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A missense mutant myostatin causes hyperplasia without hypertrophy in the mouse muscle

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Abstract

Myostatin, which is a member of the TGF-β superfamily, is a negative regulator of skeletal muscle formation. Double-muscled Piedmontese cattle have a C313Y mutation in myostatin and show increased skeletal muscle mass which resulted from an increase of myofiber number (hyperplasia) without that of myofiber size (hypertrophy). To examine whether this mutation in myostatin gene affects muscle development in a dominant negative manner, we generated transgenic mice overexpressing the mutated gene. The transgenic mice exhibited dramatic increases in the skeletal muscle mass resulting from hyperplasia without hypertrophy. In contrast, it has been reported that a myostatin mutated at its cleavage site produces hypertrophy without hyperplasia in the muscle. Thus, these results suggest that (1) the myostatin containing the missense mutation exhibits a dominant negative activity and that (2) there are two types in the dominant negative form of myostatin, causing either hypertrophy or hyperplasia.

Keywords: Mouse; Transgenic; Myostatin; GDF-8; Skeletal muscle; Dominant negative; Hypertrophy; Hyperplasia

Myostatin, also called growth differentiation factor-8 (GDF-8), is a member of the TGF-β superfamily, which is expressed specifically in developing an adult skeletal muscle [1]. Myostatin null mice show a large and widespread increase in skeletal muscle mass, due to an increase in both myofiber size (hypertrophy) and myofiber number (hyperplasia) [1]. Thus, myostatin is a negative regulator of skeletal muscle formation. Myostatin is highly conserved among species [2–5]: The predicted myostatin proteins in human, rat, mouse, pig, chicken, and turkey exhibit high homology in the biologically active carboxy-terminal region (Fig. 1A) [6]. The Piedmontese and Belgian Blue cattle commonly known as double muscling reveal mutations in myostatin [2,6,7]. The Piedmontese myostatin sequence contains a missense mutation that causes a cysteine to tyrosine substitution (C313Y) in the mature region of the protein (Fig. 1B) [2,6,7], while the Belgian Blue myostatin sequence contains a 11-nucleotide deletion that causes a frame-shift mutation (Fig. 1C) [2,6,7]. Interestingly enough, this increased muscle mass results only from hyperplasia of muscle fibers without hypertrophy. These myostatin mutants have been supposed to act as dominant negative forms. On the other hand, recently, it was reported that transgenic mice overexpressing dominant negative myostatin altering the predicted cleavage site from RSRR to GLDG (Fig. 1D) exhibited a significant increase due to hypertrophy without hyperplasia [8]. This phenotype is in contrast with the phenotype observed in the double-muscled cattle. To examine whether the Piedmontese-type

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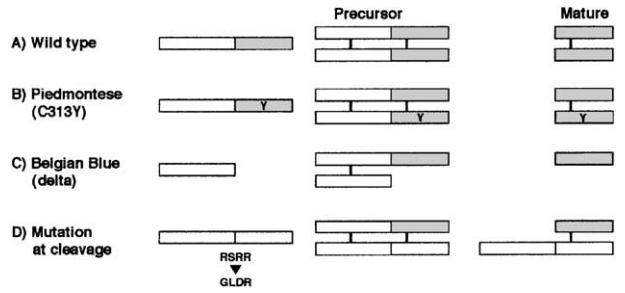

Fig. 1. Maturation processes of myostatin and its mutant froms. (A) Wild-type myostatin. (B) Belgian Blue myostatin containing a 11-nucleotide deletion. (C) Piedmontese myostatin containing a missense mutation (C313Y). (D) Myostatin mutated at its cleavage site from RSRR to GLDR.

myostatin mutant can induce hyperplasia without hypertrophy in muscle, we generated the transgenic mice overexpressing myostatin with the missense (C313Y) mutation. We found that the transgenic mice exhibit hyperplasia without hypertrophy as observed in the Piedmontese cattle. Thus, our results suggested that (1) the myostatin containing the missense mutation exhibits a dominant negative activity and (2) there are two types in the dominant negative form of myostatin, causing either hypertrophy or hyperplasia.

Materials and methods

Construction of myostatin C313Y mutant. A full length cDNA of the chicken wild-type myostatin was modified by PCR to add *Cla*I and *Xba*I sites at the 5′ and 3′ ends, respectively. The cDNA was subcloned into pZERO2 vector (Invitrogen) and digested with *Bsp*EI/*Xba*I to replace with DNA fragment encoding the C313Y mutation. The DNA fragment was obtained by PCR using mutagenic and reverse primers. The full length cDNA for myostatin C313Y mutant was confirmed by sequencing, digested with *Cla*I/*Xba*I, and then subcloned into a vector pCAG containing the modified chicken β-action promoter with the CMV-IE enhancer (CAG promoter) [9]. The resultant vector was then linearized to make a transgene. Primers for PCR are as follows:

myogenin forward, 5′-CATCGATTGACTGTAAGATCATGCA AAAGC-3′;

reverse, 5′-TTCTAGAATGGTGGATCTCACGACAG-3′;

mutagenic, 5′-GCTCCGGAGAATACGAATTTGTGTTTCTAC AG-3′.

Restriction sites are underlined and the mutation for C313Y is represented by bold.

Generation of transgenic mice. The transgene was microinjected into the male pronuclei of fertilized eggs obtained from superovulated BDF1 (C57 BL/6xDBA2 F1) female mice crossed with males of the same strain. Injected eggs were implanted into the oviducts of pseudopregnant female mice and allowed to develop. DNA was extracted from tail snips of live offspring by the proteinase K/SDS method. The integration of the transgene into the mouse genome was detected by PCR. F1 transgenic progenies were bred by crossing transgenic founder mice with BDF1 mice and used for a long-term observation of phenotypes and for repeating crossing with BDF1.

PCR analysis. DNA was extracted from tail snips of live offspring by the proteinase K/SDS method. Primers were designed within the CAG vector sequence to amplify the intervening chicken myostatin cDNA: F: 5′-CCTACA GCT CCT GGG CAA CG-3′; R: 5′-TCA AGG GGC TTC ATG ATG TC-3′. Target sequences were amplified by PCR using the mouse genomic DNA as a template with 0.025 U/μl of Ampli Taq Gold (Perkin–Elmer–Roche) under the following conditions: 9 min 94 °C, 35 cycles consisting of 1 min 94 °C, 1 min 65 °C, 1 min 72 °C, and 8 min 72 °C. PCR products were fractionated by electrophoresis in 1.2% agarose gels and visualized by staining with ethidium bromide. Transgenes were used as a control.

Histological and immunohistochemical analysis. The expression of myostatin was detected by using an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of the mouse myostatin. (Santa Cruz Biotechnology). Detection of signals was performed by using avidin–biotin complex system (Histofine SAB-PO (G) kit, Nichirei). The triceps surae, quadriceps, triceps brachii, digastric muscle from the 16–18-week-old transgenic and control mice were dissected and weighted. Thereafter, paraffin embedded sections of these tissues were prepared for histological and immunohistochemical analyses. Sections stained with hematoxylin/eosin were photographed and the images were analyzed using NIH Image.

Results

Construction and overexpression of myostatin with the missense mutation (C313Y)

A chicken myostatin with a missense mutation, identified in the Piedmontese Cattle [6] (Fig. 1B), was used for overexpression in transgenic mice so as to detect expression of the transgene in the mouse. The missense mutant myostatin is here designated as msMS, in which a tyrosine is substituted for an invariant cysteine in the mature region. To determine whether msMs is capable of blocking myostatin activity in vivo, we generated transgenic mice in which the CAG promoter containing the modified chicken β-action promoter with the CMV-IE enhancer [9] was used to drive expression of *msMS* in all tissues including skeletal muscles. Three independent lines of the transgenic mice were generated from pronuclear injection of the propeptide construct for msMS. Expression of the transgene was confirmed by a PCR analysis revealing that the amplified fragment of the chicken myostatin cDNA of the transgene had the predicted size of 1.3 kb in all three transgenic lines (data not shown). To detect the msMS protein, we carried out immunohistochemical analysis using myostatin specific antibody. Weak signals for the mouse myostatin were detected in sections of the quadriceps muscle of the control littermates, as shown in Fig. 2A, while intense signals were observed in sections of the corresponding muscle of the transgenic mice (Fig. 2B), indicating that the msMS proteins were present in the transgenic mice.

Phenotypes of skeletal muscles in transgenic mice overexpressing msMs

The three independent lines of transgenic mice appeared healthy and developed musculature. We observed the musculature of the transgenic mice and compared them with that of the control littermates after

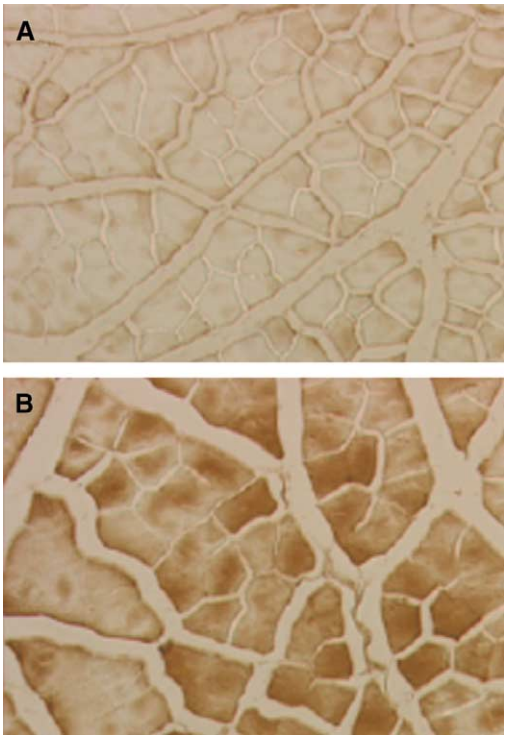

Fig. 2. Immunohistochemical analyses on the expression of myostatin with myostatin specific antibodies. (A) A section of the quadriceps muscle of a control littermate was stained weakly. (B) A section of the quadriceps muscle of a transgenic mouse was stained intensely.

skinning, as shown in Fig. 3. It was apparent that the muscles of the transgenic mice were much larger than those of control littermates (Fig. 3) in the facial (Fig. 3A and B) region, upper (Fig. 3C and D) and lower limbs (Fig. 3E and F). To measure the weight of muscle mass of transgenic mice, individual muscles (quadriceps, triceps surae, triceps brachii, and digastrics) were isolated and weighed. The average muscle mass of the transgenic mice increased about two times more than that of control littermates (Fig. 4). This tendency was found to be independent of sexuality (Fig. 4).

To determine whether the increase in skeletal muscle mass resulted from hyperplasia or hypertrophy, we carried out histological analysis of the skeletal muscles (Fig. 5): We prepared cross sections of the quadriceps muscle around its mid-portion and measured their cross sectional area. The average cross sectional area of the msMS transgenic mice (32.6 mm^2, $n = 5$) was 88% larger than that of control littermates (17.7 mm^2, $n = 5$) (Fig. 5A). The total myofiber number of quadriceps of the transgenic mice (10.6×10^3) was also much larger than the corresponding number of control littermates (6.5×10^3) (Fig. 5B). In contrast, we could not find any significant difference in the number of myofibers per square millimeter between control and msMS transgenic mice (Fig. 5C). These results demonstrate that the increase in skeletal muscle mass in the msMS transgenic mice is due to only hyperplasia without hypertrophy.

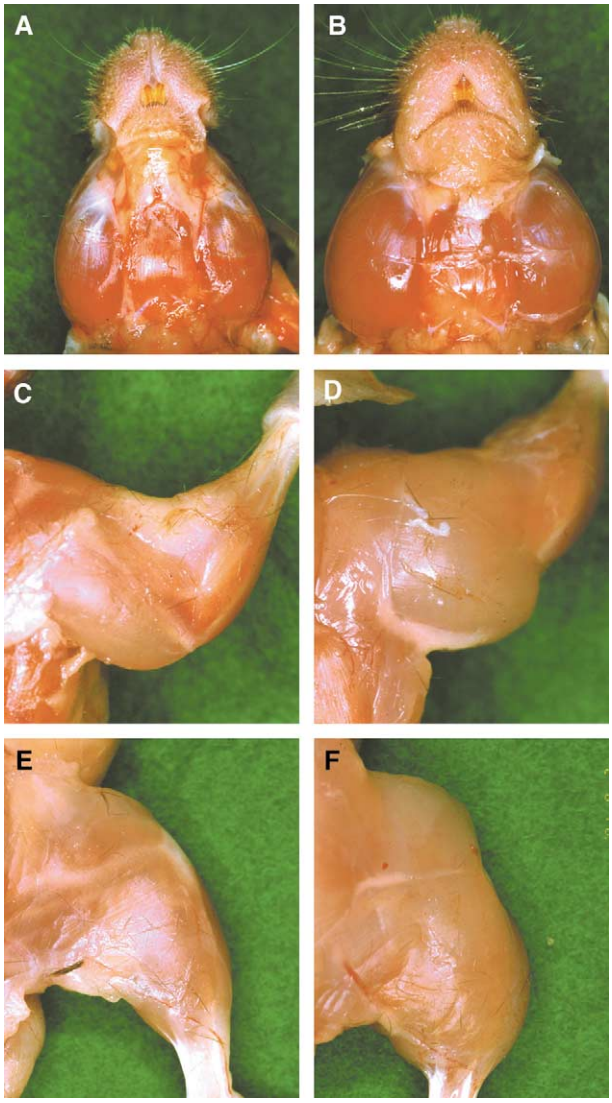

Fig. 3. Increased skeletal muscles of control littermates (A, C, E) and transgenic mice with the missense mutation (C313Y) (B, D, F). (A, B) facial region. (C, D) Skinned upper limb. (E, F) Skinned lower limb.

Discussion

We found that in transgenic mice expressing the myostatin containing the missense mutation, their skeletal muscle mass became double, and this increase was a result of muscle hyperplasia without hypertrophy. This result was consistent with the fact that the increased musculature of the Piedmontese cattle with a missense mutation (C313Y) results only from hyperplasia without hypertrophy [2]. In contrast, this result was different from a result that a dominant negative myostatin (dnMS) created by alternation of the cleavage site producing hypertrophy without hyperplasia [8]. It has been reported that in the myostatin null mice and the transgenic mice expressing high levels of the propeptide, follistatin, or a dominant negative form of activin receptor type IIB (ActR IIB), the increased muscle mass

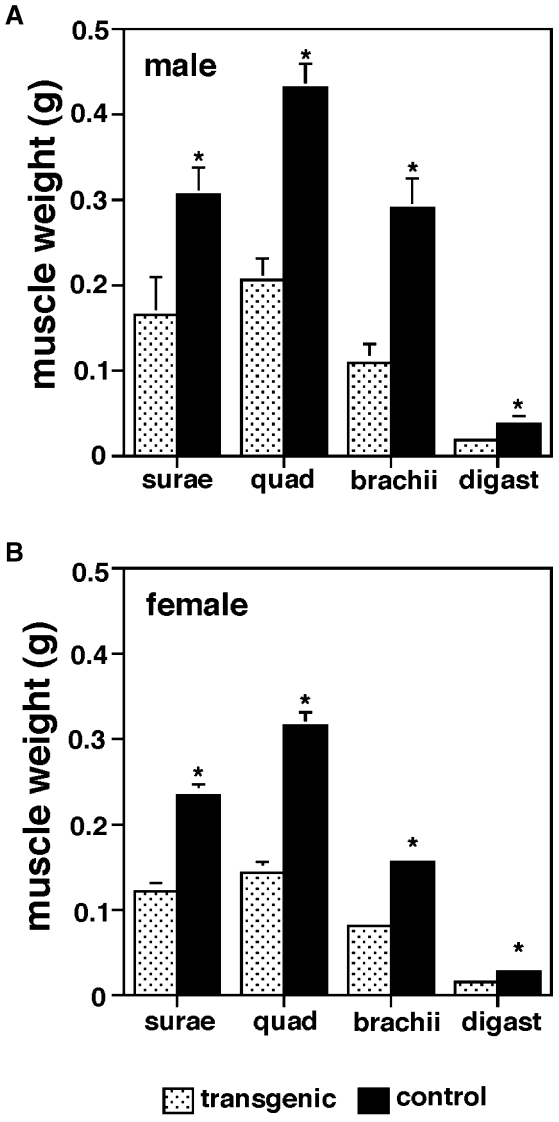

Fig. 4. Weights of individual muscle mass in male (A) and female (B) control littermates (gray) and transgenic mice with the missense mutation (C313Y) (black). An average muscle mass of transgenic mice was shown as a bar graph. The values for the transgenic mice are about twice as those of the littermates for triceps surae, quadriceps, triceps brachii, and digastric. Error bar represents SD. The asterisk indicates that the P value for transgenic mice versus control littermates is <0.0001.

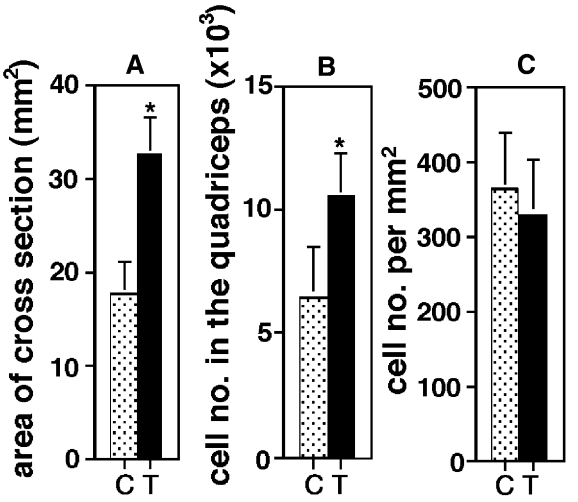

Fig. 5. Analysis of the transgenic mice with the missense mutation. (A) Average cross sectional area ($n = 5$) of the quadriceps muscle at its mid-portion for control littermates (C, dotted) and the transgenic mice (T, black). (B) The myofiber number of the quadriceps muscles from in control littermates (C, dotted, $6.5 \pm 2.1 \times 10^3$) and the transgenic mice (T, black, $10.6 \pm 1.7 \times 10^3$). (C) The myofiber number per mm^2 in cross sections of control littermates (C, dotted, 364 ± 75) and the transgenic mice (T, black, 330 ± 72), indicating that the myofiber size was not different significantly.

results from both hyperplasia and hypertrophy [1,10]. These results indicated that when normal functions of the endogenous myostatin proteins are inhibited completely, an increase in muscle mass results from both hyperplasia and hypertrophy. Thus, increase in muscle mass resulted from either hyperplasia or hypertrophy implies that the functions of the endogenous myostatin proteins are not blocked completely by msMS or dnMS.

The missense mutation (C313Y) may affect a conformation of the bioactive carboxy-terminal (C-terminal) domain of myostatin. Since the purified C-terminal domain of myostatin was reported to form dimer and to bind to the activin receptor type IIB (ActR IIB) (11), a binding mode of msMS to the ActR IIB may be changed, resulting in blocking the signaling of normal ligands including normal myostatin, activin, and other TGFβ-related factors, which may cause hyperplasia in muscle. In the case of dnMS, it is also reasonable to consider that the binding mode of dnMS to the receptor may also be changed, but differently from that of msMS, resulting in hypertrophy. Thus, the different effects on muscle phenotypes between msMS and dnMS may be attributed to the difference in the binding mode to the receptor. In any case, our finding suggests that there are at least two independent signaling pathways to regulate the number and size of muscle cells.

Acknowledgments

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