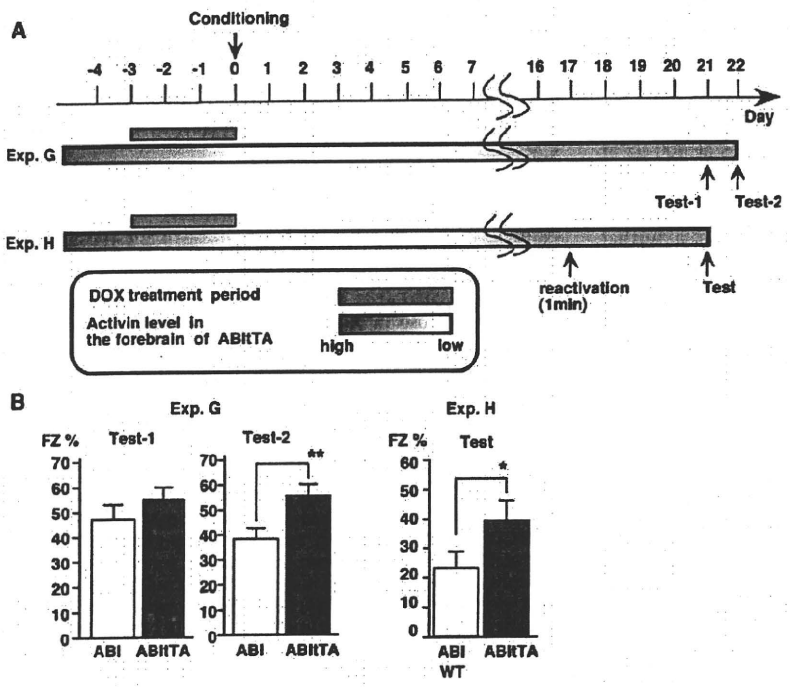


**Figure 5.** Fear memory is influenced by activin overexpression during the retrieval phase. (*A*) Experimental schedule. Horizontal axis indicates the time line. Mice were fed DOX for 3 consecutive days before conditioning (from noon of day −3 to noon of day 0, orange bar). The density of the red color indicates the predicted level of activin in the forebrain. Conditioning was performed in the afternoon of day 0. (*B*) Freezing response during test period. (FZ%) Average freezing percentage during the first 3 min in the 6-min test period. The numbers of mice used were: Experiment G (ABItTA, $n = 15$; ABI, $n = 16$); Experiment H (ABItTA, $n = 9$; ABI, $n = 5$; wild-type littermates, $n = 4$). (*) $P = 0.05$, (**) $P = 0.001$, statistically significant difference between ABItTA and ABI mice (Experiment G) or mixed genotypes ABI and wild group (Experiment H), as determined by one-way ANOVA followed by Fisher's LSD test. Error bars indicate SEM. (WT) Wild-type littermates. Experiment G: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,23)} = 4.18$, $P = 0.0526$; Test effect, $F_{(1,23)} = 0.70$, $P = 0.410$; genotype × Test, $F_{(1,23)} = 1.63$, $P = 0.214$.

addition, activin tunes GABAergic neurotransmission (Zheng et al. 2008), which affects learning and memory (Collinson et al. 2002). All of these activin functions may contribute to the prolonged synaptic plasticity that may underlie LTM formation.

Inhibition of the activin signal during retrieval resulted in a significant suppression of subsequent expression of fear memory (Fig. 4, Experiment D, Test-2). Memory retrieval triggers two opposing processes, reconsolidation or extinction (Tronson and Taylor 2007; Quirk and Mueller 2008). Whether reconsolidation or extinction follows memory retrieval depends on the experimental conditions, such as training and retrieval conditions (Suzuki et al. 2004; Tronson and Taylor 2007). When reconsolidation dominates following memory retrieval, amnesic reagents reduce subsequent memory expression (Nader et al. 2000; Tronson and Taylor 2007). We injected the protein synthesis inhibitor anisomycin 30 min after reactivation (retrieval) into wild-type mice that had been subjected to the same experimental paradigm as in Experiment E of Figure 4. We observed a reduction in the fear response in Test (Supplemental Fig. S6). This suggests that the experimental condition used in Experiment E triggers reconsolidation. In addition, we observed that there was no significant extinction effect in control mice in Experiment D ($P = 0.7839$, one-way analysis of variance [ANOVA] with Fisher's test). On the other hand, in Experiment H in control mice, the freezing level was significantly lower than that of Test-1 in Experiment G ($P = 0.008$, one-way ANOVA with Fisher's test). As the experimental condition used in Experiment H may trigger extinction by the short-time reactivation, control mice showed a lower level of freezing in Experiment H when compared with Test-1 in Experiment G. Unfortunately, we could not record the freezing level during the reactivation phase in Experiment H because of the short period of time (reactivation took a total of 1 min, which included transportation time from the home cage to the test chamber located in a soundproof room [15 sec], exposure time to the chamber [30 sec], and another transportation time from the chamber to the home cage [15 sec]). Taken together, the suppression of freezing observed in Experiments D and E could be caused by inhibition of reconsolidation. On the other hand, the constant level of freezing in ABItTA mice observed in Experiments G and H could be attributed to the inhibition of extinction. In any case, the level of activin in the brain during the retrieval phase plays a key role in the maintenance of LTM.

Recent studies show that fear memory can be weakened by inhibiting CREB (Kida et al. 2002), *zif*268 (Lee et al. 2004), C/EBPβ (Tronel et al. 2005), ERK (Kelly et al. 2003), or PKA (Tronson et al. 2006) during memory retrieval. The present study shows that activin inhibition during memory retrieval suppresses previously consolidated fear memories. Thus, activin signaling could be a promising target for treatment of disorders that are based upon strong traumatic memories, such as post traumatic stress disorder and phobias.

## Materials and Methods

### Animals

Male Wistar rats (5–6 mo old) were used for the LTP experiments. All procedures involving the use of animals complied with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee of the Mitsubishi Kagaku Institute of Life Sciences.

All behavior experiments were conducted in a blind fashion on male mice. Two weeks before behavioral analysis, animals were housed individually in plastic cages and maintained on a 12:12 h light:dark cycle. Food and water were provided ad libitum. The mice were handled daily for 7 d before behavioral analysis.

### Dentate gyrus LTP in vivo

Dentate gyrus LTP experiments with urethane-anesthetized rats were carried out as described previously (Ikegami et al. 1996; Inokuchi et al. 1996; Ikegami and Inokuchi 2000; Fukazawa et al. 2003) with the following modifications: To avoid subjecting the animals to unnecessary pain, the wound margins were locally infiltrated and anesthetized with 2% xylocaine. The monopolar recording electrode was then inserted into the hilus of the dentate gyrus (4.0 mm posterior, 2.8 mm lateral, 3.0 mm ventral to the dura). The bipolar stimulating electrode was positioned ipsilaterally to the medial perforant pathway (8.0 mm posterior, 4.0 mm lateral, 3.0 mm ventral to the dura). The stimulus intensities

were set at the current that evoked 50% of the maximum population spike amplitude and were kept constant throughout the experiment. Test stimuli were delivered at 30-sec intervals to record the fEPSP. After recording a stable basal transmission for 30 min, an HFS was delivered and the fEPSPs were recorded for 24 h. In the follistatin infusion experiments, a strong HFS was used, which consisted of five trains of 400 pulses at 400 Hz at 2-min intervals. In the activin infusion experiments, we used a weak HFS, which consisted of 50 pulses at 100 Hz. In most experiments, an HFS (100 pulses at 100 Hz) was given after the 24-h recording period and responses were recorded for a further 60 min. The initial EPSP slopes are shown as a percentage of the mean value obtained in the 15 min immediately prior to delivery of the HFS. To determine whether the magnitude of LTP differed among the groups, responses from the last 15 min block of recording for each 60 min period were compared statistically. The data are expressed as means ± standard error of the mean (SEM) and were analyzed by one-way or two-way ANOVA followed by Fisher's least significant difference (LSD) test.

## Drug infusions

Follistatin and activin were prepared from bovine follicular fluid as described previously (Nakamura et al. 1992). Follistatin and activin were dissolved in 50 mM phosphate-buffered saline (PBS, pH 7.4) containing 0.125% bovine serum albumin (BSA). Anisomycin (Sigma-Aldrich) was dissolved in equimolar HCl, diluted with saline, and adjusted to pH 7.4 with NaOH.

In the experiments shown in Figure 1, activin, follistatin, anti-activin A, or the vehicle solution (PBS containing 0.125% BSA) were infused 45 min prior to the first HFS delivery. Anisomycin was infused 60 min prior to the first HFS delivery. The drugs were infused into the left lateral ventricle (1.0 mm posterior to the bregma, 1.8 mm lateral, 3.5 mm ventral to the dura) ipsilateral to the recording site with a 27-gauge microsyringe. The drug solution (total volume of 2.0 μL) was infused at a rate of 0.5 μL/min and the microsyringe was left in place for 5 min after the injection.

In the experiments shown in Figure 2, activin, follistatin, or vehicle solution (PBS containing 0.125% BSA) were injected at 1 or 3 h after the first HFS delivery using a cannula device (brain infusion kit, Alzet) that had been implanted into the lateral ventricle and connected to a microsyringe. The drug solution (total volume of 2.0 μL) was infused at a rate of 0.5 μL/min.

## Transgenic mice

To generate the transgene vectors, we used the pBI-G plasmid (Clontech), which has a multiple cloning site, TRE promoter, a LacZ gene, and β-globin poly A and SV40 poly A sequences (Fig. 2A). We introduced a *XhoI-FseI-PacI-HindIII-AscI* site to the *NotI* site of pBI-G and refer to this plasmid as pBI-G2. Coding sequences for *activin* and *follistatin* were isolated by *AscI-XhoI* digestion from the pCaM-activin-Myc and pCaM-follistatin-Myc plasmids (Ageta et al. 2008), respectively, and inserted into the *AscI-XhoI*-digested pBI-G2 to generate the pBI-G2-activin-Myc and pBI-G2-follistatin-Myc plasmids. *AseI* fragments were isolated from pBI-G2-activin-Myc or pBI-G2-follistatin-Myc and microinjected into the pronuclei of one-cell embryos of C57BL/6J mice to produce transgenic mice (Hogan et al. 1994). Microinjected embryos were transferred to the oviducts of pseudopregnant females. We purchased tTA mice (B6; CBA-TgN [CamK2tTA]-1Mmay) from the Jackson Laboratory (Maine), and these mice were backcrossed for six generations to the C57BL/6J background mice before crossing with FBI mice. Littermate single transgenic mice, FBI and ABI, were used as controls against FBItTA and ABItTA mice, respectively. Male mice 70–100 d old were used for behavioral analysis. Mice were fed DOX (Sigma, D-9891, 6 mg/g food) for 3 consecutive days where indicated. The founder mice and offspring were identified by Southern blot analysis using the LacZ gene as a probe and PCR analysis using two independent transgene-specific primer pairs. Forward (f) and reverse (r) PCR primers for genotyping were as follows: tTA, f-5′-TGCCGCCA TTATTACGACAA-3′ and r-5′-TCCTCGCCGTCTAAGTGGAG-3′, f-5′-TTGCGTATTGGAAGATCAAG-3′ and r-5′-GATGGTAGACCC GTAATTGT-3′; FBI, f-5′-TATTCGCGTAAGGAAATCCA-3′ and r-5′-GCCGAGTTTGTCAGAAGCA-3′, f-5′-TCCTTGCTCAGTTCG GTCTT-3′ and r-5′-CCCCACTTTGCGTTTCTTCT-3′; ABI, f-5′-TA TTCGCGTAAGGAAATCCA-3′ and r-5′-GCCGAGTTTGTCAGAA AGCA-3′, f-5′-ATCTCCACATACCCGTTCTC-3′ and r-5′-CTCCC CACTTTGCGTTTCTT-3′.

## X-gal staining

Animals were sacrificed using an overdose of anesthesia, and the brains were dissected and immediately frozen on dry ice. Cryostat sections (20-μm thick) were cut and mounted onto polylysine-coated glass slides. Sections were air-dried and stored at 80°C until use in X-gal staining. X-gal staining was carried out as described (Takeuchi et al. 1995).

## CA1 LTP in slice preparation

Transversely cut hippocampal slices (300 μm) were prepared from 6- to 10-wk-old transgenic FBI and FBItTA mice, and were immersed in ice-cold artificial cerebrospinal fluid (ACSF). ACSF was saturated with 95% $O_2$/5% $CO_2$, and contained: 124 mM NaCl, 3 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM $NaH_2PO_4$, and 10 mM D-glucose. Slices were maintained in ACSF at room temperature for at least 1 h before recording. They were then transferred to a submersion chamber and perfused continuously (2 mL/min) with ACSF at 30°C. Schaffer collaterals were stimulated with 0.1-msec pulses using bipolar tungsten electrodes. fEPSPs were recorded extracellularly in the stratum radiatum of the CA1 region using glass microelectrodes filled with ACSF (tip resistance ca. 3–6 MΩ). Stimulus intensities were set to evoke 30%–50% of the maximal fEPSP slope. LTP was induced after recording a stable 15- to 30-min baseline fEPSP. The test pulse frequency was 0.05 Hz. For some of the experiments, two stimulating electrodes were positioned in the stratum radiatum layer to activate two independent sets of Schaffer collaterals. Pathway independence was assessed by applying two pulses with 100-msec interpulse intervals and confirming the absence of PPF between the pathways (collision test). After baseline stimulation the pathway stimulated at 0.05 Hz received one or three trains of tetanic stimulation at a frequency of 100 Hz for 1 sec at 20-sec intervals. The second pathways were stimulated at 0.017 Hz and served as a control.

Electrophysiological data were collected from two strains of transgenic mice on alternate days using PowerLab (ADInstruments) and three EPC series amplifiers (HEKA Electronik). Data were low-pass filtered (1 kHz) and sampled at 10 kHz. As a measure of synaptic strength, the initial slope of the evoked fEPSPs was calculated and expressed as percent change from the baseline mean. Error bars denote SEM values. All data were used for analysis unless the pipette resistance deviated more than 30% from baseline values or the stimulus artifact, or the shape of fiber volleys changed significantly. To test for group differences between LTP values across conditions, a Student's *t*-test was performed.

## Contextual fear conditioning

After the risk-taking behavior test and light/dark test, we performed the contextual fear conditioning test on each experimental group. For the experiments in Figure 4, 2 min after putting the mice into a chamber, mice received electrical footshocks three times (0.5 mA, 1 sec, intershock interval of 1 min). After the shock they remained in the chamber for an additional 2 min. For experiments in Figure 5, 2 min after putting the mice into a chamber, mice received electrical footshocks four times (0.2 mA, 0.5 sec, intershock interval of 3 sec). After the shock they remained in the chamber for an additional 4 min. To monitor the freezing response in the test, mice were again put into the same chamber for 6 min, except for Test-1 of experiment A (duration, 1 min) in Figure 4. Freezing behavior, which is defined as no movement during consecutive 2-sec intervals, was analyzed by an automated imaging system (Muromachi Kagaku). In Experiments E and H, reactivation took a total of 1 min, which included transportation

time from the home cage to the test chamber located in a soundproof room (15 sec), exposure time to the chamber (30 sec), and another transportation time from the chamber to the home cage (15 sec).

### Sensitivity to electrical stimulation

After the contextual fear-conditioning test, we measured the sensitivity of mice to footshock (Supplemental Fig. S4C). In this test, each mouse is placed in a conditioning chamber and receives 1-sec shocks of increasing intensity (Inoue et al. 2009). The interval between shocks is 10 sec. The sequence of the current used was as follows: 0.05 mA, 0.08 mA, 0.1 mA, 0.2 mA, 0.3 mA, 0.4 mA, 0.5 mA, 0.6 mA, and 0.8 mA. The minimal level of current required to elicit the stereotypical responses of running, vocalization, and jumping was determined.

### Miscellaneous methods

ELISA and analysis of neurogenesis were carried out essentially as described previously (Ageta et al. 2008). Behavioral analyses, including the risk-taking behavior and light/dark tests, were carried out as described (Ageta et al. 2008).

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# Regulation of Muscle Mass by Follistatin and Activins

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**Myostatin is a TGF-β family member that normally acts to limit skeletal muscle mass. Follistatin is a myostatin-binding protein that can inhibit myostatin activity *in vitro* and promote muscle growth *in vivo*. Mice homozygous for a mutation in the *Fst* gene have been shown to die immediately after birth but have a reduced amount of muscle tissue, consistent with a role for follistatin in regulating myogenesis. Here, we show that *Fst* mutant mice exhibit haploinsufficiency, with muscles of *Fst* heterozygotes having significantly reduced size, a shift toward more oxidative fiber types, an impairment of muscle remodeling in response to cardiotoxin-induced injury, and a reduction in tetanic force production yet a maintenance of specific force. We show that the effect of heterozygous loss of *Fst* is at least partially retained in a *Mstn*-null background, implying that follistatin normally acts to inhibit other TGF-β family members in addition to myostatin to regulate muscle size. Finally, we present genetic evidence suggesting that activin A may be one of the ligands that is regulated by follistatin and that functions with myostatin to limit muscle mass. These findings potentially have important implications with respect to the development of therapeutics targeting this signaling pathway to preserve muscle mass and prevent muscle atrophy in a variety of inherited and acquired forms of muscle degeneration. (*Molecular Endocrinology* 24: 1998–2008, 2010)**

Myostatin is a TGF-β family member that acts as a negative regulator of skeletal muscle mass (1). *Mstn* mRNA is first detectable in midgestation embryos in cells of the myotome compartment of developing somites and continues to be expressed in muscle throughout embryogenesis as well as in adult mice. Mice homozygous for a deletion of the *Mstn* gene exhibit dramatic and widespread increases in skeletal muscle mass, with individual muscles of *Mstn*-knockout mice weighing about twice as much as those of *wild-type* mice as a result of a combination of increased fiber number and muscle fiber hypertrophy. These findings suggested that myostatin plays two distinct roles to regulate muscle mass, one to regulate the number of muscle fibers that are formed during development and a second to regulate growth of those fibers. In this respect, selective postnatal loss of myostatin signaling as a result of either deletion of the *Mstn* gene (2, 3) or pharmacological inhibition of myostatin activity (4–7) can cause significant muscle fiber hypertrophy, demonstrating that myostatin plays an important role in regulating muscle homeostasis in adult mice. Moreover, genetic studies in cattle (8–11), sheep (12), dogs (13), and humans (14) have all shown that the function of myostatin as a negative regulator of muscle mass is highly conserved across species.

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Abbreviations: EDL, Extensor digitorum longus; FSTL-3, follistatin-like 3; GDF, growth differentiation factor; MHC, myosin heavy chain.

The identification of myostatin and its biological function has raised the possibility that inhibition of myostatin activity may be an effective strategy for increasing muscle mass and strength in patients with inherited and acquired clinical conditions associated with debilitating muscle loss (for reviews, see Refs. 15–17). Indeed, studies employing mouse models of muscle diseases have suggested that loss of myostatin signaling has beneficial effects in a wide range of disease settings, including muscular dystrophy, spinal muscular atrophy, cachexia, steroid-induced myopathy, and age-related sarcopenia. Moreover, loss of myostatin signaling has been shown to decrease fat accumulation and improve glucose metabolism in models of metabolic diseases, raising the possibility that targeting myostatin may also have applications for diseases such as obesity and type II diabetes. As a result, there has been an extensive effort directed at understanding the mechanisms by which myostatin activity is normally regulated and on identifying the components of the myostatin-signaling pathway with the long-term goal of developing the most effective therapeutic strategies for targeting its actions.

In this regard, considerable progress has been made in terms of understanding how myostatin activity is regulated extracellularly by binding proteins (for review, see Ref. 15). One of these regulatory proteins is follistatin (FST), which is capable of acting as a potent myostatin antagonist. Follistatin has been shown to be capable of binding directly to myostatin and inhibiting its activity in receptor binding and reporter gene assays *in vitro* (18–20). Moreover, follistatin also appears to be capable of blocking endogenous myostatin activity *in vivo*, as transgenic mice overexpressing follistatin specifically in skeletal muscle have been shown to exhibit dramatic increases in muscle growth comparable to those seen in *Mstn*-knockout mice (18, 21, 22). Finally, mice homozygous for a targeted mutation in the *Fst* gene have reduced muscle mass at birth (23), consistent with a role for follistatin in inhibiting myostatin activity during embryonic development. The fact that $Fst^{-/-}$ mice die immediately after birth, however, has hampered a more detailed analysis of the role of follistatin in regulating muscle homeostasis. Here, we show that *Fst* mutant mice exhibit haploinsufficiency, with $Fst^{+/-}$ mice having significant reductions in muscle mass accompanied by corresponding decreases in muscle function and impaired muscle regeneration. Furthermore, we show that this muscle phenotype reflects a normal role for follistatin in regulating not only myostatin but also other TGF-$\beta$ family members that cooperate with mysotatin to limit muscle growth, and we present genetic evidence that activin A may be one of these key cooperating ligands.

## Results

Because mice homozygous for a deletion of *Fst* gene die immediately after birth (23) and because many components of the myostatin-regulatory system have shown dose-dependent effects when manipulated *in vivo*, we investigated the possibility that *Fst* mutant mice might exhibit haploinsufficiency with respect to muscle growth and function. We backcrossed the *Fst* loss-of-function mutation at least 10 times onto a C57BL/6 background and then analyzed muscle weights in $Fst^{+/-}$ mice at 10 wk of age. As shown in Table 1 and Fig. 1 (*bottom panel*), $Fst^{+/-}$ mice exhibited a clear muscle phenotype, with muscle weights in $Fst^{+/-}$ mice being lower by about 15–20% compared with those of wild-type mice. These reductions in muscle weights were highly statistically significant ($P$ values ranged from $10^{-8}$ to $10^{-12}$), were seen in all four muscles that were analyzed (pectoralis, triceps, quadriceps, and gastrocnemius) as well as in both males and females, and were also apparent after normalizing for total body weights (Supplemental Table 1 and Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

These effects on muscle mass were the converse of what has been observed in mice with mutations in the *Mstn* gene and were therefore consistent with a normal role for follistatin in inhibiting myostatin activity *in vivo*. We showed previously that the higher muscle mass seen in $Mstn^{-/-}$ mice results from effects on both fiber numbers and fiber sizes (1). To determine whether both fiber numbers and fiber sizes are also affected by the *Fst* mutation, we carried out morphometric analysis of sections of the gastrocnemius muscle. As shown in Table 2, total fiber number in the gastrocnemius appeared to be unaffected in $Fst^{+/-}$ mice compared with *wild-type* controls. One difference clearly evident in hematoxylin and eosin-stained sections, however, was the increased proportion of smaller, more darkly stained fibers in muscles of $Fst^{+/-}$ mice (Fig. 2A), raising the possibility that heterozygous loss of *Fst* might affect fiber type distribution. In this respect, previous studies have shown that loss of myostatin affects the relative proportions of the different fiber types, with $Mstn^{-/-}$ mice having a decreased number of type I fibers and an increased number of type II fibers in the soleus as well as a shift in the distribution of type II fibers toward more of the glycolytic type IIb fibers in the extensor digitorum longus (EDL) (24–27). Fiber type analysis of the gastrocnemius muscle of $Fst^{+/-}$ mice revealed an opposite shift toward more oxidative fibers. In particular, the number of oxidative type I fibers was increased significantly in the gastrocnemius muscle of $Fst^{+/-}$ mice (Table 2), and most of the small darkly

**TABLE 1.** Muscle weights of mutant mice

| | | Body weight (g) | Muscle weights (mg) | | | |
|---|---|---|---|---|---|---|
| | | | Pectoralis | Triceps | Quadriceps | Gastrocnemius |
| Males | | | | | | |
| *Wild type* | (n=36) | 24.0 ± 0.3 | 74.5 ± 1.0 | 94.8 ± 1.2 | 193.3 ± 2.5 | 137.5 ± 1.7 |
| $Fst^{+/-}$ | (n=15) | 23.3 ± 0.7 | 60.7 ± 1.0[a] | 78.3 ± 1.4[a] | 157.4 ± 3.0[a] | 118.2 ± 2.0[a] |
| $Fstl3^{-/-}$ | (n=23) | 25.3 ± 0.6 | 77.2 ± 1.7 | 97.9 ± 1.6 | 200.7 ± 3.8 | 138.2 ± 2.1 |
| $Mstn^{+/-}$ | (n=17) | 27.6 ± 0.4 | 97.4 ± 1.6 | 123.2 ± 2.4 | 252.6 ± 4.5 | 181.2 ± 3.9 |
| $Mstn^{+/-}$, $Fst^{+/-}$ | (n=12) | 26.0 ± 0.4[b] | 79.3 ± 1.5[c] | 100.0 ± 2.0[c] | 197.8 ± 5.3[c] | 147.4 ± 3.2[c] |
| $Mstn^{-/-}$ | (n= 8) | 33.8 ± 0.9 | 215.1 ± 7.9 | 231.8 ± 7.3 | 407.1 ± 12.1 | 302.0 ± 10.3 |
| $Mstn^{-/-}$, $Fst^{+/-}$ | (n=10) | 31.0 ± 1.3 | 174.3 ± 10.0[d] | 206.7 ± 10.4 | 352.6 ± 17.3[e] | 274.4 ± 13.4 |
| $inh\beta A^{+/-}$ | (n=21) | 25.0 ± 0.4[f] | 80.8 ± 1.2[a] | 104.0 ± 1.6[a] | 215.0 ± 3.8[a] | 152.3 ± 2.2[a] |
| $inh\beta B^{+/-}$ | (n=13) | 24.3 ± 0.5 | 73.5 ± 1.7 | 95.8 ± 2.5 | 190.1 ± 3.9 | 134.6 ± 2.2 |
| $inh\beta B^{-/-}$ | (n=15) | 24.7 ± 0.4 | 78.9 ± 1.7[f] | 100.4 ± 2.5[f] | 201.3 ± 5.0 | 139.1 ± 2.9 |
| $inh\beta C^{+/-}$, $\beta E^{+/-}$ | (n=12) | 24.8 ± 1.2 | 76.2 ± 3.3 | 95.8 ± 2.6 | 192.2 ± 8.3 | 133.9 ± 5.7 |
| $inh\beta C^{-/-}$, $\beta E^{-/-}$ | (n=18) | 25.0 ± 0.4[f] | 76.9 ± 1.3 | 96.8 ± 1.9 | 196.9 ± 3.3 | 138.6 ± 2.0 |
| Females | | | | | | |
| *Wild type* | (n=22) | 19.0 ± 0.2 | 48.3 ± 0.8 | 69.5 ± 0.9 | 146.2 ± 1.8 | 102.5 ± 1.2 |
| $Fst^{+/-}$ | (n=14) | 17.2 ± 0.4[a] | 39.1 ± 0.9[a] | 56.6 ± 1.0[a] | 115.2 ± 2.0[a] | 84.7 ± 1.7[a] |
| $Fstl3^{-/-}$ | (n=29) | 19.1 ± 0.3 | 50.6 ± 0.8[f] | 72.5 ± 0.9[f] | 149.4 ± 1.8 | 100.7 ± 1.1 |
| $Fst^{+/-}$, $Fstl3^{+/-}$ | (n= 9) | 16.3 ± 0.5[a] | 37.3 ± 1.6[a] | 57.0 ± 2.2[a] | 113.9 ± 4.6[a] | 82.9 ± 2.8[a] |
| $Fst^{+/-}$, $Fstl3^{-/-}$ | (n= 7) | 16.9 ± 0.6[g] | 36.9 ± 1.4[a] | 57.3 ± 1.5[a] | 116.1 ± 4.0[a] | 80.3 ± 2.6[a] |
| $Mstn^{+/-}$ | (n=13) | 22.0 ± 0.4 | 64.2 ± 1.0 | 90.5 ± 1.1 | 185.1 ± 2.3 | 130.5 ± 1.8 |
| $Mstn^{+/-}$, $Fst^{+/-}$ | (n=10) | 20.6 ± 0.4[h] | 52.3 ± 1.3[c] | 74.6 ± 2.0[c] | 152.6 ± 3.5[c] | 111.7 ± 2.2[c] |
| $Mstn^{-/-}$ | (n=13) | 24.5 ± 0.4 | 111.0 ± 2.3 | 148.5 ± 1.8 | 276.8 ± 4.2 | 195.8 ± 2.4 |
| $Mstn^{-/-}$, $Fst^{+/-}$ | (n=17) | 24.3 ± 0.3 | 94.4 ± 1.6[i] | 139.2 ± 1.6[i] | 251.2 ± 3.4[i] | 186.9 ± 2.4[e] |
| $inh\beta A^{+/-}$ | (n=20) | 19.4 ± 0.2 | 52.4 ± 0.9[a] | 73.3 ± 1.3[f] | 155.3 ± 2.2[g] | 109.3 ± 1.7[g] |
| $inh\beta B^{+/-}$ | (n=15) | 19.8 ± 0.4 | 50.5 ± 0.8[f] | 70.0 ± 1.2 | 147.5 ± 2.7 | 100.8 ± 1.8 |
| $inh\beta B^{-/-}$ | (n=12) | 20.0 ± 0.6 | 51.8 ± 1.4[f] | 69.9 ± 1.2 | 144.3 ± 3.3 | 100.8 ± 2.2 |
| $inh\beta C^{+/-}$, $\beta E^{+/-}$ | (n=15) | 18.5 ± 0.7 | 49.2 ± 1.5 | 69.4 ± 2.3 | 141.0 ± 5.5 | 97.5 ± 4.5 |
| $inh\beta C^{-/-}$, $\beta E^{-/-}$ | (n=25) | 19.1 ± 0.2 | 49.9 ± 0.9 | 70.5 ± 1.0 | 150.4 ± 2.1 | 103.0 ± 1.4 |

[a] $P < 0.001$ *vs. wild type*; [b] $P < 0.01$ *vs.* $Mstn^{+/-}$; [c] $P < 0.001$ *vs.* $Mstn^{+/-}$; [d] $P < 0.01$ *vs.* $Mstn^{-/-}$; [e] $P < 0.05$ *vs.* $Mstn^{-/-}$; [f] $P < 0.05$ *vs. wild-type*; [g] $P < 0.01$ *vs. wild type*; [h] $P < 0.05$ *vs.* $Mstn^{+/-}$; [i] $P < 0.001$ *vs.* $Mstn^{-/-}$.

stained fibers that appeared to be increased in number in $Fst^{+/-}$ mice corresponded to mixed glycolytic/oxidative type IIa fibers, representing a further shift away from glycolytic type IIb fibers (Fig. 2A). We observed similar trends toward more oxidative fibers in other muscles as well, with the appearance of a significant percentage of type I fibers in the EDL ($P < 0.001$) (Fig. 2B) and an approximately 5% shift from type IIa fibers to type I fibers in the soleus, although these latter data did not reach statistical significance.

To determine whether differences in fiber sizes could account for the differences in muscle weights between $Fst^{+/-}$ and *wild-type* mice, we measured fiber diameters in representative sections of the gastrocnemius muscle. As shown in Fig. 2C and Table 2, the distribution of fiber diameters was shifted toward smaller fibers in the gastrocnemius muscle of $Fst^{+/-}$ mice compared with that of *wild-type* mice. Significantly, the shift in the distribution toward smaller fibers was observed not only in type II fibers but also in type I fibers. Hence, the overall decrease in the weight of the gastrocnemius muscle of $Fst^{+/-}$ mice appeared to result from a combination of an increase in the proportion of fiber types that are generally smaller in size and a decrease in mean fiber diameter for each fiber type.

We also analyzed the effects of heterozygous loss of *Fst* on muscle function. Previous studies have shown that inhibition of myostatin activity in mice results in increased muscle force (4). To determine whether the lower muscle mass seen in $Fst^{+/-}$ mice results in lower muscle force production, we carried out force measurements on isolated muscles. As shown in Fig. 3, twitch and tetanic force were lower in $Fst^{+/-}$ mice by 27% and 26%, respectively, in the soleus and by 28% and 17%, respectively, in the EDL, which was commensurate with the reduction in cross-sectional area. Hence, the lower muscle weights seen in $Fst^{+/-}$ mice appear to result in corresponding decreases in tetanic force production, with no statistically significant changes in specific force.

Loss of myostatin has been shown to affect not only muscle mass and strength but also the ability of the muscle to regenerate. In particular, loss of myostatin activity has been shown to result in an enhanced regenerative response to both chronic (for reviews, see Refs. 15–17) and acute (28–30) injury. We investigated the possibility that heterozygous loss of *Fst* might have the opposite effect on muscle regeneration by examining the response of the gastrocnemius muscle to cardiotoxin-induced injury. Indeed, 21 d after induction of injury, *Fst* heterozygous mice

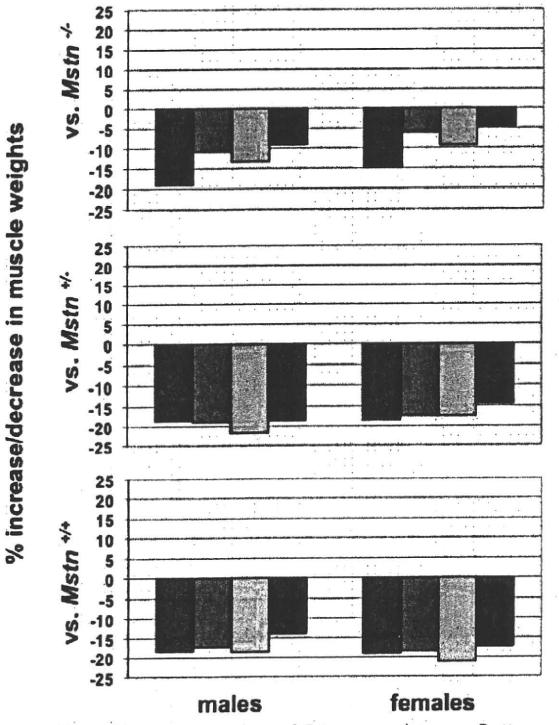


**FIG. 1.** Effect of heterozygous loss of *Fst* on muscle mass. *Bottom panel* shows percent decrease in muscle weights in $Fst^{+/-}$ mice compared with *wild-type* mice. *Middle panel* shows percent decrease in muscle weights in $Fst^{+/-}$, $Mstn^{+/-}$ mice compared with $Fst^{+/+}$, $Mstn^{+/-}$ mice. *Top panel* shows percent decrease in muscle weights in $Fst^{+/-}$, $Mstn^{-/-}$ mice compared with $Fst^{+/+}$, $Mstn^{-/-}$ mice. All calculations were made from the data shown in Table 1. Muscles analyzed were: pectoralis (*red*), triceps (*gray*), quadriceps (*blue*), and gastrocnemius (*green*).

showed clear deficits in muscle remodeling (Fig. 4A) with an almost 4-fold increase in the amount of muscle fibrosis as compared with *wild-type* mice (Fig. 4B). Interestingly, at 4 d after cardiotoxin-induced injury, *Fst* heterozygous mice showed no obvious difference in neonatal myosin-positive fibers compared with *wild-type* mice (Fig. 4A), suggesting that the defects in muscle remodeling may result from failed muscle maturation rather than impaired satellite cell function.

**TABLE 2.** Analysis of gastrocnemius/plantaris muscles

| | *Wild type* (*n* = 3) | $Fst^{+/-}$ (n = 3) | % Difference |
|---|---|---|---|
| Total fiber number | 8340 ± 323 | 8260 ± 521 | −1.0 |
| Type I fiber number | 124 ± 30 | 206 ± 21 | +66.1 |
| Percentage type I fibers | 1.47 ± 0.3 | 2.50 ± 0.2[a] | +69.7 |
| Mean fiber diameters (μm) | | | |
| Type I fibers | 32.8 ± 1.0 | 28.5 ± 1.2[a] | −13.0 |
| Type IIa fibers | 35.6 ± 2.4 | 29.9 ± 1.2[b] | −16.1 |
| Type IIb fibers | 41.9 ± 0.1 | 37.3 ± 1.3[a] | −11.0 |
| All fiber types | 41.0 ± 0.6 | 36.0 ± 1.5[a] | −12.2 |

[a] $P < 0.05$ *vs. wild type*; [b] $P = 0.08$ *vs. wild type*.

The fact that we could observe reductions in muscle mass in $Fst^{+/-}$ mice opened up the possibility of looking at genetic interactions between *Fst* and other genes encoding components of this regulatory system. We first looked at genetic interactions between *Fst* and *Fstl3*, which is another member of the follistatin gene family. Previous studies had shown that like follistatin, FSTL-3 (follistatin-like 3; also called FLRG) is capable of blocking myostatin activity *in vitro* (31) and promoting muscle growth when overexpressed *in vivo* (21, 22). What role FSTL-3 normally plays in regulating myostatin activity *in vivo* is unclear, however, as homozygous *Fstl3* mutant mice have been reported to have normal muscle mass (32). We investigated the possibility that the lack of a clear muscle phenotype in *Fstl3* mutant mice might reflect functional redundancy between FSTL-3 and follistatin. For these studies, we used a line of mice that we independently generated carrying a targeted deletion of *Fstl3*. As shown in Fig. 5A, we generated mice in which we deleted exons 3–5, which contains most of the protein-coding region, including both of the follistatin domains of FSTL-3. We then backcrossed this deletion allele at least seven times onto a C57BL/6 background for analysis.

Consistent with findings previously reported by others (32), mice homozygous for a deletion of *Fstl3* were viable and had relatively normal muscle weights (Table 1). To investigate possible functional redundancy, we analyzed the effect of crossing the *Fst* loss-of-function mutation onto an *Fstl3* mutant background. As shown in Table 1 and Fig. 5B, we observed no additive effects of the *Fst* and *Fstl3* mutations in terms of reducing muscle mass; that is, neither $Fst^{+/-}$, $Fstl3^{+/-}$ nor $Fst^{+/-}$, $Fstl3^{-/-}$ mice showed further reductions in muscle weights compared with $Fst^{+/-}$, $Fstl3^{+/+}$ mice. Although we have not ruled out the possibility that we might see effects of FSTL-3 loss in mice completely lacking follistatin, our data suggest that these two proteins are not functionally redundant in terms of regulating muscle growth. Hence, despite all of the evidence implicating FSTL-3 as a key regulator of myostatin activity *in vivo*, we were unable to uncover any effects of genetic loss of *Fstl3* on muscle mass in these studies.

We also took advantage of the muscle phenotype in $Fst^{+/-}$ mice to investigate genetic interactions between *Fst* and *Mstn*. Our rationale for these studies was that two lines of investigation had demonstrated that other members of the TGF-β family, in addition to myostatin, seem to play important roles in limiting muscle growth. In particular, both overexpression of follistatin as a muscle-specific transgene and systemic administration of a soluble form of one of the known myostatin receptors

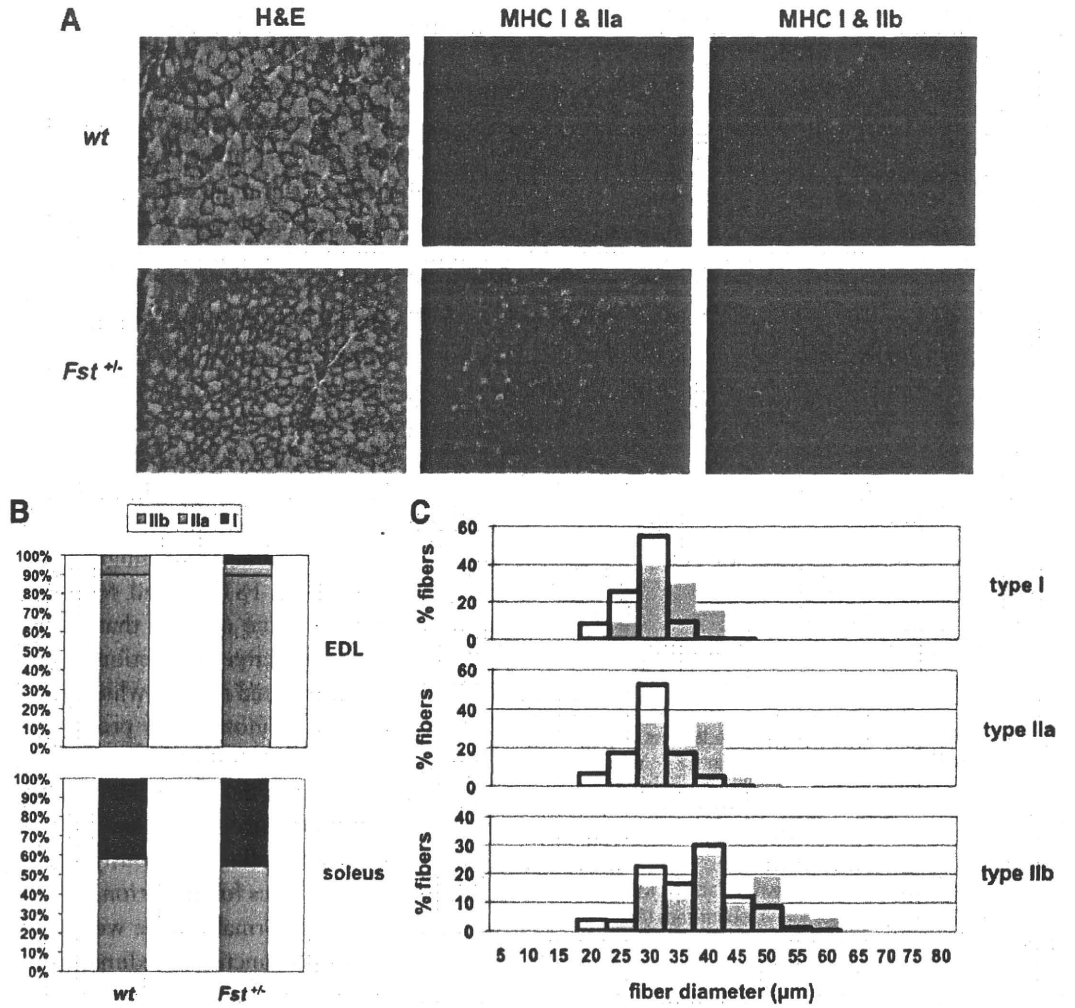


**FIG. 2.** Fiber type analysis. A, Sections of gastrocnemius muscles either stained with hematoxylin and eosin or incubated with antibodies against type I (*red*), type IIa (*green*), or type IIb (*green*) MHC isoforms. Note that muscles of $Fst^{+/-}$ mice had increased numbers of small, darkly stained fibers, which corresponded to type IIa fibers, as well as increased numbers of type I fibers. B, Fiber type distributions in the EDL and soleus muscles. Note the appearance of type I fibers and the decrease in proportion of type IIa fibers in $Fst^{+/-}$ EDL muscle. C, Distribution of type I, IIa, and IIb fiber diameters in the gastrocnemius muscle. *Solid gray bars* represent muscle fibers from *wild-type* mice, and *open black bars* represent muscle fibers from $Fst^{+/-}$ mice. Note the shift in the distributions toward fibers with smaller diameters in muscles of $Fst^{+/-}$ mice. H&E, hematoxylin and eosin; wt, wild type.

(ACVR2B) had been shown to cause increases in muscle mass not only in *wild-type* mice but also in $Mstn^{-/-}$ mice, implying that these inhibitors were exerting their effects by targeting other TGF-β family members in addition to myostatin (7, 18, 21). Hence, we sought to determine whether the reductions in muscle weights seen in $Fst^{+/-}$ mice result entirely from increased levels of myostatin signaling.

Our approach was to look for genetic interactions between *Fst* and *Mstn* by examining the effect of introducing the *Fst* mutation onto a *Mstn* mutant background. If the sole role for follistatin in regulating muscle mass *in vivo* is to block myostatin signaling, then the *Fst* mutation would be predicted to have no effect in the complete absence of myostatin. If, on the other hand, follistatin normally acts to block multiple ligands to regulate muscle mass, then the *Fst* mutation might be expected to have at least some effect on muscle mass even in a *Mstn* mutant background. As shown in Table 1 and Fig. 1, we found the latter to be the case. Specifically, heterozygous loss of *Fst* caused reductions in muscle mass in both $Mstn^{+/-}$ and $Mstn^{-/-}$ mutant backgrounds in both male and female mice. The effects of the *Fst* mutation were somewhat attenuated in the complete *Mstn*-null background, implying that part of the effect of follistatin loss in $Mstn^{+/+}$ mice likely results from loss of inhibition of myostatin signaling. Nevertheless, the fact that the *Fst* mutation had at least some effect on muscle weights even in the *Mstn*-null background implies that this residual effect resulted from loss of inhibition of other TGF-β family members in these mutant mice. Hence, these studies suggest that follistatin normally acts *in vivo* to inhibit multiple TGF-β family

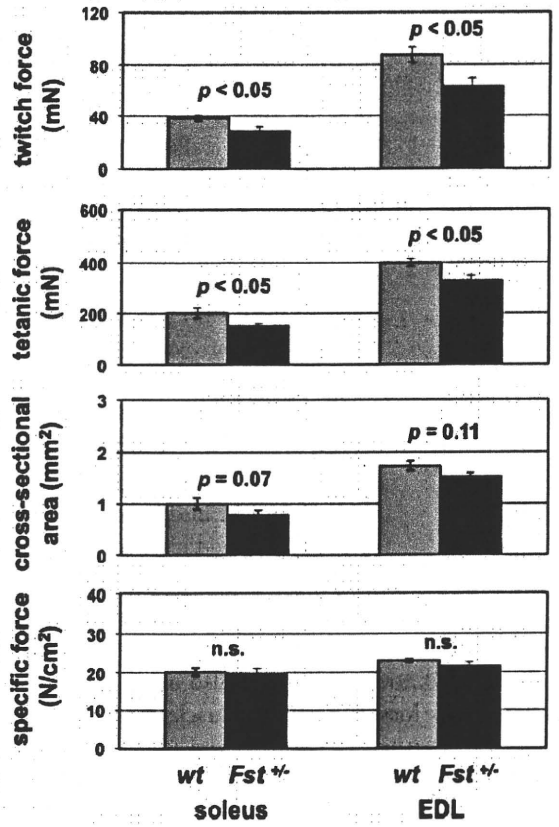


**FIG. 3.** Force measurements in the soleus and EDL muscles of *wild-type* and $Fst^{+/-}$ mice. Note the decreased twitch and tetanic force with no change in specific force in muscles of $Fst^{+/-}$ mice. n.s., Nonsignificant; $P > 0.20$ wt, wild type.

members, including myostatin, that function to limit muscle mass.

In the final set of experiments, we used genetic approaches to attempt to determine the identity of the ligand (or ligands) that cooperates with myostatin to suppress muscle growth. The TGF-$\beta$ superfamily consists of almost 40 proteins (for reviews, see Refs 34 and 35), and many could be eliminated as possible candidates based on their known binding properties, because the key ligand can be blocked both by follistatin and by the soluble ACVR2B receptor. The most obvious candidate was growth/differentiation factor (GDF)-11, which is highly related to myostatin and is also expressed in skeletal muscle; genetic studies to date, however, have not revealed any role for GDF-11 in regulating muscle (26). As a result, we decided to extend our genetic analysis to other candidate ligands. The activins, which are dimers of inhibin-$\beta$ subunits, were attractive as candidates because they had been shown to have *in vitro* activities on muscle cells (36–39). Moreover, a recent study showed that activin A is capable of inducing atrophy when overexpressed in muscle (40).

We decided to focus our initial analysis on mice carrying mutations in genes encoding the inhibin-$\beta$ subunits. In mice, four genes encoding inhibin-$\beta$ subunits have been identified, *Inh*$\beta$*A*, *Inh*$\beta$*B*, *Inh*$\beta$*C*, and *Inh*$\beta$*E* (for review, see Ref. 34). Mice carrying targeted mutations in each of these genes have been generated and characterized previously (41–44), and for *Inh*$\beta$*A* and *Inh*$\beta$*B*, we analyzed the existing mutant mouse lines. For *Inh*$\beta$*C* and *Inh*$\beta$*E*, however, we analyzed a double-mutant mouse line that we generated independently in which the exon encoding the C-terminal domain of *Inh*$\beta$*C* and the entire coding sequence of *Inh*$\beta$*E* were deleted in the same mutant allele (Fig. 5C). All of these *Inh*$\beta$ mutant alleles were backcrossed at least six times onto a C57BL/6 genetic background before analysis.

For the *Inh*$\beta$*B* and *Inh*$\beta$*C*/$\beta$*E* mutations, we were able to analyze the effect of complete loss of function, as the homozygous mutants are viable as adults. In the case of *Inh*$\beta$*A*, however, homozygous loss has been shown to lead to embryonic lethality (43); therefore, we were only able to analyze the effect of heterozygous loss of *Inh*$\beta$*A*. As shown in Table 1 and Fig. 5D, the most significant effect that we observed was, in fact, in mice heterozygous for the *Inh*$\beta$*A* loss-of-function mutation, which exhibited statistically significant increases in weights of all four muscles that were examined. The effects seen in $Inh\beta A^{+/-}$ mice were most pronounced in males, which had increases ranging from about 8–11%, with $P$ values ranging from $2 \times 10^{-4}$ to $2 \times 10^{-6}$ depending on the specific muscle. The effects in females were generally lower, with increases ranging from about 5–8%. These trends were also present after normalizing muscle weights to total body weights (Supplemental Table 1 and Supplemental Fig. 1). Mutations in each of the other genes had little or no effect, except in the case of *Inh*$\beta$*B* homozygous mutants, in which two muscles (pectoralis and triceps) also showed statistically significant increases, although the magnitude of the effects was lower than that seen in $Inh\beta A^{+/-}$ mice. These data provide the first loss-of-function genetic evidence that activin A may be one of the key ligands that functions with myostatin to limit muscle mass.

## Discussion

Follistatin is a potent myostatin inhibitor that can cause dramatic increases in muscle mass when overexpressed as a transgene in mice (18, 21, 22). Follistatin is known to play an important role in regulating muscle development, because newborn *Fst* mutant mice have a reduced amount of muscle tissue, which is readily discernible by histological analysis (23). Because homozygous *Fst* mutants die immediately after birth, however, little is known about the role that follistatin normally plays in regulating mus-

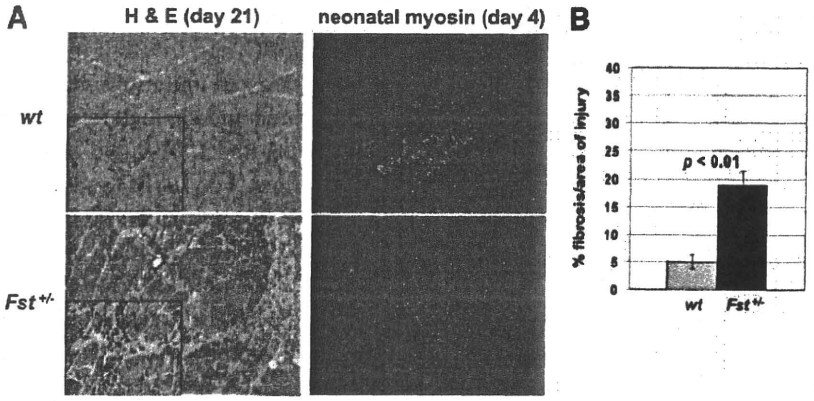


**FIG. 4.** Impaired muscle regeneration in $Fst^{+/-}$ mice. A, Sections of gastrocnemius muscles of *wild-type* and $Fst^{+/-}$ mice after cardiotoxin-induced injury. At 21 d after injury, note the centrally located nuclei characteristic of regenerating fibers in the *wild-type* injured muscle (see *inset*) and the significantly increased extent of fibrosis in the $Fst^{+/-}$ muscle. At 4 d after injury, note the presence of neonatal myosin in both *wild type* and $Fst^{+/-}$ muscle. B, Quantification of amount of fibrosis at 21 d after cardiotoxin-induced injury as assessed by measurement of percent fibrotic area relative to total injured area. H&E, Hematoxylin and eosin; wt, wild type.

cle homeostasis. Here, we have shown that the *Fst* loss-of-function mutation exhibits haploinsufficiency, with $Fst^{+/-}$ mice having lower overall muscle mass by about 15–20%. These reductions in muscle mass were highly statistically significant and resulted from a shift toward smaller diameter fibers with little or no apparent effect on total fiber number. This shift toward smaller fibers could be attributed to two distinct effects of the *Fst* mutation. First, there was a shift in the distribution of fiber types resulting in an increased proportion of smaller, more oxidative fibers in muscles of $Fst^{+/-}$ mice compared with those of *wild-type* mice. Second, for each fiber type that was examined, there was a shift toward fibers with smaller diameters in muscles of $Fst^{+/-}$ mice compared with those of *wild-type* mice. All of these effects are the opposite of what has been described in mice with absent or reduced myostatin activity, which exhibit increased muscle mass, a shift in fiber types toward more glycolytic fibers, and hypertrophy of both type I and type II fibers (1, 24–27, 45). We also found that $Fst^{+/-}$ mice exhibit an impaired muscle remodeling response to chemical injury, which also contrasts with the enhanced muscle regeneration seen in $Mstn^{-/-}$ mice (for reviews, see Refs. 15–17).

All of these findings demonstrate that follistatin normally functions to suppress activity of this signaling pathway in muscle. In this respect, an important point is that we were able to document significant effects of follistatin loss even though these mice still retained one normal copy of the *Fst* gene. Hence, the effect of follistatin is almost certainly dose dependent, as has been shown for many other components of this regulatory system, and we presume that complete loss of follistatin activity in muscle would lead to much more dramatic effects. We also investigated the possibility that the effects of follistatin loss might have been attenuated by functional compensation by the related protein, FSTL-3. FSTL-3 (also called FLRG) contains two follistatin domains (*vs.* three for follistatin itself), and like follistatin, FSTL-3 is capable of binding and inhibiting both activin and myostatin *in vitro* (31, 46–48) and increasing muscle mass when overexpressed *in vivo* (21, 22). FSTL-3 has been further implicated in the regulation of myostatin based on the fact that FSTL-3 could be detected in a complex with myostatin in both mouse and human blood samples (31). Gene-targeting studies, however, demonstrated that complete loss of FSTL-3 had no effects on muscle mass (32). Using an independently generated *Fstl3*-knockout line, we also observed no effect of homozygous loss of *Fstl3* on muscle mass, and furthermore, we were unable to detect any additive effects of the *Fst* and *Fstl3* loss-of-function mutations. Hence, we were unable to detect any evidence that follistatin and FSTL-3 are functionally redundant with respect to the regulation of muscle mass by myostatin and related proteins.

We also examined genetic interactions between *Fst* and *Mstn*. In particular, we showed that the effects of follistatin loss are seen even in mice null for *Mstn*, implying that myostatin cannot be the sole target for follistatin and that follistatin normally acts to block the activities of multiple TGF-$\beta$ family members that function to limit muscle mass. These findings are consistent with the results of two prior studies. One set of experiments was the analysis of mice treated with a soluble form of ACVR2B, which has been shown to be one of the activin type II receptors involved in mediating myostatin signaling (7, 18, 49). The soluble form of ACVR2B (ACVR2B/Fc) was shown to be capable of blocking myostatin activity *in vitro*, and administration of ACVR2B/Fc to adult mice was shown to cause dramatic muscle growth (up to 40–60% in 2 wk). Significantly, this effect was attenuated, but not eliminated, in $Mstn^{-/-}$ mice, implying that ACVR2B/Fc was targeting at least one additional ligand that also functions to block muscle growth (7). A second set of experiments was the analysis of transgenic mice overexpressing follistatin in muscle (18). As expected, based on the ability of follistatin to inhibit myostatin, these transgenic mice exhibited significant increases in muscle mass. As in the studies with the soluble ACVR2B receptor, however, the follistatin transgene could also cause increases in muscle

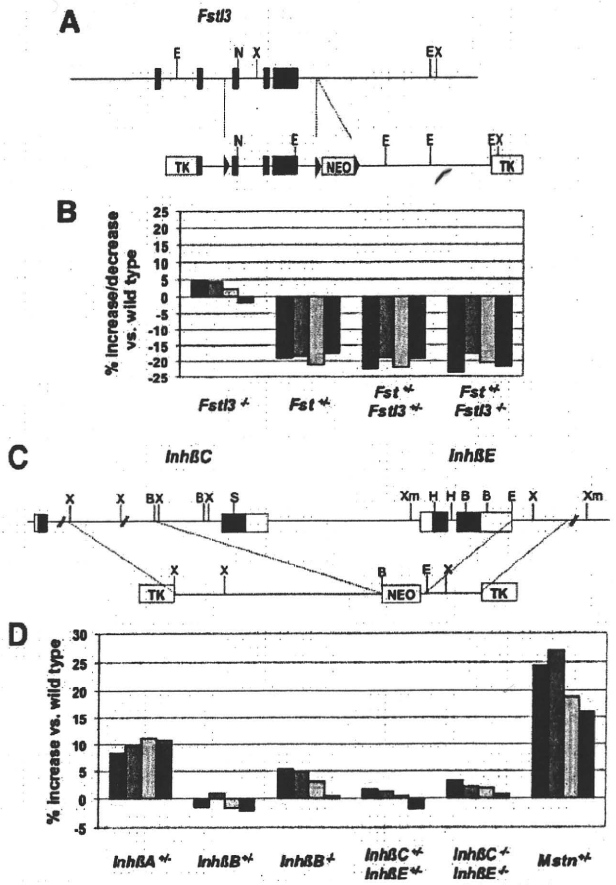


**FIG. 5.** Effect of a mutation in *Fstl3* and in genes encoding inhibin β-subunits on muscle mass. A, Diagram of *Fstl3*-targeting strategy. Mice carrying the targeted allele were crossed to Ella-cre transgenic mice (33) to generate mice in which recombination had occurred between the outside LoxP sites (denoted by *triangles*), thereby resulting in a mutant allele in which exons 3–5 were completely deleted in the germline. B, Effects of the deletion mutation in *Fstl3* either alone or in combination with heterozygous loss of *Fst*. C, Diagram of *InhβC/InhβE*-targeting strategy. D, Effect of *Inhβ* mutations in male mice. In the *bar graphs* shown in Panels b and d, *numbers* represent percent increase or decrease in muscle mass relative to *wild-type* mice and were calculated from the data shown in Table 1. Data from $Mstn^{+/-}$ mice (21) are shown for comparison. Muscles analyzed were: pectoralis (*red*), triceps (*gray*), quadriceps (*blue*), and gastrocnemius (*green*). TK, Thymidine kinase.

growth even in mice lacking myostatin (21); in fact, the follistatin transgene could cause yet another doubling of muscle mass on top of the doubling seen in the absence of myostatin (*i.e.* an overall quadrupling).

All of these studies demonstrate that at least one other TGF-β family member, in addition to myostatin, also functions to limit muscle mass *in vivo*. Thus, the capacity for increasing muscle growth by targeting this signaling pathway is much more substantial than previously appreciated. We have been using a genetic approach to determining the identity of this other ligand. An obvious candidate was GDF-11, which is highly related to myostatin (1, 50). Initial gene-knockout studies demonstrated that mice completely lacking GDF-11 exhibit multiple developmental defects and die during the perinatal period (51), which precluded a detailed analysis of the role of GDF-11 in muscle. Subsequent studies utilizing a floxed *Gdf11* allele, however, revealed no effect of *Gdf11* deletion specifically in skeletal muscle either alone or in combination with a *Mstn*-knockout mutation (26).

Perhaps the next most likely candidates were the activins, which have been shown to be capable of regulating differentiation of muscle cells in culture (36–39) and share a common receptor with myostatin (for reviews, see Refs. 15 and 34). Moreover, a recent study also suggested the possibility that activins may be involved in regulating muscle mass based on their ability to induce muscle atrophy when overexpressed *in vivo* and based on differential effects seen *in vivo* between follistatin and a follistatin variant with reduced affinity for activin (40). Finally, another recent study implicated activins as well as a number of other ligands, including GDF-11, BMP-9, and BMP-10, as possible candidates based on the fact that these could be affinity purified from serum using the ACVR2B/Fc ligand trap (38). Indeed, by analyzing mouse strains carrying targeted deletions in each of the genes encoding the inhibin β-subunits, we observed increases in muscle mass in mice heterozygous for the *InhβA* mutation, consistent with an important role for activin A in regulating muscle mass. Although further characterization of the muscles of these mice will be required to demonstrate that these effects on muscle mass result from muscle fiber hypertrophy, these data provide the first genetic loss-of-function evidence that activin A may be one of the key ligands that function with myostatin to limit muscle mass.

Although the increases in muscle mass that we observed in *InhβA* mutants were relatively modest, we believe that the overall role that activin A may play is potentially much more substantial for several reasons. First, this phenotype was observed in mice that still retained one functional copy of the *InhβA* gene. By comparison, male mice heterozygous for a mutation in *Mstn* exhibit increases in muscle weights ranging from 16–27% (21); hence, the magnitude of the effects seen in male $InhβA^{+/-}$ mice was approximately half that seen in $Mstn^{+/-}$ mice. Homozygous loss of *Mstn* results in increases in muscle weights of 100–150%, and we presume that greater loss of activin A signaling would similarly result in a significantly enhanced effect. Second, the existence of multiple *Inhβ* genes raises the possibility of functional redundancy, and in this respect, we did see some effect, albeit quite small, in *InhβB* homozygous mutants. Third, a mutation in the *InhβA* gene affects the production of both activin A (as well as activin AB) and inhibin A, which

share the βA-subunit. Given that activins and inhibins generally have counteracting activities, it is perhaps fortuitous that we were able to see any phenotype at all in $Inh\beta A^{+/-}$ mice, because the mutation would lead to decreases in both activin A and an inhibitor of activin signaling. We believe that the likely explanation is that whereas activins are believed to act mostly via a paracrine mechanism, inhibins appear to be capable of regulating signaling in an endocrine manner (for reviews, see Refs. 52–54), and the predominant circulating form of inhibin is known to be inhibin B (for reviews, see Refs. 55 and 56). Hence, the *Inhβ A* mutation would be predicted to reduce levels of activin A but have only a minimal effect on circulating inhibin levels.

Clearly, additional studies will be required to elucidate the precise roles that all of the activin isoforms may play in regulating muscle growth and function in different physiological states. It is interesting to note, however, that circulating levels of activin A in humans have been shown to increase during aging, and conversely, circulating levels of inhibin B have been shown to decrease during aging (57–61), raising the intriguing possibility that enhanced activin signaling during aging may be a key contributing factor in the etiology of age-related sarcopenia. Understanding how muscle homeostasis is coordinately regulated by myostatin and by activins under both normal and pathological conditions will be essential for developing the most optimal strategies to tap the full potential of targeting this general signaling pathway to preserve muscle mass and prevent muscle atrophy in a variety of clinical settings associated with debilitating loss of muscle function.

## Materials and Methods

Targeting constructs were generated from 129 SvJ genomic clones and used to transfect R1 embryonic stem cells (kindly provided by A. Nagy, R. Nagy, and W. Abramow-Newerly). Blastocyst injections of targeted clones were carried out by the Johns Hopkins Transgenic Core Facility. All mice were backcrossed at least six times onto a C57BL/6 background before analysis. All analysis was carried out on 10-wk-old mice, except for the force measurements and cardiotoxin studies, which were carried out on 14-wk-old mice. All animal experiments were carried out in accordance with protocols that were approved by the Institutional Animal Care and Use Committees at the Johns Hopkins University School of Medicine and the University of Pennsylvania School of Dental Medicine.

For measurement of muscle weights, muscles were dissected from both sides of the animal and weighed, and the average weight was used. For morphometric analysis, the gastrocnemius muscle was sectioned to its widest point using a cryostat, and fiber diameters were measured as the shortest width passing through the center of the fiber. Measurements were carried out on 84 type I fibers, 150 type IIa fibers, and 150 type IIb fibers per muscle, and mean fiber diameters for each type were calculated for each animal. For plotting the distribution of fiber sizes, data from all three mice in each group were pooled. Measurements were also carried out on 250 fibers of mixed types randomly selected from five representative areas of each section (every attempt was made to analyze the same five regions from muscle to muscle) to estimate overall mean fiber diameters.

For isolated muscle mechanics, mice were anesthetized with ketamine/xylazine. Muscles were removed and placed in a bath of Ringers solution gas equilibrated with 95% $O_2$/5% $CO_2$. Sutures were attached to the distal and proximal tendons of the EDL and soleus muscles. Muscles were subjected to isolated mechanical measurements using a previously described apparatus (Aurora Scientific, Ontario, Canada) (62). After optimal length (Lo) was determined by supramaximal twitch stimulation, maximal isometric tetanus was measured in the muscles during a 500-msec stimulation. For histological analysis, samples were rinsed in PBS, blotted, weighed, covered in mounting medium before freezing in melting isopentane, and then stored at −80 C. Muscle cross-sectional areas were determined using the following formula: cross-sectional area = m/(Lo × L/Lo × 1.06 g/cm$^3$), where m is muscle mass, Lo is muscle length, L/Lo is the ratio of fiber length to muscle length, and 1.06 is the density of muscle (63). L/Lo was 0.45 for EDL and 0.69 for soleus.

For muscle fiber typing, 10-μm frozen cross-sections taken from the midbelly of each muscle were subjected to immunohistochemistry for laminin (rabbit antilaminin Ab-1; Neomarkers, Fremont, CA) to outline the muscle fibers. Fiber typing was performed with antibodies recognizing myosin heavy chain (MHC)2a (SC-71), MHC 2b (BF-F3), and MHC 1 (BAF-8) as previously described (64). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Images were acquired on an epifluorescence microscope (Leica, Deerfield, IL) and analyzed for the proportion of myosin-positive fibers using image analysis software (OpenLab, Improvision; Coventry, UK).

For skeletal muscle injury studies, 250 μl of cardiotoxin (10 μM *Naja nigricollis*; Calbiochem, La Jolla, CA) were injected into the gastrocnemius, and muscles were harvested 4 d or 21 d after induction of injury. Quantification of areas of fibrosis per area of muscle injury was performed using Nikon's NIS elements BR3.0 software (Laboratory Imaging; Nikon, Melville, NY).

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Disclosure Summary: Under a licensing agreement between MetaMorphix, Inc. (MMI) and the Johns Hopkins University, S.-J.L. is entitled to a share of royalty received by the University on sales of the factors described in this paper. S.J.L. and the University own MMI stock, which is subject to certain restrictions under University policy. S-J.L., who is the scientific founder of MMI, is a consultant to MMI on research areas related to the study described in this paper. The terms of these arrangements are being managed by the University in accordance with its conflict of interest policies. Y.-S.L., T.Z., A.S., M.M., K.T., R.D., and E.B. have nothing to declare.

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基礎老化研究　34(4);5－11,2010

【総　説】

# 老化や疾患における骨格筋の萎縮と治療への応用

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**要約**

老化や難治性筋疾患等に伴って、骨格筋は形態的変化および機能低下が生じる。老化では特に速筋型のタイプII筋線維の萎縮が顕著に見られ、脂肪沈着や繊維化が見られる。骨格筋の再生を担うのは筋衛星細胞であるが、老化に伴い、筋再生能力の低下を来たす。近年、骨格筋量調節因子であるマイスタチンやアクチビンの機能制御により、筋萎縮が防げる事が明らかとなってきた。マイスタチン阻害によって、筋萎縮の防止、繊維化の抑制、体脂肪量の減少、脂肪肝抑制が期待されており、老化、筋疾患、悪液質への応用も現実味を帯びてきた。更に、脂肪細胞の起源に関する研究に近年大きな進展があり、骨格筋内の異所性脂肪変性を担う細胞群や脂肪組織での脂肪産生細胞の起源が明らかとなってきた。本総説では、老化、筋疾患等に伴って生じる筋萎縮の病態と治療法開発の現状、そして、脂肪細胞の起源と異所性脂肪変性に関する最新の知見を筆者らの研究を中心に紹介する。

**キーワード**：sarcopenia, muscle atrophy, myostatin, muscular disease, ectopic fat formation

## 1. はじめに

筋骨格系は、骨格筋、腱、骨、軟骨、脂肪組織、結合組織から構成され、生体の最重量を占める組織である。とりわけ、骨格筋は成人の身体中の重量の約40%を占め、関節運動を協調させた円滑な運きや姿勢の維持に関与している。熱産生による体温の維持やインスリンの標的臓器として血糖調節にも重要な役割を果たしている。骨格筋は、老化や各種病態によって劇的な変化と機能低下を示す。筋骨格系組織の機能低下は、運動能力低下に直結し、日常生活動作（ADL）や生活の質（QOL）を低くするため、その対策は医学的に重要である。

骨格筋は、筋膜に包まれた筋線維と結合組織で構成されている。筋線維は、多くの筋芽細胞が融合した結果、多核を形成する筋細胞で形成されるが、分裂して新たな筋線維を生み出す事は出来ない。その役割を担うのは、筋線維の基底膜直下に存在する単核の筋衛星細胞（サテライト細胞）である。筋衛星細胞は骨格筋の発生に寄与すると共に、筋線維の損傷時には、増殖、分化、融合といった経過をたどり、筋再生に重要な働きをしている。筋線維は、遅筋線維（タイプI）と速筋線維（タイプIIA, IIB, IIX）に分けられ、遅筋線維はミトコンドリアを豊富に含み、酸化系酵素の活性が高く、収縮速度は遅いが疲労しにくい性質を持つ。ミオグロビンを多く含み赤筋線維とも呼ばれる。速筋線維は、解糖系酵素活性が高く収縮速度は早いが、特にIIB, IIX線維は疲労しやすい。無酸素状態での代謝に依存しており、白筋線維とも呼ばれる。

脊髄前角に細胞体を持つα運動ニューロンとそれに支配される筋線維を運動単位と呼ぶ。身体内には400を超える骨格筋が存在し、遅筋線維と速筋線維が混在しているがそれぞれの骨格筋では、その比率は異なっている。トレーニング等で、筋線維を肥大させたり、線維タイプをIIB型からIIA型への変換させることで持久力を改善させる事は可能と考えられている。

骨格筋組織は生体内で隣接する骨格筋内の細胞のみならず、身体内の脂肪組織や内臓臓器、神経組織との間で、サイトカインの産生と受容、神経連絡を介して相互連携しており、生体の恒常性維持に重要な役割を果たしている。

最近の研究で、老化や筋疾患によって生じる筋萎縮を抑制可能であることが示されている。特に、骨格筋から産生されるマイオスタチンと呼ばれる分子を阻害する治療法は、有望視されている。骨格筋は、再生医療の分野からのアプローチも期待されている。骨格筋は再生能力の高い組織であり、筋衛星細胞が筋再生に大きな役割を担っている。筋衛星細胞以外にも独特の分化能や特徴を持った前駆細胞が存在し、骨格筋の恒常性維持に寄与している。本総説では、骨格筋の老化や疾患における変化を述べると共に、筋萎縮の防止法や最近発見された脂肪前駆細胞群について紹介する。

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## 2. 骨格筋の形成と筋萎縮

1) 骨格筋の形成

横紋筋である骨格筋は、胎児期の体節に由来し、体節中の筋前駆細胞が運命決定され筋分化に寄与している。筋前駆細胞は遊走と増殖を行ない、細胞融合と成熟過程を経て多核の筋線維を形成する。Helix-loop-helix構造を持ったMyoDファミリー転写因子群が骨格筋の分化制御に決定的な役割を果たしており、筋分化決定因子(muscle determining factor, MRF)と呼ばれる。MyoDとMyf5は主として筋芽細胞への分化決定と維持に働き、myogeninとMRF4は主に筋芽細胞から筋管細胞への最終分化に関与する [1]。

Pax7は、胚期や周産期の骨格筋形成に必須の分子であり、生後は筋衛星細胞の生存に必須であると考えられている [2]。筋衛星細胞は、筋細胞の筋形質膜と基底膜の間に存在し、成体の筋再生を担う細胞であり、Pax7は筋衛星細胞のマーカーとして頻用されている。胎生期の皮筋節のPax3/Pax7陽性細胞が筋衛星細胞の起源であることが示されている [2]。長らく、Pax7の筋衛星細胞での発現が筋再生に必須であると考えられていたが、マウスの実験から、生後2-3週以降では、Pax7は筋衛星細胞による筋再生に必須ではない事が示された [3]。なお、試験管内で、筋分化を示す細胞には、筋衛星細胞以外に、骨髄等の間葉系幹細胞、血管由来の前駆細胞、iPS細胞等がある。

2) 骨格筋形成におけるマイオスタチンの役割

生体内の個々の組織の大きさを決定するための抑制因子が存在するという仮説があり、カローン仮説と呼ばれている [4]。骨格筋のカローンの最有力候補が骨格筋から産生され筋量を負に制御しているマイオスタチンである。マイオスタチンは、1997年に機能が明らかになったTGF－βファミリーに属するサイトカインである [5]。マイオスタチンが遺伝子変異等で欠損したり、マイオスタチン阻害分子を投与すると、骨格筋量が増大する。遺伝子破壊マウスやウシ、羊、Whippetと呼ばれる短距離レースの犬などで遺伝子レベルでのマイオスタチン阻害動物の例が報告されている [5-8]。羊の場合は、マイオスタチン遺伝子の3'翻訳領域が変異することで、骨格筋特異的なmiRNAであるmiRNA1やmiRNA 206の標的部位となることで、発現が減少する [8]。ヒトのマイオスタチン変異も報告されており、筋量と筋力が増大し脂肪量が少ない [9]。マイオスタチンが筋量を調節する詳細な分子機構は不明点も多いが、筋線維構成分子のタンパク分解系の亢進が作用機構の一つだと想定されている。マイオスタチン阻害は、筋衛星細胞の分化調節へ影響を及ぼすが、その寄与は当初考えられていたより少ないとする報告もある [10,11]。マイオスタチンとタンパク質の一次構造上類似したアクチビンAも筋量の調節に関与している [12]。マイオスタチンの作用が骨格筋に特異的であるのに比較して、アクチビンの作用は全身におよぶ。アクチビンは下垂体からの卵胞刺激ホルモンの分泌作用を基に精製されたホルモンであるが、生殖系への作用以外にも、神経栄養因子としての作用や記憶への関与など多彩な作用を有している [13]。アクチビンとマイオスタチンの阻害分子としてホリスタチン（FSTN）が知られている [14]。我々は、FSTNに由来しアクチビンへの阻害がないマイオスタチン阻害因子を開発した [15]。開発因子やFSTNには成体の筋量を増加させる効果がある [15,16]。FSTN等のマイオスタチン阻害因子の遺伝子導入や因子の投与で筋量を増大させ、筋ジストロフィーモデル動物の病態を軽減可能である事が確認されている [15,16]。

3) 骨格筋萎縮の分子機構

骨格筋萎縮は、筋肉構成タンパク質の合成と分解の正常なバランスが崩れた状態で引き起こされる。老化に伴う筋萎縮はサルコペニア（sarcopenia）と呼ばれる。加齢の初期の筋萎縮には、筋自体の寄与が大きい。形態学的には、筋線維数は加齢に伴って40%もの減少が見られる。横断面積の縮小もII型筋線維に顕著に見られ、I型とII型筋線維が混在した筋線維が増える。また、同系統の筋線維が束になって存在する筋線維のグループ化が生じる。この原因としては、高齢者ほど運動単位数が減少する事による神経原性変化に起因する事が示唆されている。つまり、老化では、筋原性萎縮と運動単位の減少による神経原性変化の両者が生じる。さらに、細胞死も関与すると考えられている。サルコペニアの特徴は、ゆっくりと筋力や筋持久力が低下していく事である。一方、重症の脊椎損傷による半身麻痺や脳梗塞などで長期の寝たきり（ベッドレスト）やギプス固定で生じる筋萎縮は廃用性筋萎縮と呼ばれるが、遅筋型のタイプI線維が優位に萎縮し、速筋優位となる。

筋萎縮は、加齢に伴って生じる以外にも、筋ジストロフィーを代表とする遺伝性の神経筋疾患で見られる。さらには、癌や感染症、慢性心不全、肝不全の末期に生じる悪液質（カヘキシー、cachexia）では、筋萎縮と共に脂肪細胞の萎縮が生じて体の恒常性が維持出来ない。悪液質では、食思不振、体重減少、栄養障害、疲労が見られる。TNFαやIL-6などのサイトカインの発現の変化が病態に関与するが、現状では治療法は存在しない。

蛋白質分解系には、ライソソーム系、カルパイン系、ユビキチン／プロテアソーム系があるが、骨格筋萎縮では、筋特異的ユビキチンリガーゼの発見がなされ、主にユビキチン／プロテアソーム活性の上昇によるタンパク分解の亢進の解析が大きく進展した [17]。MuRF1(muscle RING finger-1)とMAFbx-1/atrogin-1は、坐骨神経切断等の筋萎縮で発現上昇する骨格筋と心筋特異的なユビキチンリガーゼであり、筋萎縮時に活性化する [17]。これらの遺伝子破壊マウスや阻害で、筋萎縮に抵抗性を示す事から筋萎縮を担う重要分子であると考えられている [17]。IGF-1 (insulin-like growth factor-1)は強力に筋形成を促すが、その下流で、Akt-1がリン酸化により活性化する。Akt-1が活性化すると、S6キナーゼ系によりタンパク質合成が上昇すると共に、転写因子のFOXO (forkhead box O)がリン酸化する。リ

ン酸化FOXOは核内へ移行せず、その標的遺伝子であるMuRF1とMAFbx-1の発現が抑制される。ベッドレスト等の廃用性筋萎縮においては、IGF-1が低下し、Akt-1が抑制され、FOXOは脱リン酸化状態になり、核移行して、MuRF1とMAFbx-1の発現を高め、筋タンパク分解が亢進するモデルが提唱されている [18]。マイオスタチンは、IGF-1と拮抗して作用する事で、筋萎縮を促進する [19]。

サルコペニアにおける筋萎縮は、廃用性筋萎縮とは異なり、加齢に伴い緩やかに生じる現象である。廃用性筋萎縮とは異なった分子機構が示唆されている。MuRF1とMAFbx-1の関与については、相反する報告もあり、明らかではない。サルコペニアにおいて、筋衛星細胞を活性化する因子が減少する事が知られている。老化によって、筋衛星細胞の増殖能が低下し、筋再生能が弱まり、線維化や脂肪化が生じる。老齢マウスでは、筋衛星細胞の筋分化への寄与が弱まり、Wnt経路の活性が高まり、線維化を起こす [20]。これは、循環血液を含めた細胞環境が加齢によって異なることに起因することが分かっている [21]。

4) 筋ジストロフィー

代表的な難治性筋疾患に筋ジストロフィーがあり、現在では40種を超える病型に分類することが出来る。筋ジストロフィーの中で最も頻度が高く症状も重篤なのが、X連鎖性劣性遺伝性疾患のデュシェンヌ型ジストロフィー（DMD）である。DMDの責任分子であるジストロフィン遺伝子は79個のエクソンから成り、ゲノムサイズが2.3Mb、mRNAのサイズが14Kbにおよび、タンパク質の分子量が40万を超える巨大分子である。そのため遺伝子治療は難しく、現在に至るまで有効な治療法はない。ジストロフィンは、ジストロフィン、ジストログリカン、ラミニンからなる複合体（ジストロフィン／グライコプロテイン複合体、DGC）の主な構成要素であり、細胞内ではアクチンフィラメントと結合している。ジストロフィンが存在しないと、DGC自体が膜に集積出来ないため、筋膜の修復に支障を来たし、軽度の運動負荷でも筋崩壊し、筋萎縮と筋力低下を起こす。筋線維の大小不同、円形化、中心核の増加が生じる。筋線維の変性・破壊と再生を繰り返しながら、進行性の筋萎縮が生じる。末期では骨格筋内に脂肪化や繊維化が見られる。

## 3. マイオスタチン阻害による筋萎縮の治療法開発

老化や筋ジストロフィーによる筋萎縮は治療が難しいと考えられて来たが、近年の分子生物学、細胞生物学的手法の発展に伴って、有望な治療法が開発されつつある。本総説では、萎縮した筋肉量を取り戻し、筋力を増加させるマイオスタチン阻害療法を紹介する。実験動物レベルでは、デュシェンヌ型、肢帯型等多くの筋ジストロフィーの病型でマイオスタチン阻害療法の有効性が示されている [15,16,22-27]。しかしながら、メロシン欠損型など病型によっては、病態を悪化させる場合もあるので注意が必要である [28]。筋ジストロフィーは、遺伝病なので、マイオスタチン阻害療法と原因遺伝子を補う手法とを組み合わせるとより有効性が増すと考えられている [29]。

マイオスタチン阻害の候補分子としては、マイオスタチンに結合して活性を阻害するマイオスタチン前駆体ペプチド、ホリスタチンやその改変分子、マイオスタチン阻害抗体、マイオスタチン受容体であるアクチビンタイプIIB受容体の細胞外領域（ACVR2B-Fc）等がある [30]。なお、ACVR2B-Fcとホリスタチンは、マイオスタチン阻害のみならず、アクチビンの阻害作用も有している。現状では、欧米を中心に多くの阻害抗体が開発されている。また、ACVR2B-Fcに強力な筋肥大効果が見出され、筋ジストロフィーに対する治験が行なわれつつある段階にある。マイオスタチン阻害やアクチビン阻害による筋量増加治療法は、サルコペニアや悪液質による筋萎縮にも効果があることが期待されている [31-34]。老齢マウスにおいて、マイオスタチン阻害によって、筋衛星細胞の活性化や筋損傷時でのマクロファージの遊走が促進されることで、筋再生が促進され、サルコペニアが改善するという興味深い報告がある [31]。さらに、ホリスタチン遺伝子をアデノウイルスベクターで1回投与しただけで、長期発現とサルコペニア抑制効果がある [33]。また、マイオスタチンやアクチビンの阻害分子であるホリスタチンを用いた遺伝子導入による治療が霊長類モデル動物を用いて筋力増加と筋力増強効果が証明されている [34]。ヒトへの応用も実現することが期待される。遺伝子変異によるマイオスタチン阻害では、主に速筋線維が増加し、筋線維の肥大と筋線維数増加の両方が見られる [5]。一方、最終分化が終わった後の成体のマイオスタチン阻害による筋肥大は、必ずしも速筋線維優位ではなく、筋線維数の増加よりも筋線維の肥大効果が大きいと考えられている。また、マイオスタチン阻害により、骨格筋の繊維化も抑制される [22-24]。

癌による悪液質では、脂肪量の低下と共に筋量低下が生じるが、ACVR2B-Fc投与によって、主にマイオスタチンを阻害する事で筋量増加と生存率の改善が見られている [32]。癌の悪液質に陥った骨格筋では、ジストロフィンの発現が低下し、正常なDGCが形成されないとの報告もあり、悪液質による筋萎縮と筋ジストロフィーによる筋疲弊に共通点がある可能性が示唆されている [35]。

## 4. 骨格筋と脂肪組織との臓器間相互作用について

1) 異所性脂肪沈着と疾患

内臓脂肪、皮下脂肪に加えて、第3の脂肪として異所性脂肪が近年注目されている。異所性脂肪蓄積とは、肝臓、血管、腎臓などの脂肪組織以外の組織や細胞中に脂肪細胞や脂質が蓄積した状態である。異所性脂肪細胞の脂質は毒性を持ち、細胞の機能障害や細胞死を起こして、糖尿病、動脈硬化、脂質代謝異常をきたす。外側広筋内の異所性脂肪蓄積とインスリン抵抗性との関係が解析されている。体重減少と運動を行なうと、脂肪細胞の油滴サイズは減少し、インスリン抵抗性を改善しうる [36]。

異所性脂肪の中には、組織内の細胞に脂質が蓄積する場合と、脂肪細胞そのものが出現する場合があり、骨格筋は後者の代表的な組織である。筋ジストロフィーの病態の一つにも、骨格筋内脂肪沈着があり、病態の進行を把握する指標として有用である。

脂肪細胞の起源については間葉系幹細胞に由来すると考えられていたものの、不明点も多かったが、近年細胞レベルでの詳細な解析がなされている。筋衛星細胞は、多分化能を有し脂肪細胞にも分化するとの報告もあるが、我々の詳細な解析では、脂肪分化能は検出出来ないほど低いことが示された。骨格筋内に存在する単核細胞を網羅的に単離し、その脂肪分化能を検討したところ、筋衛星細胞とは異なる、血小板由来増殖因子受容体α（PDGFRα）陽性の間葉系前駆細胞に強い脂肪細胞への分化能があることが分かった [37]。生体内への移植実験の結果では、この間葉系前駆細胞のみが脂肪細胞への分化能を持つことが示された [37]（図1及び表紙参照）。なお、移植細胞の脂肪分化は、脂肪変性条件下でのみ見られ、筋再生条件下では見られなかった。細胞の微小環境の重要性が示唆される。さらに、筋衛星細胞由来の筋線維と共培養したところ、PDGFRα陽性細胞の脂肪分化は抑制された。PDGFRα陽性細胞は、骨格筋の間質、特に、血管の近傍に存在していた。これらの結果から、骨格筋に存在するPDGFRα陽性細胞が異所性脂肪沈着に関わる主要細胞であり、筋衛星細胞と共に骨格筋の恒常性に関与するものと結論された [37]。筋ジストロフィー等の筋疾患やメタボリック症候群で骨格筋内に沈着する新たな細胞を発見した成果であり、治療標的にもなりうる細胞であり非常に興味深い細胞である。一方、我々とは別に、筋損傷時に活性化され、筋形成を促す新たな細胞集団が骨格筋内に発見された [38]。FAP (fibro/adipogenic progenitor) 細胞と名付けられた細胞で、筋肉内や皮下から細胞を移植すると脂肪分化が見られた。PDGFRα陽性細胞と同様に、筋肉内投与では脂肪変性条件下で、脂肪細胞に分化した。FAP細胞自身は、筋線維を産生しないが、筋前駆細胞の分化率を高める作用を有していた。細胞表面マーカーや細胞の性質から見て、我々の解析したPDGFRα陽性細胞と極めて類似した細胞であると考えられる [38]。筋衛星細胞による筋形成とPDGFRα陽性細胞による脂肪細胞産生のバランスが骨格筋の恒常性に重要だと考察される。

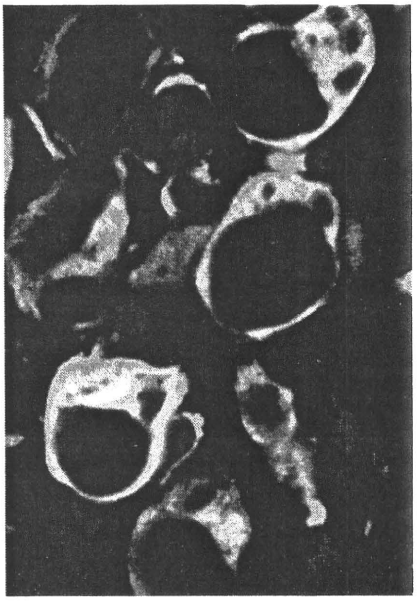

**図1.（カラー図は表紙に掲載）**
脂肪変性条件下での骨格筋内移植による間葉系前駆細胞の脂肪細胞への分化
GFPを全身に発現しているマウスの骨格筋からPDGFRα陽性の間葉系前駆細胞（緑）を単離し、脂肪変性条件下で野生型マウスの前頸骨筋に移植を行なった。PDGFRα陽性細胞が、PPARγ（赤）やペリリピン（紫）で染色される脂肪細胞の分化に寄与することが観察される。なお、核はDAPI(青)で染色されている。この結果から、骨格筋内脂肪沈着を起こすのはPDGFRα陽性の間葉系前駆細胞であること、そして、脂肪変性には骨格筋組織内で細胞がおかれる微小環境が重要であることが示された。

ヒトの骨格筋には、上記の細胞以外にも、骨髄同様に多くの治療用あるいは疾患の治療標的となる細胞集団が存在している。CD56陽性細胞は、増殖能が高く、その一部がCD56陽性細胞に由来するCD15陽性細胞と共に、間葉系細胞系譜のマーカーを発現する。この点で、骨髄由来間葉系前駆細胞と類似している。分化培養系では、CD56陽性細胞、CD15陽性細胞共に、骨分化と軟骨細胞分化能力を有しているが、前者は筋分化能を有し、一方後者のCD15陽性細胞は脂肪細胞分化能を有する点で違いが見られる [39]。

一方、異所性脂肪の解析の進展と共に、脂肪組織の脂肪細胞を生み出す細胞の解析も近年、急速な展開を見せている。脂肪組織は白色脂肪組織と褐色脂肪組織に分類されるが、両者は組織学的にも機能的に異なっており、発生過程にも違いがある。白色脂肪は、全身に存在し、余剰エネルギーを中性脂肪として貯蓄している。最近、白色脂肪組織内の壁細胞が白色脂肪の供給源となることが示された。脂肪組織に存在するLin(-)CD29(+)CD34(+)Sca-1(+) 細胞や血管近傍のPPARγ陽性細胞が脂肪細胞の前駆細胞として働く [40, 41]。

褐色脂肪細胞は、肩甲骨周囲、腋下などに存在し、ミトコンドリアに存在するUCP1（uncoupling protein 1, 脱共役タンパク質1）を介して熱産生に働く。エネルギー消費の役割を担う脂肪細胞である。褐色脂肪細胞は、白色脂肪細胞よりも骨格筋細胞系譜に類似した遺伝子発現を示す。Myf 5は、筋形成に関わる筋芽細胞のマーカーであるが、Myf 5陽性細胞から筋芽細胞と褐色脂肪細胞が分化するという報告がなされた [42]。その際に重要な働きをするのが、PRDM16と呼ばれるZinc finger型の転写因子である。筋芽細胞にPRDM16を発現させると褐色脂肪細胞に分化する。逆に、PRDM16活性が弱いとMyf 5陽性細胞の褐色脂肪細胞への分化能が低下し、筋分化が活性化する。PRDM16は、PPARγと結合しその転写活性を上昇させる働きがある[42]。身体中に存在する褐色脂肪以外に、人為的に褐色脂肪細胞を産生させることが可能である。例えば、ノルアドレナリンのβ3アゴニストを投与すると、白色脂肪内に褐色脂肪が出現する。寒冷刺激も褐色脂肪産生に働く。興味深い事に、これらの褐色細胞は、Myf 5陽性細胞由来ではない。

従って、褐色脂肪の起源は単一ではなく、Myf 5陽性細胞以外に、白色脂肪細胞と褐色脂肪細胞を生み出す共通の脂肪前駆細胞が存在する可能性があると考えられている [43]。

2) マイオスタチンが仲介する骨格筋と脂肪細胞の相互作用（図2）

骨格筋と脂肪細胞は生体内で相互に連携し生理的作用を発揮している。マイオスタチンのノックアウトマウスやマイオスタチン阻害によって、筋量が増加すると共に、全身の体脂肪量が減少する事が知られている [44]。実際、我々が開発したホリスタチンに由来するマイオスタチン阻害因子を発現させたマウスの脂肪組織は、対照と比較して内臓脂肪量、皮下脂肪量共に著しく低下し脂肪細胞が肥大化しない。高脂肪食を負荷しても脂肪量の上昇は抑制され、特に脂肪肝は全く見られない。マイオスタチン阻害によって筋量が増加し、二次的に脂肪組織や肝臓に影響が現れると考えられている。脂肪肝形成阻害の分子機構として、ステアリルCoA不飽和化酵素の低下、オレイン酸等の不飽和脂肪酸の減少が観察された。マイオスタチン阻害療法は、筋量を適切に維持する事で、体脂肪の蓄積を防ぎ、脂肪肝の形成も阻止出来る可能性が示されたと言える。

## 5. おわりに

我が国は、超高齢化社会を迎えており、筋骨格系の機能を維持しながら、健康に老いる事が重要になっている。本総説では、骨格筋の萎縮機構、マイオスタチン制御による筋量増加作用と近年急速な展開が見られる脂肪細胞を生み出す新たな細胞群について紹介した。こういった研究によって、老化、神経筋難病、悪液質の病態が明らかとなり、治療法開発に繋がる事を期待したい。また、骨格筋や脂肪細胞の基になる幹細胞の解析は、筋疾患、肥満／糖尿病における脂肪細胞の動態に対する理解を深め、新たな再生医療の開発にもつながることが期待される。

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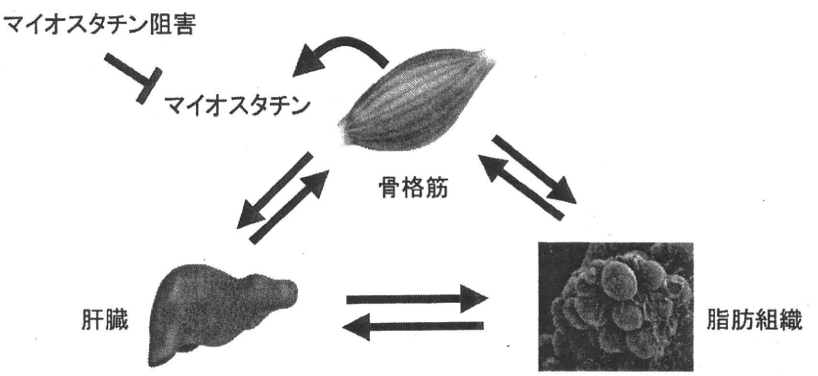


**図2. マイオスタチン阻害による組織間のクロストーク。**
マイオスタチンは、骨格筋から産生されて、血液中を循環する細胞増殖因子である。マイオスタチンは、骨格筋形成を強力に抑制する。マイオスタチンの活性を阻害すると骨格筋量の増加が見られる。それと共に、脂肪細胞が肥大化せず、脂肪量が減少する。高脂肪食を負荷しても、脂肪肝の形成が見られないことも分かっている。骨格筋組織は生体内で隣接する骨格筋内の細胞のみならず、脂肪組織や内臓臓器との間で、サイトカインの産生と受容を介して相互連携しており、生体の恒常性維持に重要な役割を果たしている。マイオスタチンの活性制御によって、骨格筋－脂肪組織－肝臓の組織間相互作用を介して、体内の脂肪動態が影響を受ける。