

Dominant negative myostatin produces hypertrophy without hyperplasia in muscle

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Abstract Myostatin, a TGF- β family member, is a negative regulator of muscle growth. Here, we generated transgenic mice that expressed myostatin mutated at its cleavage site under the control of a muscle specific promoter creating a dominant negative myostatin. These mice exhibited a significant (20–35%) increase in muscle mass that resulted from myofiber hypertrophy and not from myofiber hyperplasia. We also evaluated the role of myostatin in muscle degenerative states, such as muscular dystrophy, and found significant downregulation of myostatin. Thus, further inhibition of myostatin may permit increased muscle growth in muscle degenerative disorders.

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Key words: Muscle development; Muscle regeneration; Muscular dystrophy; TGF- β

1. Introduction

Skeletal muscle development and regeneration are regulated positively by a variety of growth and transcription factors that affect both muscle stem cell proliferation and myotube differentiation [1–3]. In particular, growth factors such as FGFs, IGFs and TGF- β s play a critical role in promoting myogenesis [4–9]. More recently, a negative regulator of skeletal muscle growth, myostatin, was described [10–12]. Myostatin, also known as growth differentiation factor 8 (GDF-8) is a member of the TGF- β superfamily [10–12]. Mice completely lacking myostatin showed a profound increase in skeletal muscle growth with both an increase in myofiber size and an increase in myofiber number [11]. Thus, myostatin function was implicated in both hypertrophy and hyperplasia of muscle.

TGF- β family members undergo dimerization and cleavage in the extracellular milieu to release the mature, active form derived from the carboxyl-terminus [13,14]. We generated transgenic mice expressing myostatin lacking its normal cleavage site. We predicted that this mutant would function in a dominant negative manner since this approach has been successful for the study of other TGF- β s [15]. Mice expressing this mutant myostatin exhibited an increase in skeletal muscle mass as a result of widespread muscle hypertrophy. No increase in hyperplasia was noted in mice expressing dominant

negative myostatin (dnMS) suggesting that the hypertrophic effect of myostatin was independent of its hyperplastic effect. Interestingly, muscle hypertrophy was independent of known transcription factors that have previously been implicated in muscle hypertrophy, including myogenin, GATA-2 and MEF-2C. Since positive regulators of muscle growth have been shown to be upregulated in muscle undergoing extensive regeneration after damage [16,17], we predicted that a negative regulator of muscle growth would be downregulated to attenuate its suppressive effect and allow regeneration to proceed. Consistent with this, we found myostatin transcript level decreased in two models of muscular dystrophy in which a significant degree of regeneration takes place after repetitive cycles of degeneration [18,19]. Therefore, myostatin is a negative regulator of muscle hypertrophy, and its normal function can be inhibited through a dominant negative mechanism.

2. Materials and methods

2.1. Generation of dnMS transgenic mice

The 5' and 3' ends of murine myostatin were amplified separately with the following: (1) M5F 5'-TGGCGGCCGCCACCATGATGCAAAAACCTG-3', and M5R 5'-GTCTCCGTCTAGACCCTTGGGTGTGTCTGT-3'; (2) M3F 5'-AAGGGTCTAGACGGAGACTTTGGGCTTGAC-3', and M3R 5'-GAGTCGACTATAGATCCTCTTCGCTGATCAGCTTCTGCTCTGAGACCCACAGCGGTCTA-3'. The PCR products were digested with *Xba*I and ligated to *Sma*I-digested Bluescript II (KS), (Stratagene, LaJolla, CA, USA). The normal cleavage site, amino acid sequence RSRR, was altered to GLDG, and a *c-myc* epitope sequence was included in M3R. Polyadenylation and termination signals from the bovine growth hormone gene were added. The 6.5 kb MCK promoter was inserted into a *Xho*I site upstream of dnMS [20,21]. Sequences were verified using cycle sequencing. The prokaryotic vector sequences were removed and the DNA was purified as described [22]. Transgenic mice were generated by microinjection into the pronucleus of fertilized single cell CD1 embryos as described [23]. The transgene copy number was estimated in founder mice by Southern blot analysis of tail DNA with an α -³²P labeled DNA probe containing the first 810 bp of the myostatin coding sequence (nucleotides 107–817 of GenBank accession number U84005). This probe detected both the dnMS transgene and the endogenous myostatin gene.

2.2. Northern blot analysis

dnMS mRNA expression was detected with the same probe used for Southern blotting. To detect endogenous myostatin and GDF-11 mRNA expression, 3' UTR sequences were used. For myostatin, nucleotides 1387–1883 of GenBank accession number U84005 were used as a probe. For GDF-11, nucleotides 1183–1584 of GenBank accession number AF092734 were used. Probes used to detect mRNA expression of myogenin, GATA-2 and MEF-2C correlated to the following regions: for myogenin, nucleotides 757–1229 of GenBank

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accession number X15784; for GATA-2, nucleotides 1981–2620 of GenBank accession number NM_008090; and for MEF-2C, nucleotides 520–1160 of GenBank accession number L13171. Probe sequences were all generated by RT-PCR and verified with direct sequencing of PCR fragments. Signal strength was quantified using ImageQuant software and a STORM 860 phosphorimager (Amersham-Pharmacia Biotech, Piscataway, NJ, USA).

2.3. Immunoblotting, histology and immunocytochemistry

dnMS protein was detected with a polyclonal anti-*c-myc* antibody (Upstate Biotechnology, Lake placid, NY, USA). Myostatin and dnMS were detected with an antibody specific to myostatin that recognizes both the precursor and amino-terminal processed form of myostatin [24]. Goat anti-rabbit secondary was from Jackson ImmunoResearch (West Grove, PA, USA), and signal strength was quantified using ECL Plus and a STORM 860 (Amersham-Pharmacia Biotech). Coomassie blue stained gels were quantified using Kodak ID 2.0.2 (Eastman-Kodak, Rochester, NY, USA). Quadriceps, gastrocnemius, triceps brachialis, extensor digitorum longus (EDL) and soleus muscles from 16-week-old mice were isolated and weighed, blinded to genotype. Immunostaining was performed as described [25]. Three sections from the midportion of each muscle were stained with polyclonal anti-dystrophin AB6–10 [26] that was detected with Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Counterstaining with 4,6-diamidino-2-phenylindole (DAPI) was included in the mounting medium (Vector Laboratories, Burlingame, CA, USA). Sections were photographed on a Zeiss Axiophot epifluorescence microscope. For sections stained with hematoxylin/eosin or Masson trichrome, the images were acquired on a Kodak digital science DC 120 zoom digital camera and analyzed using NIH Image.

3. Results

3.1. Expression of dnMS in transgenic mice

As shown in Fig. 1A, a dnMS was engineered by altering the predicted cleavage site from RSRR to GLDG (residues 264–267 of GenBank accession number U84005). A *c-myc* epitope tag, EQKLISEDDL, was added to the carboxyl-terminus of the mutant myostatin. dnMS was expressed using a

6.5 kb fragment of the muscle-specific creatine kinase (MCK) promoter [20,21]. Four independent lines of transgenic mice were generated on an outbred CD1 background. The transgene copy number was 3, 15, 10 and 10 for lines 1, 2, 3 and 4, respectively. Northern blot analysis demonstrated that dnMS mRNA was expressed in all four transgenic lines (Fig. 1B, upper panel) at the predicted size of 1.7 kb. The native myostatin transcript was 2.9 kb, and required longer exposure times for visualization (data not shown). Despite varying copy number, the amount of dnMS transcript was similar from all four lines.

Immunoblotting of skeletal muscle extracts with anti-*c-myc* antibody detected dnMS protein expression as the expected 50 kDa size in the transgenic mice (Fig. 1C). The relative level of dnMS protein expression was about 113, 100, 136 and 169 for lines 1, 2, 3 and 4, respectively (the lowest was considered as 100%). The 50 kDa band was consistent with the uncleaved form of dnMS. No smaller bands were detected indicating dnMS was resistant to cleavage. Because the MCK promoter can express in both skeletal and cardiac muscle [21], we examined the expression of dnMS in cardiac muscle. Abundant dnMS mRNA was detected in the hearts of all four transgenic lines (data not shown). However, expression of dnMS protein was not seen with the anti-*c-myc* antibody. Correspondingly, the hearts from the dnMS mice were not hypertrophied.

To further investigate whether dnMS interfered with endogenous myostatin processing, we evaluated the amount of processed myostatin in both wild type and dnMS muscle. For these experiments, we used an antibody directed at the amino-terminus of myostatin. This antibody recognized both normal myostatin as well as mutant myostatin. The right panel of Fig. 1D shows an absence of full length, uncleaved myostatin (50 kDa) in the normal control consistent with fully cleaved myostatin. In dnMS muscle, the processed form (37 kDa) was reduced by 40% compared to normal muscle. The lanes were

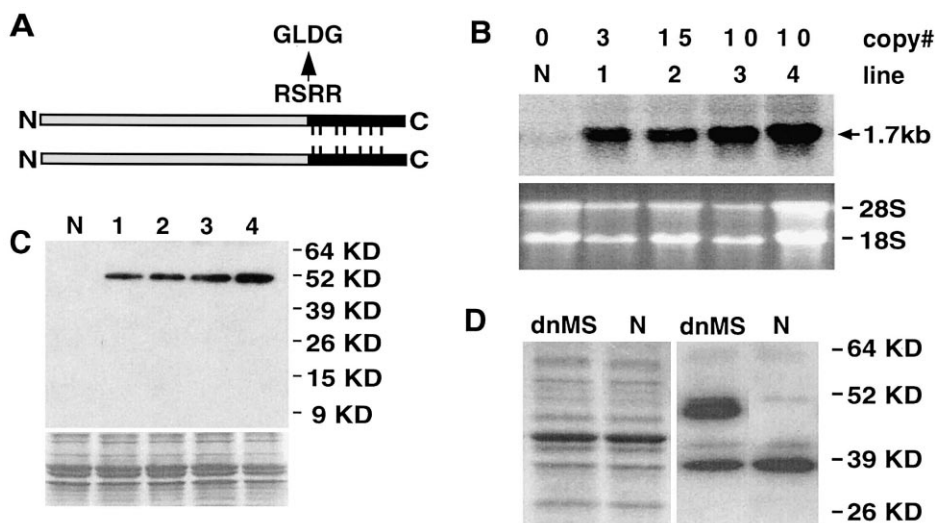
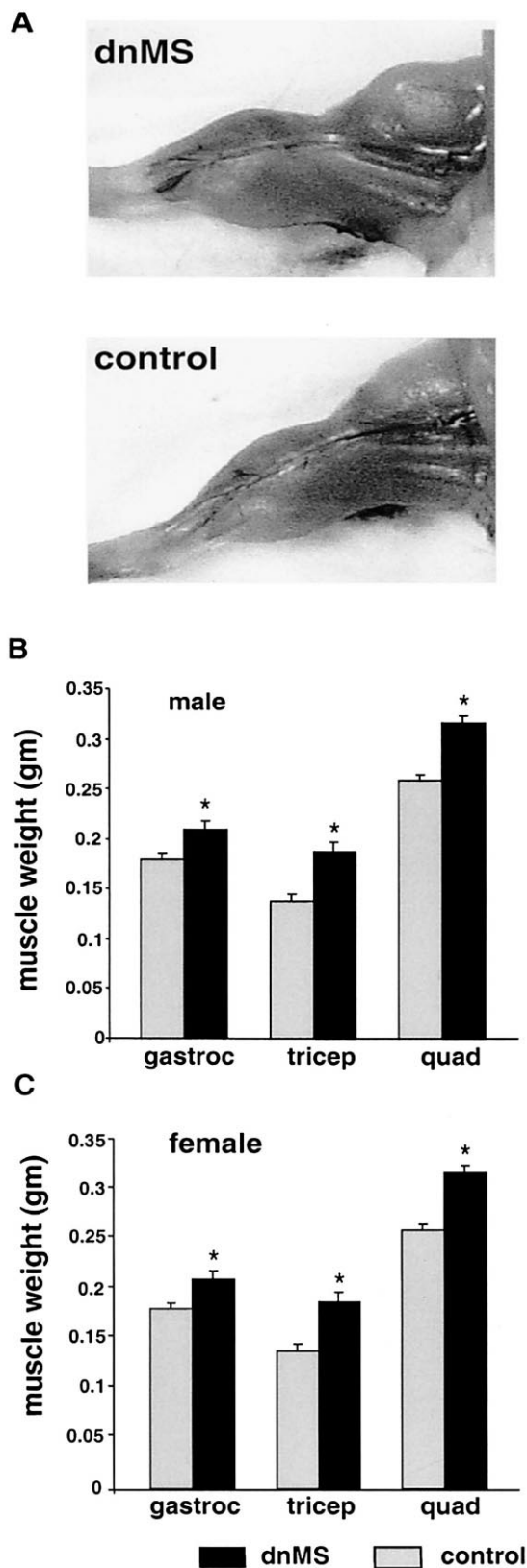


Fig. 1. Expression of dnMS in transgenic mice. A: The cleavage site of myostatin was mutated from RSRR to GLDG to produce dnMS. The hatchmarks indicate the positioning of disulfide bridges that permit dimerization. B: Expression of dnMS mRNA (arrow) is shown in lanes 2, 3, 4 and 5 representing transgenic lines 1, 2, 3 and 4. Ribosomal RNA is shown on the lower panel as an RNA loading control. C: Immunoblot analysis of dnMS protein expression in skeletal muscle. The anti-*c-myc* antibody detects only the 50 kDa dnMS in transgenic lines 1, 2, 3 and 4, but not in normal muscle (N). The carboxyl-terminus of processed dnMS would be detected at 13 kDa, and is not present. Coomassie blue staining (lower panel) shows an equal amount of protein loading. D: Immunoblot analysis of myostatin expression in dnMS and normal skeletal muscle. Using an antibody that detects the amino-terminus of myostatin, a reduction of the processed form (37 kDa band) was consistently present in dnMS muscle compared to normal control (N) (right panel). The reduction of processed myostatin ranged from 23 to 40% and paralleled the increase in muscle mass. Coomassie blue staining was used to normalize protein content (left panel).



equally loaded and normalized to the actin content (Fig. 1D, left panel). Furthermore, the reduction in processed myostatin was consistent among all four lines of dnMS transgenic mice ranging from 23 to 40% consistent with the increase in muscle

Fig. 2. Characterization of mice expressing dnMS. A: A freshly de-skinned hindlimb from a myostatin transgenic mouse (dnMS) shows more pronounced musculature than a control, gender-matched littermate (control). B: Individual muscle mass comparison in male mice. The average muscle mass from male transgenic mice was 17, 35 and 23% greater than the control group for gastrocnemius, triceps and quadriceps, respectively. In female mice (C), the increase was 16, 21 and 27.5% for gastrocnemius, triceps and quadriceps, respectively. $n=10$ for each muscle group; Error bar=S.E.M. The asterisk indicates the P value for dnMS mice versus control littermates is <0.01 .

mass (see below). Thus, dnMS had an inhibitory effect on endogenous myostatin processing.

3.2. Characterization of dnMS transgenic mice

All four lines of mice bearing the dnMS transgene appeared

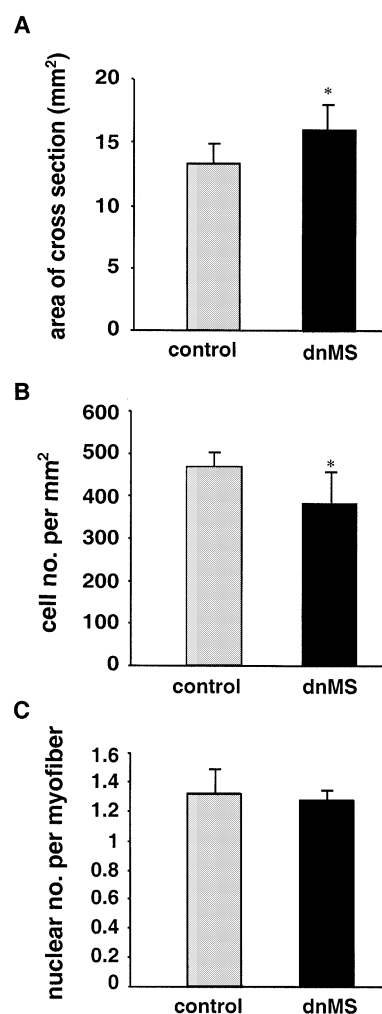


Fig. 3. Analysis of dnMS mice. A: The cross sectional area of muscle from dnMS transgenic mice was 20% larger than control littermates. Cross sectional areas were determined from quadriceps muscle taken at its midportion. $n=5$ in each group. B: Myofiber number per mm^2 in cross sections from transgene bearing mice was 379 ± 76 versus 467 ± 31 in control littermates indicating that the number of myofibers per section was less due to increased myofiber size. C: The number of nuclei per myofiber remained similar between dnMS mice and their control littermates indicating no increase in nuclear number and an absence of hyperplasia. Error bar=S.D. The asterisk indicates the P value for dnMS mice versus control littermates is <0.01 .

healthy and in the expected Mendelian ratio. Fig. 2A shows a representative comparison of deskinmed dnMS transgenic and wild type littermate hind limbs. The transgenic mice frequently had muscles that were visibly more developed than their wild type littermates. The pronounced musculature was consistently observed in dnMS transgenic mice of all four lines and in both male and female dnMS transgenic mice. As shown in Fig. 2B, the average muscle mass of male transgenic mice was increased 17, 35 and 23% for gastrocnemius, triceps brachialis and quadriceps respectively over littermate controls. Individual muscle mass from female transgenic mice was also greater than littermates (16% for gastrocnemius, 21% for triceps and 28% for quadriceps) (Fig. 2C).

To investigate whether the increase in skeletal muscle mass in dnMS transgenic mice arose from hypertrophy and/or hyperplasia, we quantified muscle fiber size and myonuclear number. Fig. 3A shows that the average cross sectional area of dnMS quadriceps muscle was 20% larger than the control muscle ($15.33 \pm 1.96 \text{ mm}^2$ for transgenic and $12.33 \pm 1.52 \text{ mm}^2$) at its mid-portion. Fig. 3B demonstrates that dnMS muscle averaged 379 ± 76 myofiber/ mm^2 while wild type littermates averaged 467 ± 31 myofiber/ mm^2 . In dnMS mice, the smaller number of myofibers per given area reflected an increase in myofiber size indicating hypertrophy. Moreover, a comparison of fast (EDL) and slow (soleus) muscle cross sectional areas indicated that hypertrophy occurred in both muscle types (Table 1). As a measure of hyperplasia, the number of nuclei per myofiber was calculated. Both wild type and dnMS mice showed the same number of nuclei per myofiber (Fig. 3C, 1.31 ± 0.15 for wild type and 1.28 ± 0.06 for dnMS) in each section indicating that hyperplasia was not a significant contributor to the increase in muscle size seen in dnMS transgenic mice.

We examined whether hypertrophy in dnMS muscle relied on the upregulation of transcription factors previously implicated in muscle hypertrophy [6–9,27]. Quantitative Northern blot analysis was performed on total RNA from normal and mutant gastrocnemius muscle ($n=9$ for each). No significant difference was detected in the expression of MEF-2C, myogenin or GATA-2 (Fig. 4A). Thus, dnMS-mediated hypertrophy occurred by mechanisms that did not involve an increase in these transcription factors.

3.3. Myostatin was downregulated in regenerating muscle from muscular dystrophy mice

To examine whether myostatin expression was altered in skeletal muscle undergoing regeneration, mice with muscular dystrophy were examined. *mdx* mice lack dystrophin and are a model for Duchenne muscular dystrophy [28] while *gsg*^{-/-} mice lack γ -sarcoglycan and serve as a model for limb girdle muscular dystrophy [29]. These animal models exhibit repeated and continuous rounds of degeneration accompanied

Table 1
Comparison of fast (EDL) and slow (soleus) muscle cross sectional area

	Cell number per mm^2	
	wild type	dnMS
EDL	549 ± 32	430 ± 92^a
soleus	546 ± 32	432 ± 36^a

$n=5$ for each muscle group.

^aIndicates *P* value for dnMS versus wild type is <0.01 .

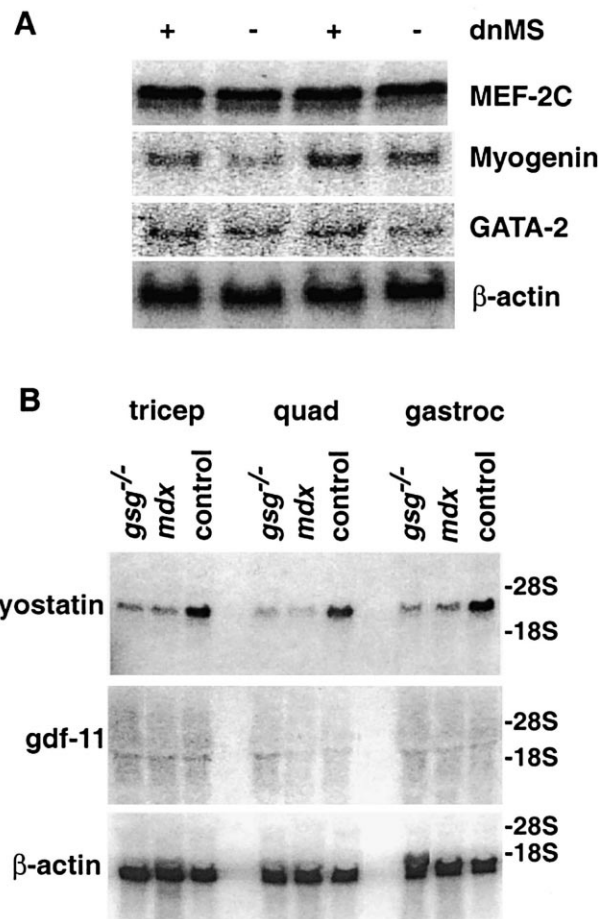


Fig. 4. A: mRNA expression of MEF2C, myogenin and GATA-2 in muscles from normal (–) and dnMS transgenic (+) mice. The results from two different mice in each group are shown. The same blot was also hybridized with β -actin probe and used for normalization. B: Myostatin mRNA expression in muscle from normal mice and mice with muscular dystrophy. Myostatin mRNA expression was significantly decreased in *gsg*^{-/-} and *mdx* mice (top panel). GDF-11, a homolog of myostatin, was not affected (middle panel). The same blot was also hybridized with β -actin probe for normalization (bottom panel).

by regeneration [29,30]. Eight-week, gender-matched mice of each strain, *mdx*, *gsg*^{-/-} and wild type, were studied. As shown in Fig. 4B, myostatin expression was significantly reduced in *mdx* and *gsg*^{-/-} mice (upper panel), while GDF-11, a homolog of myostatin was not affected (middle panel).

4. Discussion

We found that mice expressing dnMS showed up to a 35% increase in skeletal muscle mass, and this increase was a result of muscle hypertrophy. Interestingly, hypertrophy equally affected slow and fast fibers. Notably, hyperplasia was not a significant contributor to the increase in muscle mass. In comparison to dnMS mice, mice fully null for myostatin showed muscle masses that were nearly double that of normal muscle [11]. In addition, this marked increase in muscle mass was associated with both hypertrophy and hyperplasia [11]. The difference in muscle mass seen in dnMS and null myostatin mice likely results from incomplete dominance of dnMS so

that dimerization and cleavage of normal myostatin is not fully blocked in dnMS mice. Expression from the dnMS transgene produced a 23–40% decrease in processed myostatin, and this degree of reduction paralleled the increase in muscle mass. Thus, lower levels of myostatin inhibition may affect hypertrophy, while higher levels of myostatin inhibition may be required to alter hyperplasia. We cannot exclude that dnMS may interfere with other closely related TGF- β s whose normal function is to promote muscle growth. Additionally, the MCK promoter that directed expression of dnMS may express later in development, beyond the developmental window in which skeletal muscle cell proliferation is complete. Supporting this, the muscle creatine kinase gene was noted to be highly expressed in fetal but not embryonic myotubes [31]. Nevertheless, it appears that dnMS effectively separates the regulation of myofiber size from the regulation of myofiber number in a manner distinct from that seen in myostatin null mice.

The *double muscled* phenotype has arisen in several different strains of cattle and is associated with at least two different myostatin mutations. One mutation resulted in a truncated myostatin protein similar to the dnMS we engineered here [10,32]. This breed displayed a 20–25% increase in muscle mass, similar to that observed in dnMS mice [10]. A second mutation was also associated with the double muscled phenotype in cattle, resulting in a missense mutation in the active portion of myostatin [10]. The increase in muscle mass associated with this breed is also 20–25%. Based on our observations, it is likely that these mutants exhibit their effect in a dominant negative manner.

It is not known what transcription factors might mediate the hypertrophic effect of dnMS. We tested three transcription factors, MEF-2C, myogenin and GATA-2, whose functions have been previously well established in muscle hypertrophy. The observation that these three transcription factors were not increased in hypertrophic muscle from dnMS transgenic mice indicates that alternative signaling pathways and/or other transcription factors mediate myostatin-inhibited hypertrophy. We also found myostatin expression was significantly reduced in two strains of mice that undergo muscle degeneration coupled with extensive regeneration. Skeletal muscle development and regeneration are highly paralleled processes under balanced regulation. Myostatin is well positioned as a negative regulator of these processes. Inhibiting myostatin may be a useful tool in the treatment of muscle degenerative diseases such as the muscular dystrophies or aging-associated muscle degeneration. The identification and demonstration of a dominant negative form of myostatin offers a more facile molecular mechanism to interfere with myostatin function in diseased tissues.

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