Supporting Information

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SI Text

Production and Injection of AAV Vector. The myostatin propeptide construct, prepared by PCR amplification of C57Bl6 cDNA, using the primers 5′-CCGCTCGAGATGATGCAAAAACTG-CAAATG-3′ and 5′-CCGGGATCCCTATTAGTCTCTC-CGGGACCTCTT-3′, was introduced into an AAV-2-based vector between the 2 inverted terminal repeat and under the control of the cytomegaly virus promoter using the XhoI and BamHI sites.

The AAV myostatin propeptide and AAV-muSeAP (34) vectors were produced in human embryonic kidney 293 cells by the triple-transfection method using the calcium phosphate precipitation technique with either the pAAV2 propeptide plasmid or the pAAV2 muSeAP plasmid, the pXX6 plasmid coding for the adenoviral sequences essential for AAV production, and the pRepCAp plasmid coding for AAV1 capsid. The virus was then purified by 2 cycles of cesium chloride gradient centrifugation and concentrated by dialysis. The final viral preparations were kept in PBS solution at −80 °C. The particle titer (number of viral genomes) was determined by a quantitative PCR.

Vector was injected into tibialis anterior muscle (TA) of female C57Bl6 mice using 50 μL of AAV-prop virus containing a single-vector dose of 10^{11} to 4×10^{11} viral genome. The contralateral TA was injected with 50 μL AAV-muSeAP with the same viral quantity. Alternatively, vector was injected into male *Myf5*$^{nlacZ/+}$ mice on a C57BL/6 background. Age and number of injected animals are shown in Table 2.

Myofiber Culture. Freshly isolated myofibers were placed in 24-well Primaria plates (Marathon) coated with 1 mg/mL of Matrigel (Collaborative Research) and incubated in growth medium (DMEM with 10% (vol/vol) horse serum (PAA Laboratories) and 0.5% (vol/vol) chicken embryo extract (ICN Biomedicals) at 37 °C in 5% CO_2. Cultures were supplemented with J16-antibody at a concentration of 168 μg/mL (gift from L.A. Whittemore) or with recombinant myostatin at 100 ng/mL (R&D Systems). Myofibers and cells were fixed in 4% PFA/PBS for 5−20 min and myoblasts counted under an inverted microscope. The following numbers of cultures were performed: 258 cultures from 8 EDL muscles from 2-month-old mstn$^{-/-}$ mice; 220 cultures from 7 EDL muscles from 2-month-old C57BL/6 wild-type mice; 127 cultures treated with J16 from 4 EDL muscles from 2-month-old mstn$^{-/-}$ mice; 210 cultures from 5 EDL muscles from 1-year-old mstn$^{-/-}$ mice; 152 cultures from 4 EDL muscles from 1-year-old C57BL/6 wild-type mice; 134 cultures treated with J16 from 4 EDL muscles from 1-year-old mstn$^{-/-}$ mice. Forty-one cultures were treated with recombinant myostatin and compared to 44 nontreated control cultures.

Verification of Recombinant Myostatin Activity. The biological effect of recombinant myostatin (concentration as stated in the results) was tested on high-density cultures of mouse embryonic mesenchyme, which were carried out as described previously (37). Micromass cultures were fixed in 4% PFA for 10 min, washed in PBS, incubated in 0.5% Triton X-100/PBS for 5 min, and preincubated in 20% goat serum in PBS for 20 min. Primary antibodies used were anti-Pax-7 [Developmental Studies Hybridoma Bank (DSHB)], anti-MHC (clone A4.1025, DSHB) and anti-MyoD (Santa Cruz). Recombinant myostatin (10 ng/mL and 100 ng/mL) inhibited the formation of Pax-7 and MyoD expressing muscle precursors and of myosin heavy chain positive cells (data not shown).

Immunohistochemistry and Analysis of Revertant Fibers. EDL muscles were weighed, mounted in OCT (BDH), and frozen in melting isopentane cooled in liquid nitrogen. Unfixed 10-μm transverse cryosections from the midbelly region of EDL muscles from *mstn*$^{-/-}$*mdx* mice and *mstn*$^{+/+}$*mdx* littermates were cut over a distance of 1 mm and every tenth section analyzed. Sections were blocked in 10% goat serum for 30 min and thereafter incubated for 1 (1/2) hours with primary antibodies P7, which detects dystrophin positive revertant fibers (28). Subsequently cryosections were washed in PBS, incubated with biotinylated swine anti-rabbit IgG (DAKO) for 1 h, washed in PBS, and incubated in streptavidin Alexa Fluor 594 conjugate (Molecular Probes) for 1 h. Muscle fibers were regarded as dystrophin positive (revertant fibers, RF) when more than half the membrane circumference was stained on cross-sections. RFs adjacent to each other were characterized as a single cluster (at least 3 RFs). The total number of myofibers per cross-section, the maximal number of RFs, the number of revertant clusters, the number of RFs in each cluster, and the number of isolated single RFs were quantified and compared. For number of mice see supporting information (*SI*) Table S2.

Analysis of Myofibers Containing Centrally Located Nuclei. Unfixed cryosections from the midbelly region of EDL muscles from *mstn*$^{-/-}$*mdx* mice and *mstn*$^{+/+}$*mdx* littermates were blocked in 10% goat serum for 30 min and thereafter incubated with antibody against dystroglycan-β (Novo Castra) for 1 (1/2) hours. Cryosections were then washed in PBS, incubated in biotinylated rabbit anti-mouse IgG (DAKO), then incubated with an avidin-biotin complex (Vector Laboratories) and stained using a nickel enhanced DAB/hydrogen peroxide reaction (Vector Laboratories) according to manufacturer's protocols. Subsequently, cryosections were counterstained with hemalum for the detection of myonuclei. Total number of myofibers and number of myofibers containing centrally located nuclei were determined on 3–4 fields of view per section (a total of 305–786 myofibers for each mouse). For numbers and ages of mice see Table S2.

Histological Analysis of AAV-Injected Muscles. AAV vector was injected as stated above. After killing of injected mice, tibialis anteriors were dissected from both legs, weighted, mounted in OCT, and frozen in isopentane cooled in liquid nitrogen.

For fiber analysis, transverse cryostat sections (8 μm) were obtained and laminin labeling performed (see below). Ellix software (Microvision) was used to analyze the number and CSA of fibers.

For analysis of myonuclear number, transverse cryostat sections (10 μm) were fixed in 2% paraformaldehyde for 30 min and incubated 1 hour in a blocking solution (BSA 1%, sheep serum 1%, triton X-100 0.1%, and sodium azide 0.001%). Sections were then incubated with a rabbit antibody directed against laminin (Dako, Z0097, 1/300) and with a Cy3-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, 111–165-144, 1/200). Slides were mounted in a mounting solution (mowiol/dabco/hoechst). Morphometric analyses were performed using Metamorph software (Roper Scientific). The unit of cross-sectional area was arbitrary and given in pixels. Hoechst positive nuclei encompassed by laminin staining were considered as myonuclei. Satellite cells, which are also located at this

position and thus included in the counts, were considered too rare to influence the results and, on independent counts, did not differ significantly between strains.

LacZ-expressing nuclei after AAV injection into *Myf5*$^{nlacZ/+}$ mice were visualized on transverse cryostat sections (10 μm) of injected TA muscles using standard protocols for X-Gal staining. Most X-Gal positive nuclei were found in a juxta-sarcolemmal position and considered as satellite cells. Occasion X-Gal positive nuclei found within muscle fibers, were not considered to be satellite cells and not included in the counts. X-Gal positive nuclei and myofiber number were counted on whole cross-sections of TA (2 sections per muscle) using DIC microscopy (Zeiss).

Real-Time Quantitative PCR. Real-time PCR was performed according to standard protocols on a DNA Engine Opticon 2 System (Bio-Rad). One hundred nanograms of RNA, DNase treated, were reverse transcribed and 1/3 dilutions of the reaction were used for each PCR. PCR was performed using the following primers: fwd = *5′ CAT TTG TGC ATT TTG GGT GT* and rev = *5′ ACC AAA TCT TCC CCT TGC TT* for Acvr2a; fwd = *5′ CTT CTC TGG GGA TCG CTG T* and rev = *5′ CAG CCG CTT GTC CTG TTC* for Acvr2b; fwd = *5′ GCC TTC TAC GCA CCT GGA C* and rev = *5′ TAG TAG GCG GTG TCG TAG CC* for MyoD; fwd = *5′ GTT AAG CAG TAC AGC CCC AAA* and rev = *5′ AGG GCA TAT CCA ACA ACA AAC TT* for HPRT1. PCR programs were 10 min at 95 °C, followed by 40 cycles of 15 seconds at 95 °C, and 30 seconds at 62 °C. The thermal denaturation protocol was run at the end of PCR to determine the number of products. Reactions were run in duplicate with 2 biological repeats for each time point. The cycle number at which the reaction crossed an arbitrarily placed threshold ($C\tau$) was determined for each gene. The relative amount of mRNA was determined by $2^{-\Delta C}\tau$. Relative gene expression was first normalized to HPRT1 expression and secondly normalized to MyoD expression, a normalization which, with the entry of satellite cells into quiescence, would tend to understate the drops in receptor transcript.

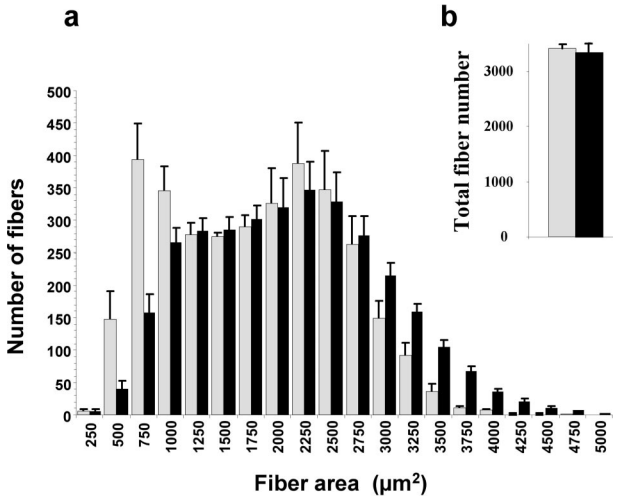

Fig. S1. Overexpression of myostatin propeptide induces muscle fiber hypertrophy. (*a*) Fiber-size distribution in the TA muscles 2 months after injection of AAV myostatin propeptide (black bars) and AAV-muSeAP (gray bars). Each bar represents the mean from six C57Bl6 female mice. (*b*) Number of muscle fiber profiles per midbelly section of above TA muscles: AAV myostatin propeptide (black bars) and AAV-muSeAP (gray bars).

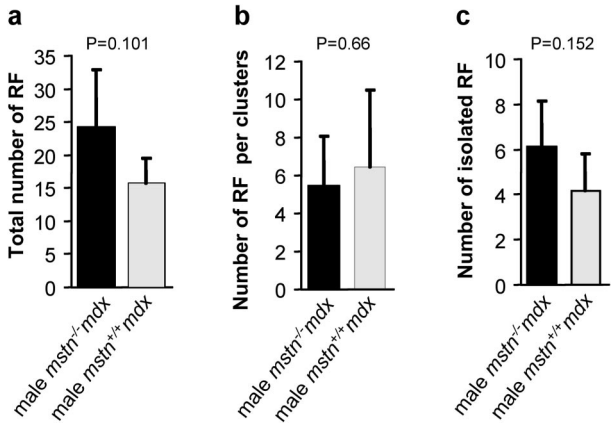

Fig. S2. Quantification of revertant fibers (RFs) per cross-section of EDL muscles from *mstn*$^{-/-}$*mdx* mice compared to *mstn*$^{+/+}$*mdx* mice. (*a*) Number of RFs per cross-section of EDL muscles from *mstn*$^{-/-}$*mdx* mice (black bar) compared to *mstn*$^{+/+}$*mdx* (gray bar), normalized per 1,000 myofibers, ($P = 0.101$). (*b*) Number of RFs per cluster per cross-section of EDL muscles from *mstn*$^{-/-}$*mdx* mice (black column) compared to *mstn*$^{+/+}$*mdx* (gray bar), ($P = 0.66$). (*c*) Number of isolated RFs per cross-section of EDL muscles from *mstn*$^{-/-}$*mdx* mice (black bar) compared to *mstn*$^{+/+}$*mdx* (gray bar), normalized per 1,000 myofibers ($P = 0.152$).

Table S1. Cellular morphometric properties of *Compact* ($BEH^{c/c}$) and $BEH^{+/+}$ control EDL muscles

Variable	Female age (months)	$BEH^{c/c}$ EDL	$BEH^{+/+}$ EDL	*P**
Number of myofibers	2	1589.3 ± 189.5 (3)	1160.3 ± 84.6 (3)	0.023*
Fiber area (μm^2)	2	3192 ± 1096.8 (139 fibers from 3 mice)	1651.2 ± 751.3 (181 fibers from 3 mice)	<0.001*
Number of satellite cells per fiber	2	4.4 ± 3.4 (85 fibers from 2 mice)	7.5 ± 4.5 (35 fibers from 1 mouse)	<0.001*
Myonuclei/fiber	2	281 ± 72.4 (232 fibers from 5 mice)	313.1 ± 74.2 (155 fibers from 4 mouse)	<0.001*
Fiber length (μm)	2	5,195.5 ± 683.8 (152 fibers from 4 mice)	4,503.8 ± 553.4 (62 fibers from 3 mice)	<0.001*

Values are given as means together with standard deviation; number of muscles examined is given in parentheses. *, for statistical analysis unpaired *t*-test was used and $P < 0.05$ considered as significant.

Table S2. Cellular properties of *Mstn*$^{-/-}$*mdx* and *Mstn*$^{+/+}$*mdx* EDL muscles

Variable	Male age (months)	*Mstn*$^{-/-}$*mdx* EDL	*Mstn*$^{+/+}$*mdx* EDL	*P**
Wet weight (mg)	15	33 ± 1.7 (11)	19.2 ± 2.1 (10)	<0.001*
Number of myofibers	15	1582 ± 233 (6)	1213 ± 350 (4)	0.078*
Number of satellite cells per fiber	15	11.8 ± 5.67 (221 fibres from 6 mice)	12.2 ± 6.18(268 fibres from 6 mice)	0.44*
Percentage of fiber-containing central nuclei	15	32.1 ± 7.33 (6)	37.5 ± 7.44 (5)	0.25*
Number of total revertant fibers (RFs)	15	24.2 ± 8.6 (6)	15.6 ± 3.8 (4)	0.101*
Number of isolated RFs	15	6.1 ± 2.0 (6)	4.2 ± 1.6 (4)	0.152*
Number of clusters of RFs	15	2.4 ± 0.82 (6)	1.4 ± 0.47 (4)	0.065*
Number of RFs per cluster	15	5.5 ± 2.6 (6)	6.4 ± 4.1 (5)	0.66*

Values are given as means together with standard deviation; number of muscles examined is given in parentheses. *, for statistical analysis unpaired *t*-test was used and $P < 0.05$ considered as significant.