

Inhibition of myostatin in adult mice increases skeletal muscle mass and strength

Lisa-Anne Whittemore,* Kening Song, Xiangping Li, Jane Aghajanian, Monique Davies, Stefan Girgenrath, Jennifer J. Hill, Mary Jalenak, Pamela Kelley, Andrea Knight, Rich Maylor, Denise O'Hara, Adele Pearson, Amira Quazi, Stephanie Ryerson, Xiang-Yang Tan, Kathleen N. Tomkinson, Geertruida M. Veldman, Angela Widom, Jill F. Wright, Steve Wudyka, Liz Zhao, and Neil M. Wolfman

Musculoskeletal Sciences Department, Wyeth Research, 200 CambridgePark Drive, Cambridge, MA 02140, USA

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Abstract

A human therapeutic that specifically modulates skeletal muscle growth would potentially provide a benefit for a variety of conditions including sarcopenia, cachexia, and muscular dystrophy. Myostatin, a member of the TGF- β family of growth factors, is a known negative regulator of muscle mass, as mice lacking the myostatin gene have increased muscle mass. Thus, an inhibitor of myostatin may be useful therapeutically as an anabolic agent for muscle. However, since myostatin is expressed in both developing and adult muscles, it is not clear whether it regulates muscle mass during development or in adults. In order to test the hypothesis that myostatin regulates muscle mass in adults, we generated an inhibitory antibody to myostatin and administered it to adult mice. Here we show that mice treated pharmacologically with an antibody to myostatin have increased skeletal muscle mass and increased grip strength. These data show for the first time that myostatin acts postnatally as a negative regulator of skeletal muscle growth and suggest that myostatin inhibitors could provide a therapeutic benefit in diseases for which muscle mass is limiting.

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Myostatin, also known as growth and differentiation factor-8 (GDF-8), is a member of the TGF- β superfamily of secreted growth factors. A number of growth factors in this family have been shown to mediate growth and differentiation during development [1,2]. In addition, some TGF- β family growth factors have a role in proliferation and regeneration of adult tissue. Myostatin is unique among the TGF- β superfamily because its expression is almost exclusively restricted to the skeletal muscle lineage [3]. During embryogenesis, myostatin is expressed in the myotome compartment of the somites and later in fetal development it is expressed in the developing limb muscles. In adult mice myostatin is expressed in all skeletal muscle, though it is prefer-

entially expressed in muscle composed primarily of fast twitch fibers over muscles composed primarily of slow twitch fibers (unpublished observation and [4]).

Myostatin knockout mice have two- to threefold greater muscle mass than their wild type littermates. The increased muscle mass is the result of fiber hypertrophy and hyperplasia [3]. In addition, the myostatin knockout mice accumulate less fat than their wild type littermates [5,6], which may be a secondary consequence of increased muscle mass since a similar effect is seen in other genetic models of muscle hypertrophy [7,8]. Aside from having increased muscle and decreased fat, the myostatin knockout mice appear normal and healthy. Thus it appears that myostatin is a specific negative regulator of skeletal muscle mass.

Since myostatin is expressed in both developing and adult muscles, it is not clear whether it regulates muscle

* Corresponding author. Fax: 1-617-665-5386.

E-mail address: lwhittemore@wyeth.com (L.-A. Whittemore).

mass during development or in adults. The question of whether or not myostatin regulates muscle mass in adults is critical from a scientific and therapeutic perspective.

If myostatin regulates muscle mass in adults, it is likely to do so by regulating satellite cells or muscle stem cells. Furthermore, from a therapeutic perspective, pharmacological inhibition of myostatin may provide a novel and safe approach to the treatment of diseases in which functional muscle mass is limiting.

There is currently no direct evidence that myostatin regulates muscle mass in adults, but several studies suggest that this might be the case. Zimmers et al. [9] have recently reported that systemic over-expression of myostatin in adult mice leads to wasting. Their studies suggest that dysregulation of myostatin may have a detrimental effect on adult muscle. Several groups have observed a negative correlation between myostatin levels and muscle mass in adult rodents under conditions of muscle hypertrophy or atrophy [4,10–14] and in humans under conditions of muscle loss [15–17]. These observations are consistent with the hypothesis that myostatin negatively regulates muscle mass in adults, but do not exclude the alternative possibility that changes in myostatin levels result from changes in muscle size.

In order to address directly the question of whether or not pharmacological inhibition of myostatin specifically increases muscle mass, we developed a neutralizing monoclonal antibody to myostatin and administered it to mice. This treatment leads to an increase in muscle size and in grip strength. The increase in muscle size is the result of fiber hypertrophy. Aside from the increase in fiber size, the muscle appears histologically normal. Furthermore, the myostatin antibody treated mice have normal organ size and histology, and normal serum parameters. Thus inhibition of myostatin in adults specifically increases skeletal muscle size without obvious side effects.

Methods

Monoclonal antibody preparation. Myostatin knockout mice were immunized with recombinant myostatin purified from CHO cell conditioned media [18] and hybridoma cells were generated using standard techniques [19]. Hybridoma cells secreting anti-myostatin antibodies were identified by solid and solution phase ELISA [20] to recombinant myostatin. We then used standard ELISA techniques [20] and a pGL3-(CAGA)₁₂ reporter assay to determine the IC₅₀ with which selected antibody clones inhibited the binding of myostatin to its receptor, ActRIIB [18]. For the ActRIIB ELISA, ActRIIB.Fc chimera was purchased from R&D Systems (Minneapolis, MN; catalogue #339-RB-100). The pGL3-(CAGA)₁₂ reporter assay was performed as described [21].

Direct binding assay. Synthetic peptides representing the amino terminal of myostatin or Gdf11 were conjugated to bovine serum albumin (BSA) using a Pierce conjugation kit (Pierce; catalogue #77116ZZ) following the manufacturer's protocol. The BSA conjugated peptides were coated on 96-well flat-bottomed assay plates at 1 µg/ml in 0.2 M sodium carbonate buffer overnight at 4 °C. The plates

were washed and blocked with PBS, 1 mg/ml BSA, and 0.05% Tween for 1 h at room temperature. JA16 (5 nM) was serially diluted (1:2) and the dilutions were added to the ELISA plate and incubated for 30 min at RT. After four washes, a secondary antibody (Goat anti-murine IgG (H+L)-HRP, Calbiochem, San Diego, CA; catalogue #401215) was added at a 1:1000 dilution and incubated for 30 min at RT. Plates were washed four times, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added (KPL, Gaithersburg, MD; catalogue #50-76-04). Colorimetric measurements were done at 450 nm in a Molecular Devices (Sunnyvale, CA) microplate reader.

Competition ELISA. In order to assay for relative binding to myostatin and Gdf11, purified recombinant mature myostatin was coated on 96-well flat-bottomed assay plates at 1 µg/ml in 0.2 M sodium carbonate buffer overnight at 4 °C. Plates were then blocked with 1 mg/ml BSA and washed following standard ELISA techniques [20]. JA16 at 0.75 ng/ml was preincubated with serial dilutions of either myostatin or Gdf11 for 30 min and then added to the blocked ELISA plates, incubated for 1 h, and washed. The amount of bound JA16 was detected as described above for the direct binding assay.

Animals. C57Bl/6 male mice and BALB/c female mice (7–8 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were dosed weekly with antibody (JA16 or an isotype matched anti-snake venom protease as a control) by intraperitoneal injection at 60 mg/kg/week. Vehicle for injection was PBS, pH 7.2. Mice were weighed weekly during the treatment period. A PIXImus small animal densitometer (GE Medical Systems; Waukesha, WI) was used to carry out dual energy X-ray absorptiometry analysis (DEXA). Mice were anesthetized with isoflurane and subjected to 5 min DEXA scans at the beginning and end of the study. Grip tests were performed with a grip strength meter (model 1027csx) purchased from Columbus Instruments (Columbus, OH). Untrained mice were tested five times in succession without rest and the results of the five tests were averaged for each mouse. Serum samples were taken at the end of the study, frozen, and sent to AniLytics (Gaithersburg, MD) for analysis of the following parameters: calcium, phosphorus, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatine kinase, urea nitrogen, creatinine, cholesterol, triglycerides, total protein, albumin, insulin, glucose, and leptin. At the end of the study, a portion of the quadriceps (rectus femoris), the gastrocnemius, triceps, and the EDL were dissected and weighed by investigators who had no knowledge of the treatment group for each mouse.

Histological analysis. Muscles were removed from the mice and either fixed in formalin and embedded in paraffin or frozen. Paraffin sections were stained with hematoxylin and eosin. For fiber counting and cross-sectional area measurement, muscles were cryosectioned and stained with an antibody to dystrophin (Novocastra Laboratories, Ontario, Canada; catalogue #NCL-DYSB) to delineate the sarcolemma. Morphometric analysis was done on sections of EDL from the mid-belly of the muscles by investigators who had no knowledge of the treatment group for each mouse.

Statistical analysis. Error bars indicate standard error of the mean. (**) indicates that $P < 0.01$, and (*) indicates that $P < 0.05$, for a Student's *t* test comparing the JA16 group to the control antibody group or vehicle group.

Results

In order to determine if myostatin has a role in regulating muscle growth postnatally, we developed an inhibitor of myostatin that could be administered pharmacologically to adult mice. The inhibitor that we used in these studies is a monoclonal antibody called JA16. This antibody was generated in myostatin knockout mice by immunizing with purified recombinant

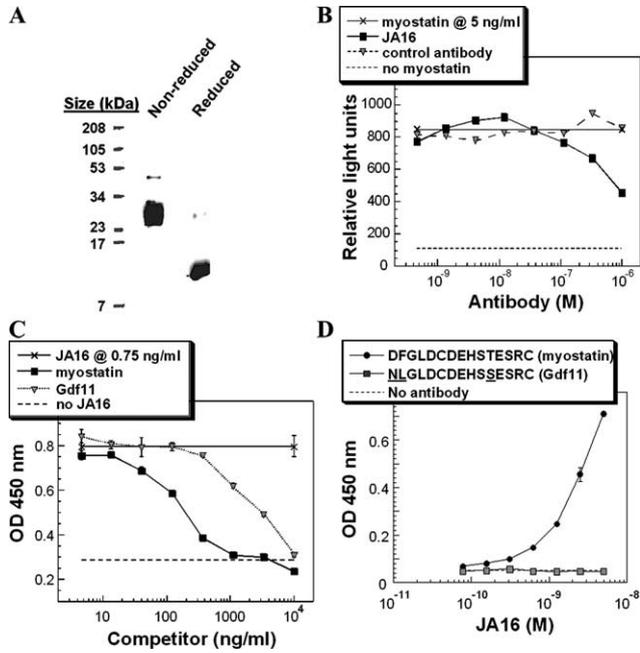


Fig. 1. Binding of JA16 to myostatin. (A) Western blot analysis showing binding of JA16 to recombinant myostatin. (B) pGL3-(CAGA)₁₂ reporter assay showing inhibition of myostatin signalling. (C) Competition ELISA showing differential binding of JA16 to myostatin and Gdf11. (D) Direct binding of JA16 to synthetic peptides representing the amino terminal of mature myostatin and Gdf11.

human myostatin protein, which is identical in amino acid sequence to mature murine myostatin (C-terminal polypeptide) [22]. Western blot analysis (Fig. 1A) shows that JA16 binds to recombinant myostatin, which appears as a band at 25 kDa under non-reducing conditions and 12.5 kDa under reducing conditions. These sizes correspond to the predicted molecular weights of dimeric and monomeric mature myostatin, respectively, based on the nucleotide sequence [3]. We also measured the neutralizing activity of JA16 using a pGL3-(CAGA)₁₂ reporter assay [21] and determined that JA16 blocks myostatin induced signaling with an IC₅₀ of approximately 500 nM (Fig. 1B). Thus, in addition to binding recombinant myostatin, high concentrations of JA16 block myostatin signaling.

Table 1
Summary of in vivo studies with JA16

Study	Strain	Sex	Age (weeks)	Dose (mg/kg/week)	Duration (weeks)	Difference in muscle mass			
						Quad (%)	Gastroc (%)	EDL	Triceps
1	C57Bl/6	M	5–8	60	15	30**	23**	ND	ND
2	BALB/c	F	7–8	60	4	21**	19**	ND	ND
3	BALB/c	F	7–8	60	2	13*	14*	ND	ND
	BALB/c	F	7–8	60	4	19**	14**	ND	ND
4	BALB/c-SCID	F	7–8	60	4	17**	13**	13**	ND
5	BALB/c	F	7–8	60	8	26**	21**	25**	ND
6	BALB/c	F	24	60	5	13**	11**	ND	12*
	BALB/c	F	8	60	5	17**	14**	ND	16**

ND, not determined.

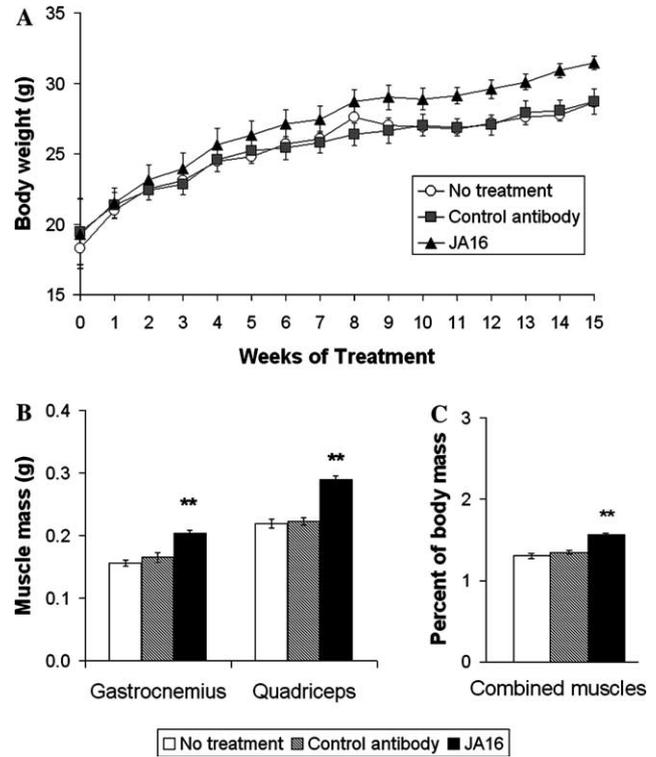


Fig. 2. JA16 treatment of C57Bl/6 mice for 15 weeks. (A) Body weight during the treatment period. (B) Dissected muscle mass at the end of the study *n* = 6 for each group.

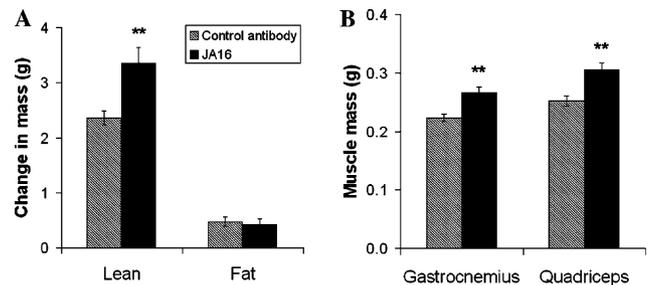


Fig. 3. JA16 treatment of BALB/c mice for four weeks. (A) DEXA scan analysis showing the change in lean body mass and total body fat mass during the treatment period. (B) Dissected muscle mass at the end of the study *n* = 8 for each group.

We have previously shown by affinity purification and mass spectrometry that JA16 binds specifically to myostatin in mouse serum where myostatin circulates as a latent complex with its propeptide [23]. Under these conditions, JA16 does not bind to other circulating TGF- β family members. Because Gdf11, the most closely related TGF- β family member, is 90% identical to myostatin [24] we compared the affinity of JA16 for myostatin and Gdf11 by competition ELISA. In this assay, myostatin was 10-fold more effective than Gdf11 at inhibiting the binding of JA16 to myostatin (Fig. 1C). In addition, we mapped the epitope to which JA16 binds using synthetic peptides representing the entire myostatin protein and found that JA16 binds specifically to amino terminal peptides where Gdf11 differs from myostatin by three of 15 amino acids (data not shown). In a direct binding assay we found that JA16 binds to a peptide from the amino terminal of myostatin (DFGLDCDEHSTESRC), but does not bind to the

analogous peptide from the amino terminal of Gdf11 (Fig. 1D). These results suggest that JA16 specifically binds to myostatin over other TGF- β family members, including Gdf11.

JA16 was administered to mice in order to determine if blocking myostatin increases muscle mass postnatally. Five to eight-week-old C57Bl/6 male mice were randomized with respect to body weight and placed into treatment groups of six mice each. Mice were treated for 15 weeks with JA16 or with an isotype matched control antibody at 60 mg/kg/week (Table 1, study 1). The average body weight for each group of mice increased during the study, but the JA16 treated mice gained approximately 10% more weight than the control or untreated mice (Fig. 2A). At the end of the study the quadriceps and gastrocnemius muscles were dissected and weighed. The mass of the muscles for the JA16 treated mice was greater than the mass of the muscles for the control antibody treated mice. The

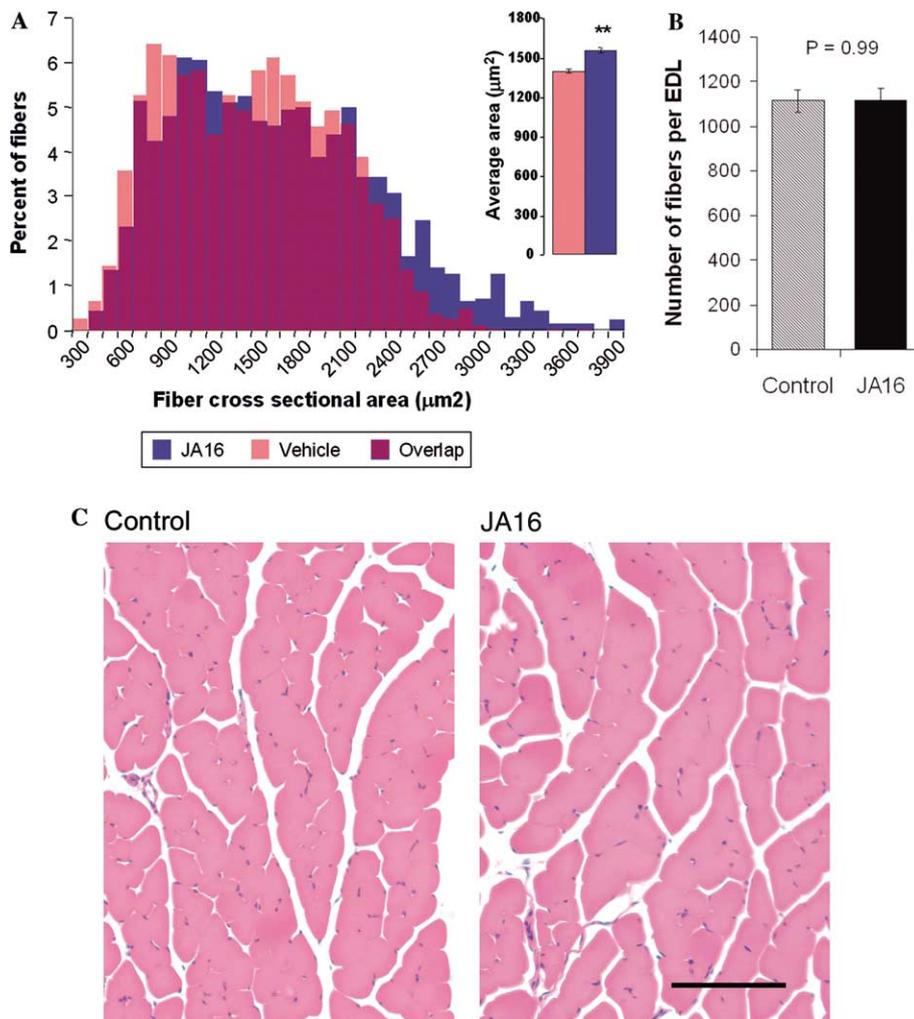


Fig. 4. Morphometric analysis of EDL from BALB/c mice treated with JA16 for four weeks. (A) Cross-sectional area of individual fibers from the EDL. A total of 1260 and 1440 fibers from JA16 and vehicle treated mice, respectively, were measured. The inset graph shows the average fiber area for each group. (B) Fiber counts from the EDL $n = 7$ for each group. (C) Hematoxylin and eosin staining of the EDL. The bar indicates 100 μm .

quadriceps was 30% larger and the gastrocnemius was 23% larger (Fig. 2B). In order to exclude the possibility that the increase in muscle mass is due to an overall increase in size of the mice, we calculated the muscle size relative to body weight. The normalized muscle size is greater in JA16 treated mice than in control mice (Fig. 2C), indicating that the muscles are larger relative to the rest of the body. We also weighed the epididymal fat pad, inguinal fat pad, kidney, liver, and heart, and found no difference between groups in organ tissue mass. Likewise we did not detect histological changes in any of these organs nor were there any changes in serum parameters. These results suggest that pharmacological inhibition of myostatin specifically increases skeletal muscle mass.

In a short-term study we treated 7–8-week-old BALB/c female mice with JA16 for four weeks (Table 1, study 2). These mice were subjected to dual energy X-ray absorptiometry (DEXA) analysis at the beginning and end of the study in order to measure changes in lean body mass and total body fat mass [25]. During the course of the study, the JA16 treated mice gained more lean body mass than control antibody treated mice (Fig. 3A). The gain in fat mass for each group was indistinguishable (Fig. 3A). At the end of the study the difference in muscle mass between JA16 treated and control antibody treated mice was 21% for the quadriceps and 19% for the gastrocnemius (Fig. 3B). In a separate experiment we have detected a difference in muscle mass as early as two weeks after JA16 treatment (Table 1, study 3). Thus, inhibition of myostatin for as little as 2–4 weeks leads to an increase in muscle mass.

Skeletal muscle is composed of post-mitotic, multinucleated fibers. The increase in muscle mass observed with anti-myostatin treatment could result from increased muscle fiber number or size. The muscles of myostatin knockout mice are both hypertrophic and hyperplastic, while the muscles of transgenic mice carrying a dominant negative version of myostatin expressed from a muscle-specific creatine kinase (MCK) promoter are hypertrophic, but not hyperplastic [26]. One explanation for this apparent discrepancy is that muscle hyperplasia observed in myostatin knockout mice is an early developmental effect which does not occur in the dominant negative transgenic mice because the MCK promoter is activated later in development than the endogenous myostatin gene. In order to determine the effect of postnatal myostatin inhibition on muscle fibers, we measured fiber cross-sectional area and counted myofibers in the extensor digitorum longus (EDL) of JA16 treated and vehicle treated mice. In this experiment mice were treated with JA16 or vehicle for 4 weeks. The difference in muscle mass between the EDL of JA16 and vehicle treated mice was 13% (Table 1, study 4). The difference in average fiber cross-sectional area was 11% (Fig. 4A) while there was no sig-

nificant difference in fiber number between JA16 and vehicle treated mice (Fig. 4B). These data indicate that the increase in muscle mass with JA16 treatment was the result of fiber hypertrophy and not hyperplasia. Aside from the increase in fiber size, the muscles from JA16 treated mice appeared histologically normal (Fig. 4C).

In order to determine if the increase in muscle size with myostatin inhibition is accompanied by an increase in strength, we performed grip strength tests on mice treated with JA16 for 8 weeks (Table 1, study 5). The mass of the gastrocnemius, quadriceps, and EDL was greater in JA16 treated mice than in vehicle treated mice by 21%, 26%, and 25%, respectively (Fig. 5A). When mice were subjected to a grip test, the peak force for the JA16 treated mice was 10% greater than the peak force for vehicle treated mice (Fig. 5B). These results suggest that inhibition of myostatin not only leads to an increase in muscle mass but also to an increase in grip strength.

The results in 7–8-week-old mice indicate that blocking myostatin postnatally leads to an increase in muscle mass. The mice used in these studies, though sexually mature, were still rapidly growing. In order to determine if blocking myostatin increases muscle mass in adult mice that are no longer growing, we compared the response of 24-week old mice to the response of eight-week-old mice. Following a five-week treatment with JA16 the difference in muscle mass between JA16 treated and vehicle treated 24-week old mice was 13%, 11%, and 12% for the quadriceps, gastrocnemius, and triceps, respectively (Table 1, study 6). The magnitude of this effect is less than the magnitude of the effect in the younger mice where there was a 17%, 14%, and 16% difference in the mass of the quadriceps, gastrocnemius, and triceps, respectively (Table 1, study 6). Nevertheless, these results clearly indicate that blocking myostatin increases muscle mass in adult mice that are no longer growing.

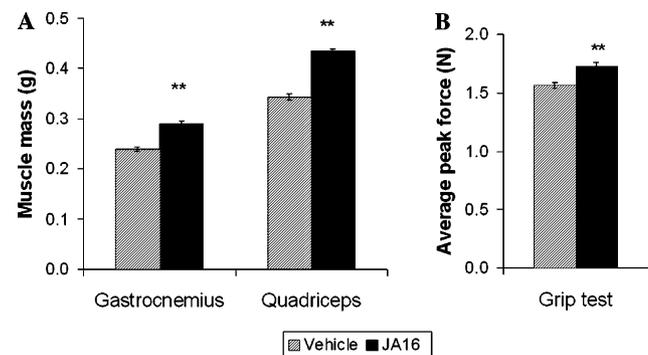


Fig. 5. JA16 treatment of BALB/c mice for eight weeks. (A) Dissected muscle mass at the end of the study. (B) Grip strength after seven weeks of treatment $n = 8$ for each group.

Discussion

The results of these studies demonstrate that inhibition of myostatin in adults for as little as 2–4 weeks leads to an increase in skeletal muscle size. The increase in muscle mass, which varies from 13% to 30%, is highly reproducible and occurs in both male and female mice of a variety of strains. The effect is seen in young adults as well as 24-week-old mice. Mice treated with the myostatin antibody have normal organ size and histology, and normal serum parameters, suggesting that inhibition of myostatin in adults specifically increases skeletal muscle size without side effects.

Interestingly, we do not detect a change in fat pad mass with myostatin inhibition. This result differs from the results reported for the myostatin knockout mice in which fat accumulation is decreased in the absence of myostatin [5,6]. Since the increase in muscle mass observed in our studies is approximately one order of magnitude less than the increase seen in the myostatin knockout mice, the effect on fat could reflect a quantitative rather than qualitative difference. Alternatively, the decrease in fat accumulation may be dependent on loss of myostatin expression during development.

Based on these results, one model for the function of myostatin in adult muscle is that it maintains satellite cells or muscle stem cells in a quiescent state. Reduced myostatin activity would lead to activation of these cells and fusion into existing fibers, leading to fiber hypertrophy. Several *in vitro* studies support this model. Treatment of C₂C₁₂ myoblasts with recombinant myostatin blocks their proliferation [27,28]. Likewise expression of the myostatin gene in C₂C₁₂ cells blocks proliferation and myogenic differentiation [29]. We are currently investigating whether inhibition of myostatin *in vivo* with JA16 affects satellite cell number or activation.

Myostatin is highly conserved in sequence and in function across species. The amino acid sequence of murine and human myostatin is identical [3], as is the pattern of mRNA expression [3,16]. There are several naturally occurring myostatin mutations in cattle, which have been linked to the double muscled phenotype [22,30–32]. This conservation of sequence and function suggests that inhibition of myostatin in humans is likely to have a similar effect to inhibition of myostatin in mice. Furthermore, several groups have investigated the level of myostatin in a variety of human disease states and in some cases there is a correlation between elevated myostatin levels and muscle loss [15–17].

There are several muscle diseases for which a myostatin inhibitor may provide a novel therapeutic approach. Sarcopenia, or age related muscle atrophy, affects many elderly people, and for some, this increases the risk of injury and impairs their ability to live a normal life [33]. Increased muscle mass could restore

muscle strength and prevent injuries. Cachexia is a form of wasting that affects 50% of cancer patients [34]. Increased muscle strength in cachectic patients may improve quality of life, improve response to cancer therapy, and increase life span. There are also a variety of muscular dystrophies, including Duchenne muscular dystrophy, for which increased skeletal muscle may provide a therapeutic benefit. We have demonstrated that treatment of normal mice with a myostatin antibody significantly increases skeletal muscle mass and leads to increased grip strength. Others have used the same antibody and made similar observations in developing mice with muscular dystrophy [35]. These results confirm that pharmacological inhibitors of myostatin may provide significant therapeutic benefit for these devastating muscle diseases.

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