 weeks are required for up to 60% increase of muscle mass by soluble form of ActRIIB in mice studies [24]. Since myostatin regulates skeletal muscle mass even in adults [44], myostatin inhibition would ameliorate muscle atrophy by aging (sarcopenia), disuse atrophy and even cachexia [32].

B. Bone formation

BMPs are identified by their ability to induce ectopic bone formation [15]. Activin is also involved in bone remodeling and tooth development. Activin is an essential cofactor for osteoclastgenesis and powerfully synergizes with receptor activator of NF-kappaB ligand (RANKL) for induction of osteoclast-like cells [45, 46]. Activins are also potent inhibitors for mineralization in osteoblast, whereas follistatin, the activin antagonist, increased mineralization [47]. The osteogenic signal by BMPs is transmitted through a combination of BMP type II receptors (BMPRII, ActRIIA and ActRIIB) and BMP type I receptors (ALK1, 2, 3, 6). Fibrodysplasia ossificans progressiva (FOP) is a

disabling genetic disorder that leads to the formation of a second (heterotopic) skeleton. It is the most catastrophic disorder of heterotopic ossification in humans. Recently, a recurrent mutation in the juxtamembrane glycine-serine domain of the ALK2 (ACVR1, ActRIA) was reported in all sporadic and familial cases of classic FOP [48]. The ACVR1 617G>A mutation is recurrent in all the affected FOP patients including Japanese and Taiwanese [49] (Fig. 1). Since the mutated molecule for FOP was identified, gene diagnosis will become possible and a therapeutic strategy could be developed in near future.

C. Cancers

The involvement of TGF-β in the regulation of growth of cancer cells is well characterized [14]. TGF-β has a dual role in tumorigenesis. TGF-β inhibits growth of cancer cells in early phase of tumor formation, and TGF-β regulates metastasis in the later phase of cancer progression. Mutation of TGF-β signaling pathway including TGF-βRII, Smad4 (Dpc4) and Smad2 have been reported in various cancers including colorectal and pancreatic cancers [50–52].

The importance of activins and their receptors in cancer biology is also well recognized. Expression of activin receptors is dramatically altered in cancers [53]. Furthermore, somatic mutations of signal transduction pathways through activin receptors have been characterized in colon and pancreatic cancers [54, 55] (Table 2). In the case of ACVR2, 8-bp polyadenine tracts were identified as targets for frameshift mutations in gastrointestinal cancers with microsatellite instability [54] (Fig. 1). Deletion and frame-shift mutations of ACVR1B were identified in pancreatic cancer xenograft [55]. In addition, several truncated ACVR1B receptor isoforms, that are not functionally active, are exclusively expressed in human pituitary tumors [56].

Inhibin is an antagonist hormone for activins. In mice deficient of the inhibin α gene, sex cord-stromal tumors developed as early as 4 weeks of age both in males and females [57]. The tumors cause an activin-dependent cachexia and wasting syndrome and affects mordality due to the absence of inhibin [58]. Activins secreted from tumors activate ACVR2 in excess and cause apoptosis of hepatocytes [57].

V. Inhibitors of Signaling through Activin Receptors

As discussed above, signaling through activin receptors is a target of multiple diseases (Table 2). Therefore, various strategies are possible to regulate signaling through activin receptors for therapies against various diseases.

A. Dominant negative forms of activin receptors

ActRIIs (ActRIIA and ActRIIB) are primary ligand-binding receptors for activins, myostatin and GDF11. Soluble forms of ActRIIs containing the extracellular domain lacking transmembrane and cytoplasmic ki-

Table 2. Activin receptors and Online Mendelian Inheritance in Man (OMIM)

Receptors	Other names	Gene locus in human	Ligands	Relationship to diseases	OMIM
ACVR2	ActRII ActRIIA	2q22.2-23.3	Activins, BMPs	Gastrointestinal cancers	102581
ACVR2B	ActRIIB	3p22-21.3	Activins, nodal GDF11, myostatin	Left-right axis malformation	+602730
ACVR1	ALK2 ACVRLK2	2q23-24	BMPs	Fibrodysplasia ossificans progressiva (FOP, OMIM135100)	102576
ACVR1B	ALK4 ACVRLK4	12q13	Activins, nodal GDF11, myostatin	Pancreatic carcinoma Pituitary tumor	601300
ACVR1C	ALK7	2q24.1-3	Activin AB, activin B, nodal		608981
TGFBR1	ALK5	9q33-34	TGF-βs GDF11, myostatin	Aortic aneurysm Loeys-Dietz syndrome	190181

Gene locus and relationship to human diseases of the listed activin receptors are available from OMIM database.

nase domains retain ligand-binding activity similar to wildtype receptors. Soluble ActRIIs are useful to block actions of ligands that bind to ActRIIs. To enhance stability *in vivo*, the Fc domain of IgG was fused at the COOH-termini of ActRIIs. As mentioned above, soluble ActRIIB has a strong muscle enhancing activity [24]. When 6 week-old female mice were injected with ActRIIB/Fc, a 39–61% increase of skeletal muscle mass was achieved [24]. It is likely that soluble ActRIIB/Fc may inhibit the activities of various ligands including myostatin, activins, GDF11 and other unidentified ligands. Whether soluble ActRII has a similar activity to soluble ActRIIB remains to be determined. Since activin signaling through ActRII is involved in the development of pancreatic islets, apoptosis of hepatocytes, tubulogenic morphogenesis of vessels and osteogenesis, soluble ActRII would affect digestive organs, endocrine pancreas, vasculogenesis and osteogenesis [45, 46, 59–61].

Interestingly, the pseudo-receptor BMP and activin membrane-bound inhibitor, called BAMBI, was identified [62]. BAMBI shares 53% similarity with BMP type I receptor, ALK3, and lacks an intracellular serine/threonine kinase domain. BAMBI interacts with the intracellular domain of several type I receptors for TGF-β, activin and BMPs, and inhibits the formation of active receptor signaling complex. In this sense, BAMBI serves as an endogenous dominant negative receptor. Interestingly, the expression level of BAMBI was aberrantly elevated in most colorectal and hepatocellular carcinomas by activation of β-catenin signaling [63]. This suggests that β-catenin interferes with TGF-β and activin-mediated growth arrest by inducing the expression of BAMBI, and this may contribute to colorectal and hepatocellular tumorigenesis [63].

B. Ligand binding proteins

Many of the TGF-β superfamily member have distinct ligand-binding proteins. They are important regulators of signaling for the TGF-β superfamily. Follistatin is the prototype of activin-binding protein [64, 65] and is a cysteine-rich single chain protein that exhibits a high affinity activin binding activities. Mice lacking the follistatin gene showed musculoskeletal and cutaneous abnormalities due to excess signaling of activins and other related ligands [66]. In fact, follistatin not only inhibits activins but also myostatin and GDF11 [32, 43].

The crystallographic structures of the follistatin-activin complex were reported [67, 68]. The activin-inhibiting fragment of follistatin comprises two consecutive follistatin domains. They encircle activin, neutralizing the ligand by burying one-third of its residues and receptor binding sites. Both type I and type II receptor binding sites of activin are blocked by follistatin binding to activin, explaining the strong activin inhibiting actions of follistatin [67, 68]. The follistatin-related gene, called FLRG, is a recently characterized follistatin-domain containing protein. Like follistatin, FLRG binds and neutralizes activin, myostatin and a subset of BMPs [69, 70]. Both in human and mouse sera, FLRG proteins were shown to be associated with myostatin [71]. FLRG gene deletion indicates that FLRG is involved in glucose metabolism and fat homeostasis by regulating the action of activin, myostatin and related ligands [72]. Noggin, chordin, and DAN/Cer family are BMP inhibitors that regulate BMP activities [73]. Each BMP inhibitor shows a preferential binding to BMP family members.

C. Cripto

The nodal co-receptor Cripto plays a regulatory role in signaling through activin receptors [6, 74]. Cripto can bind both to nodal and activin receptors and is an essential component of nodal signaling. Interestingly, Cripto also binds and inhibits activin B, and functional blocking of Cripto by monoclonal antibody enhances activin B signaling and suppresses tumor cell growth [74]. Cripto also has TGF-β binding activity and can reduce association of TGF-β with its type I receptor and inhibts TGF-β signaling [75].

D. Chemical kinase inhibitors

SB431542 was the first chemical inhibitor of TGF-β type I receptor kinase to be developed [76]. SB431542 is selective for inhibition of ALK4/5/7. Chemical TGF-β type I receptor inhibitors may offer novel drug options for cancer therapy by preventing cell proliferation, angiogenesis, metastasis and fibrosis [76, 77]. Other chemical TGF-β inhibitors, SB505124, LY364947, A-83-01, Ki26894 have also been subsequently developed [78–81].

TGF-β type I receptor kinase inhibitors inhibit TGF-β, activins, nodal and myostatin that signal

through ALK4/5/7. Since intracellular kinase domains of ALK4/5/7 are structurally very similar, SB431542 and chemical TGF-β type I receptor inhibitors do not discriminate ALK4/5/7 and inhibit all three receptors. Thus, it must be kept in mind that undesirable side effects may occur by inhibiting multiple ligands.

VI. Perspectives

Signaling through activin receptors controls cell growth and differentiation, hormonal homeostasis, and development and regeneration of musculoskeletal system. Understanding the regulation of signaling through activin receptors should shed light on the mechanism of this diverse signal transduction network. Activin, myostatin, GDF11 and nodal signal through receptor combinations of ActRIIs and ALK4/5/7. TGF-β-specific Smads, Smad2/3, act downstream of activin, myostatin, GDF11 and nodal. ActRIIs are shared with BMP6 and BMP7. BMPs signal through a combination of type II receptors, ActRIIs and BMPRII, and type I receptors, ALK1/2/3/6. BMP-specific Smads, Smad1/5/8 are downstream effectors for BMPs. Extracellular ligand binding proteins are important regulators of the TGF-β superfamily. Follistatin and related proteins are potent inhibitors of activins and myostatin, whereas BMP inhibitors control BMP activities throughout development and in adulthood.

To inhibit signaling through activin receptors, various strategies are possible. In section V, dominant negative acrivin receptors, ligand-binding proteins, Cripto and chemical kinase inhibitors are listed as inhibitors of activin receptor signaling, since they are useful therapeutic tools for human diseases. Small interfering RNAs for components of activin receptors may also become promising therapeutic tools to inhibit activin receptor signaling. In addition, functions of signaling through activin receptors are regulated by intracellular receptor binding proteins. Adaptor protein Dok-1 associates both with activin receptors and Smads, and is indispensable for activin-induced apoptosis [82]. Activin type II receptors are unique among serine/threonine kinase receptors in that PDZ protein-binding motifs are found at their COOH-terminus (Fig. 1). Two PDZ proteins, activin receptor interacting proteins 1 and 2 (ARIP1 and 2), were identified by two-hybrid screening system to bind with ActRIIs [83, 84]. ARIP1 is likely to act as a scaffolding molecule for ActRIIs in the submembraneous regions [83]. ARIP2 is involved in endocytosis and trafficking of ActRIIs [84]. FKBP12 that associates with the GS domain for type I receptors might have inhibitory role of receptor activation [85].

Signaling through activin receptors are targets of multiple human diseases including muscular dystrophy, bone formation and cancers. Inhibition of myostatin is one of the plausible options of therapy against muscular dystrophies. Abnormalities of activin receptor signaling are common in cancers of digestive and endocrine organs. Disregulation of the activin signaling pathway has a role in pathogenesis of tumors and regulation of this pathway would have therapeutic potential in cancer.

In summary, the signaling pathway through activin receptors and its involvement in intractable muscular diseases and cancer are highlighted in this review. It is hoped that therapeutic interventions targeted to this signaling pathway through activin receptors would enter clinic trials in near future.

References

1. Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J (1986) Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* 321: 776–779.
2. Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R (1986) Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. *Nature* 321: 779–782.
3. Tsuchida K (2004) Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. *Curr Drug Targets Immune Endocr Metabol Disord* 4: 157–166.
4. Harrison CA, Gray PC, Vale WW, Robertson DM (2005) Antagonists of activin signaling: mechanisms and potential biological applications. *Trends Endocrinol Metab* 16: 73–78.
5. McDowell N, Gurdon JB (1999) Activin as a morphogen in Xenopus mesoderm induction. *Semin Cell Dev Biol* 10: 311–317.

6. Shen MM (2007) Nodal signaling: developmental roles and regulation. *Development* 134: 1023–1034.
7. Lee SJ, McPherron AC (1999) Myostatin and the control of skeletal muscle mass. *Curr Opin Genet Dev* 9: 604–607.
8. McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 387: 83–90.
9. Kambadur R, Sharma M, Smith TP, Bass JJ (1997) Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. *Genome Res* 7: 910–916.
10. Schuelke M, Wagner KR, Stolz LE, Hubner C, Riebel T, Komen W, Braun T, Tobin JF, Lee SJ (2004) Myostatin mutation associated with gross muscle hypertrophy in a child. *N Engl J Med* 350: 2682–2688.
11. Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibe B, Bouix J, Caiment F, Elsen JM, Eychenne F, Larzul C, Laville E, Meish F, Milenkovic D, Tobin J, Charlier C, Georges M (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet* 38: 813–818.
12. Liu JP (2006) The function of growth/differentiation factor 11 (Gdf11) in rostrocaudal patterning of the developing spinal cord. *Development* 133: 2865–2874.
13. Esquela AF, Lee SJ (2003) Regulation of metanephric kidney development by growth/differentiation factor 11. *Dev Biol* 257: 356–370.
14. Massagúe J, Gomis RR (2006) The logic of TGFbeta signaling. *FEBS Lett* 580: 2811–2820.
15. Miyazono K, Maeda S, Imamura T (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev* 16: 251–263.
16. Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T (1999) Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. *J Cell Sci* 112 (Pt 20): 3519–3527.
17. Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin CH, Miyazono K (1995) Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J Cell Biol* 130: 217–226.
18. Reissmann E, Jornvall H, Blokzijl A, Andersson O, Chang C, Minchiotti G, Persico MG, Ibanez CF, Brivanlou AH (2001) The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev* 15: 2010–2022.
19. Tsuchida K, Nakatani M, Yamakawa N, Hashimoto O, Hasegawa Y, Sugino H (2004) Activin isoforms signal through type I receptor serine/threonine kinase ALK7. *Mol Cell Endocrinol* 220: 59–65.
20. Mathews LS, Vale WW (1991) Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell* 65: 973–982.
21. Attisano L, Wrana JL, Cheifetz S, Massagúe J (1992) Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. *Cell* 68: 97–108.
22. ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K (1993) Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. *Oncogene* 8: 2879–2887.
23. Tsuchida K, Sawchenko PE, Nishikawa S, Vale WW (1996) Molecular cloning of a novel type I receptor serine/threonine kinase for the TGF beta superfamily from rat brain. *Mol Cell Neurosci* 7: 467–478.
24. Lee SJ, Reed LA, Davies MV, Girgenrath S, Goad ME, Tomkinson KN, Wright JF, Barker C, Ehrmantraut G, Holmstrom J, Trowell B, Gertz B, Jiang MS, Sebald SM, Matzuk M, Li E, Liang LF, Quattlebaum E, Stotish RL, Wolfman NM (2005) Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proc Natl Acad Sci USA* 102: 18117–18122.
25. Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L (2003) Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol Cell Biol* 23: 7230–7242.
26. Andersson O, Reissmann E, Ibanez CF (2006) Growth differentiation factor 11 signals through the transforming growth factor-beta receptor ALK5 to regionalize the anterior-posterior axis. *EMBO Rep* 7: 831–837.
27. Greenwald J, Groppe J, Gray P, Wiater E, Kwiatkowski W, Vale W, Choe S (2003) The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. *Mol Cell* 11: 605–617.
28. Thompson TB, Woodruff TK, Jardetzky TS (2003) Structures of an ActRIIB:activin A complex reveal a novel binding mode for TGF-beta ligand:receptor interactions. *Embo J* 22: 1555–1566.
29. Greenwald J, Vega ME, Allendorph GP, Fischer WH, Vale W, Choe S (2004) A flexible activin explains the membrane-dependent cooperative assembly of TGF-beta family receptors. *Mol Cell* 15: 485–489.
30. Toda T, Kobayashi K, Kondo-Iida E, Sasaki J, Nakamura Y (2000) The Fukuyama congenital muscular dystrophy story. *Neuromuscul Disord* 10: 153–159.
31. Engvall E, Wewer UM (2003) The new frontier in muscular dystrophy research: booster genes. *Faseb J* 17: 1579–1584.
32. Tsuchida K (2006) The role of myostatin and bone morphogenetic proteins in muscular disorders. *Expert Opin Biol Ther* 6: 147–154.
33. Bogdanovich S, Krag TO, Barton ER, Morris LD,

Whittemore LA, Ahima RS, Khurana TS (2002) Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 420: 418–421.

34. Wagner KR, McPherron AC, Winik N, Lee SJ (2002) Loss of myostatin attenuates severity of muscular dystrophy in mdx mice. *Ann Neurol* 52: 832–836.
35. Lee SJ (2004) Regulation of muscle mass by myostatin. *Annu Rev Cell Dev Biol* 20: 61–86.
36. Bogdanovich S, Perkins KJ, Krag TO, Whittemore LA, Khurana TS (2005) Myostatin propeptide-mediated amelioration of dystrophic pathophysiology. *Faseb J* 19: 543–549.
37. Ohsawa Y, Hagiwara H, Nakatani M, Yasue A, Moriyama K, Murakami T, Tsuchida K, Noji S, Sunada Y (2006) Muscular atrophy of caveolin-3-deficient mice is rescued by myostatin inhibition. *J Clin Invest* 116: 2924–2934.
38. Nishi M, Yasue A, Nishimatu S, Nohno T, Yamaoka T, Itakura M, Moriyama K, Ohuchi H, Noji S (2002) A missense mutant myostatin causes hyperplasia without hypertrophy in the mouse muscle. *Biochem Biophys Res Commun* 293: 247–251.
39. Bartoli M, Poupiot J, Vulin A, Fougerousse F, Arandel L, Daniele N, Roudaut C, Noulet F, Garcia L, Danos O, Richard I (2007) AAV-mediated delivery of a mutated myostatin propeptide ameliorates calpain 3 but not alpha-sarcoglycan deficiency. *Gene Ther* 14: 733–740.
40. Parsons SA, Millay DP, Sargent MA, McNally EM, Molkentin JD (2006) Age-dependent effect of myostatin blockade on disease severity in a murine model of limb-girdle muscular dystrophy. *Am J Pathol* 168: 1975–1985.
41. Li ZF, Shelton GD, Engvall E (2005) Elimination of myostatin does not combat muscular dystrophy in dy mice but increases postnatal lethality. *Am J Pathol* 166: 491–497.
42. Holzbaur EL, Howland DS, Weber N, Wallace K, She Y, Kwak S, Tchistiakova LA, Murphy E, Hinson J, Karim R, Tan XY, Kelley P, McGill KC, Williams G, Hobbs C, Doherty P, Zaleska MM, Pangalos MN, Walsh FS (2006) Myostatin inhibition slows muscle atrophy in rodent models of amyotrophic lateral sclerosis. *Neurobiol Dis* 23: 697–707.
43. Lee SJ, McPherron AC (2001) Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* 98: 9306–9311.
44. Whittemore LA, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P, Knight A, Maylor R, O'Hara D, Pearson A, Quazi A, Ryerson S, Tan XY, Tomkinson KN, Veldman GM, Widom A, Wright JF, Wudyka S, Zhao L, Wolfman NM (2003) Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochem Biophys Res Commun* 300: 965–971.
45. Fuller K, Bayley KE, Chambers TJ (2000) Activin A is an essential cofactor for osteoclast induction. *Biochem Biophys Res Commun* 268: 2–7.
46. Koseki T, Gao Y, Okahashi N, Murase Y, Tsujisawa T, Sato T, Yamato K, Nishihara T (2002) Role of TGF-beta family in osteoclastogenesis induced by RANKL. *Cell Signal* 14: 31–36.
47. Eijken M, Swagemakers S, Koedam M, Steenbergen C, Derkx P, Uitterlinden AG, van der Spek PJ, Visser JA, de Jong FH, Pols HA, van Leeuwen JP (2007) The activin A-follistatin system: potent regulator of human extracellular matrix mineralization. *Faseb J* 21: 2949–2960.
48. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Brown MA, Kaplan FS (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. *Nat Genet* 38: 525–527.
49. Nakajima M, Haga N, Takikawa K, Manabe N, Nishimura G, Ikegawa S (2007) The ACVR1 617G>A mutation is also recurrent in three Japanese patients with fibrodysplasia ossificans progressiva. *J Hum Genet* 52: 473–475.
50. Venkatasubbarao K, Ahmed MM, Swiderski C, Harp C, Lee EY, McGrath P, Mohiuddin M, Strodel W, Freeman JW (1998) Novel mutations in the polyadenine tract of the transforming growth factor beta type II receptor gene are found in a subpopulation of human pancreatic adenocarcinomas. *Genes Chromosomes Cancer* 22: 138–144.
51. Moskaluk CA, Hruban RH, Schutte M, Lietman AS, Smyrk T, Fusaro L, Fusaro R, Lynch J, Yeo CJ, Jackson CE, Lynch HT, Kern SE (1997) Genomic sequencing of DPC4 in the analysis of familial pancreatic carcinoma. *Diagn Mol Pathol* 6: 85–90.
52. Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 271: 350–353.
53. Risbridger GP, Schmitt JF, Robertson DM (2001) Activins and inhibins in endocrine and other tumors. *Endocr Rev* 22: 836–858.
54. Hempen PM, Zhang L, Bansal RK, Iacobuzio-Donahue CA, Murphy KM, Maitra A, Vogelstein B, Whitehead RH, Markowitz SD, Willson JK, Yeo CJ, Hruban RH, Kern SE (2003) Evidence of selection for clones having genetic inactivation of the activin A type II receptor (ACVR2) gene in gastrointestinal cancers. *Cancer Res* 63: 994–999.
55. Su GH, Bansal R, Murphy KM, Montgomery E, Yeo CJ, Hruban RH, Kern SE (2001) ACVR1B (ALK4, activin receptor type 1B) gene mutations in pancreatic carcinoma. *Proc Natl Acad Sci USA* 98: 3254–3257.
56. Alexander JM, Bikkal HA, Zervas NT, Laws ER, Jr., Klibanski A (1996) Tumor-specific expression and al-

ternate splicing of messenger ribonucleic acid encoding activin/transforming growth factor-beta receptors in human pituitary adenomas. *J Clin Endocrinol Metab* 81: 783–790.

57. Coerver KA, Woodruff TK, Finegold MJ, Mather J, Bradley A, Matzuk MM (1996) Activin signaling through activin receptor type II causes the cachexia-like symptoms in inhibin-deficient mice. *Mol Endocrinol* 10: 534–543.
58. Li Q, Karam SM, Coerver KA, Matzuk MM, Gordon JI (1998) Stimulation of activin receptor II signaling pathways inhibits differentiation of multiple gastric epithelial lineages. *Mol Endocrinol* 12: 181–192.
59. Shiozaki S, Tajima T, Zhang YQ, Furukawa M, Nakazato Y, Kojima I (1999) Impaired differentiation of endocrine and exocrine cells of the pancreas in transgenic mouse expressing the truncated type II activin receptor. *Biochim Biophys Acta* 1450: 1–11.
60. Ichikawa T, Zhang YQ, Kogure K, Hasegawa Y, Takagi H, Mori M, Kojima I (2001) Transforming growth factor beta and activin tonically inhibit DNA synthesis in the rat liver. *Hepatology* 34: 918–925.
61. Hayashi Y, Maeshima K, Goto F, Kojima I (2007) Activin a as a critical mediator of capillary formation: interaction with the fibroblast growth factor action. *Endocr J* 54: 311–318.
62. Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massague J, Niehrs C (1999) Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature* 401: 480–485.
63. Sekiya T, Adachi S, Kohu K, Yamada T, Higuchi O, Furukawa Y, Nakamura Y, Nakamura T, Tashiro K, Kuhara S, Ohwada S, Akiyama T (2004) Identification of BMP and activin membrane-bound inhibitor (BAMBI), an inhibitor of transforming growth factor-beta signaling, as a target of the beta-catenin pathway in colorectal tumor cells. *J Biol Chem* 279: 6840–6846.
64. Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H (1990) Activin-binding protein from rat ovary is follistatin. *Science* 247: 836–838.
65. Tsuchida K, Sunada Y, Noji S, Murakami T, Uezumi A, Nakatani M (2006) Inhibitors of the TGF-beta superfamily and their clinical applications. *Mini Rev Med Chem* 6: 1255–1261.
66. Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A (1995) Multiple defects and perinatal death in mice deficient in follistatin. *Nature* 374: 360–363.
67. Thompson TB, Lerch TF, Cook RW, Woodruff TK, Jardetzky TS (2005) The structure of the follistatin: activin complex reveals antagonism of both type I and type II receptor binding. *Dev Cell* 9: 535–543.
68. Harrington AE, Morris-Triggs SA, Ruotolo BT, Robinson CV, Ohnuma S, Hyvonen M (2006) Structural basis for the inhibition of activin signalling by follistatin. *Embo J* 25: 1035–1045.
69. Hayette S, Gadoux M, Martel S, Bertrand S, Tigaud I, Magaud JP, Rimokh R (1998) FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders. *Oncogene* 16: 2949–2954.
70. Tsuchida K, Arai KY, Kuramoto Y, Yamakawa N, Hasegawa Y, Sugino H (2000) Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-beta family. *J Biol Chem* 275: 40788–40796.
71. Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y (2002) The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *J Biol Chem* 277: 40735–40741.
72. Mukherjee A, Sidis Y, Mahan A, Raher MJ, Xia Y, Rosen ED, Bloch KD, Thomas MK, Schneyer AL (2007) FSTL3 deletion reveals roles for TGF-beta family ligands in glucose and fat homeostasis in adults. *Proc Natl Acad Sci USA* 104: 1348–1353.
73. Gazzerro E, Canalis E (2006) Bone morphogenetic proteins and their antagonists. *Rev Endocr Metab Disord* 7: 51–65.
74. Adkins HB, Bianco C, Schiffer SG, Rayhorn P, Zafari M, Cheung AE, Orozco O, Olson D, De Luca A, Chen LL, Miatkowski K, Benjamin C, Normanno N, Williams KP, Jarpe M, LePage D, Salomon D, Sanicola M (2003) Antibody blockade of the Cripto CFC domain suppresses tumor cell growth *in vivo*. *J Clin Invest* 112: 575–587.
75. Gray PC, Shani G, Aung K, Kelber J, Vale W (2006) Cripto binds transforming growth factor beta (TGF-beta) and inhibits TGF-beta signaling. *Mol Cell Biol* 26: 9268–9278.
76. Laping NJ, Grygielko E, Mathur A, Butter S, Bomberger J, Tweed C, Martin W, Fornwald J, Lehr R, Harling J, Gaster L, Callahan JF, Olson BA (2002) Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. *Mol Pharmacol* 62: 58–64.
77. Halder SK, Beauchamp RD, Datta PK (2005) A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* 7: 509–521.
78. DaCosta Byfield S, Major C, Laping NJ, Roberts AB (2004) SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 65: 744–752.
79. Peng SB, Yan L, Xia X, Watkins SA, Brooks HB, Beight D, Herron DK, Jones ML, Lampe JW, McMillen WT, Mort N, Sawyer JS, Yingling JM (2005) Kinetic characterization of novel pyrazole TGF-beta receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition. *Biochemistry* 44: 2293–2304.