Review Article
Factors Affecting Morphology of Skeletal Muscles

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Abstract

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Myostatin is a TGF-ß family member. It plays a negative role in regulating the growth of the skeletal muscles. The effects of the myostatin mutation in adipose tissue are an indirect effect of the lack of myostatin signaling in skeletal muscle. An elucidation of the mechanism by which myostatin regulates fat metabolism *in vivo* will ultimately require the analysis of genetically manipulated animals in which components of the myostatin signaling pathway have been blocked specifically in either skeletal muscle or adipose tissue. Although the expression level of the transgene in adipose tissue was extremely low compared with the level in skeletal muscle, it is possible that this low-level expression was sufficient to block myostatin signaling in fat. Several data suggest that myostatin inhibitors such as follistatin and the myostatin propeptide, or activin type II receptor inhibitors may be effective muscle-enhancing agents for both human and agricultural applications.

Myostatin, growth factors, heparin, rabbit, skeletal muscles

Several factors influence the morphological and physiological processes of the living organism as a whole. Growth factors (GF) are among the factors that may influence the skeletal muscles positively or negatively.

Growth factors are proteins that bind to receptors on the surface of a cell, with the primary result of activating cellular proliferation and differentiation (Kambadur et al. 1997). Many growth factors are quite vague, stimulating cellular division in numerous different cell types, while others are specific to a particular cell-type. A list of some growth factors, their descriptions and principal activities are described by Kambadur et al. (1997).

Myostatin is the most up-to-date discovery. It is a transforming growth factor–ß (TGF-ß) family member. It plays an important role in regulating skeletal muscle growth. Myostatin is expressed initially in the myotome compartment of developing somites and continues to be expressed in the myogenic lineage throughout development and in adult animals (McPherron et al. 1997).

The myostatin sequences in rats, porcine, murine, chicken, turkey, and humans are identical in the biologically active C-terminal portion of the molecule following the proteolytic processing site (McPherron and Lee 1997). The function of myostatin also appears to be conserved across species, as mutations in the myostatin gene have been shown to result in the double muscling phenotype in cattle breeds, such as Belgium Blue, Piedmontese (Grobet et al. 1998).

Many authors of myostatin studies have considered that interfering with the activity of myostatin in humans may reverse muscle wasting diseases associated with muscular

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dystrophy such as, AIDS and cancer. Some predict that manipulation of this gene could produce heavily muscled farm animals. Indeed, current research is underway to investigate and develop these potentialities (Gonzalez-Cadavid et al. 1998; Grobet et al. 1998; McPherron et al. 1997).

Of course, manipulation of myostatin gene in humans may be a key to reversing muscle-wasting conditions. However, currently we have too little knowledge regarding the role of myostatin in muscle growth regulation. It is imperative that research demonstrates the loss of myostatin search must also prove that over-expression or administration of myostatin causes loss of muscle mass.

We do not fully understand the roles of myostatin in exercise-induced muscle hypertrophy or regeneration following muscle injury. Until we do, it may be premature to blame the lack of hypertrophy in weightlifters on over-expression of myostatin. Nor does research support the claim that a top bodybuilder's muscle mass gains are the consequence of a detected mutation in the myostatin gene (Ferrell et al. 1999). Researches do not simply advocate blaming genetic myostatin variations as a source of significant differences in human phenotypes.

The influence of myostatin on body fat deposit

Lee and McPherron crossed myostatin-free mice with each of two types of obese mice to get doubly engineered offspring. The second- generation mice were deficient in myostatin and also had a genetic change causing obesity. One line of fat mice, officially named "obese", eats excessively because they lack the hormone leptin. The other fat mice eat too much because their production of a protein called "agouti" is abnormal (Klebig et al. 1995).

By examining amounts of fat and muscle in mice, the scientists discovered that slim and obese mice without myostatin gained less fat, as they grew older, even though they ate about the same amounts of food as other mice. In fact, "mighty mice" outweigh their counterparts when young, but by 10 months of age or so weighed the same or less than other mice, which had bulked up with fat (Shaoquan et al. 1998).

By the time they reached middle age at 10 months, mice lacking myostatin had 70 percent less fat than regular mice. Among "agouti" mice, myostatin-free animals had about half the fat as others, while the "obese" mice without myostatin had roughly two-thirds of the fat of their myostatin-producing counterparts (Marsh et al. 1997).

The myostatin-free mice were also healthier than their myostatin-producing, engineered-to-be-obese relatives. Both the "obese" and "agouti" mice are models of type 2, or "adult onset" diabetes because they develop the disease's major symptom, which is resistance to the hormone insulin but on the other hand, "mighty" versions of these animals did not (McPherron and Lee 1997).

These proofs suggest that mice without myostatin, in addition to having less fat, may have enough extra muscle mass to make up for decreasing sensitivity to insulin as they gain weight. In type 2 diabetes, tissues, especially muscle, stop responding to insulin and hence do not use sugar from the blood (McPherron and Lee 1997).

For mice genetically altered to get fat, knocking out a particular gene keeps them both leaner and healthier. Gene of the "lean" muscle is the blueprint for myostatin, a protein known to limit muscle growth (Grobet et al. 1998; McPherron et al. 1997). Previous Hopkins studies found that mice without myostatin are muscle-bound "mighty mice". Now the scientists show that mice without the protein, even mice that usually become obese, gain much less fat as they grow (McPherron et al. 1997).

Inhibiting myostatin raise the meat animals

Myostatin may also be a good target for agricultural applications, since blocking the activity of myostatin might increase the efficiency of meat production and decrease the fat content (Fig. 1).

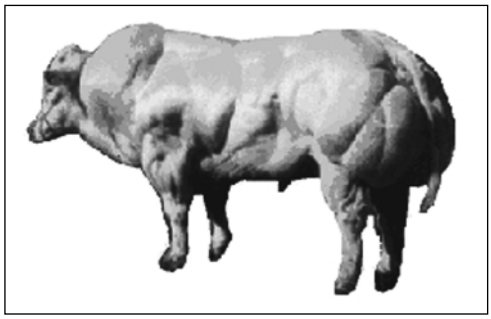

Fig. 1 A double muscle cattle breed with visible subcutaneous structures

In myostatin knockout mice, an increase in muscle mass has been shown. The result was due to increases in both fiber number and fiber size (McPherron and Lee 1997).

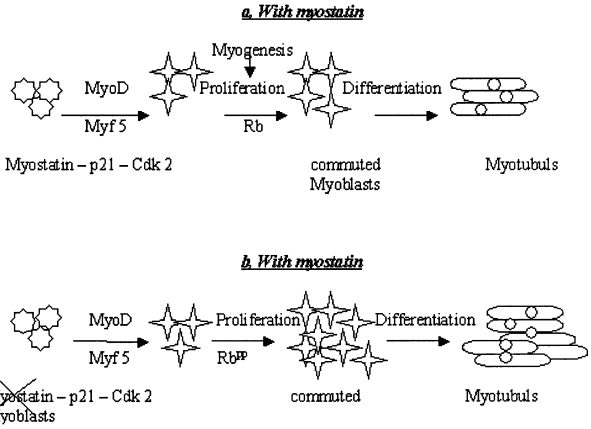

Fig. 2. Schematic expression of the comparison of new muscle cell development with/without the interferences of myostatin (Tesfaye et al. 2003)

These approaches were used to explore other possible strategies for inhibiting myostatin. Initially, the effect of myostatin propeptide was investigated. In case of TGF-ß, it is known that the C-terminal dimer is held in an inactive latent complex with other proteins, including its propeptide (Miyazono et al. 1988), and that the propeptide of TGF- ß can have an inhibitory effect on TGF- ß activity both *in vitro* (Gentry and Nash 1990) and *in vivo* (Böttinger et al. 1996).

The observation that the myostatin C-terminal dimer and propeptide copurified raised the possibility that myostatin may normally exist in a similar latent complex and that the myostatin propeptide may have inhibitory activity. Secondly, the effect of follistatin, which has been shown to be capable of binding and inhibiting the activity of several TGF- ß family members, was examined. In particular, follistatin can block the activity of GDF-11 (Gamer et al. 1999), which is highly related to myostatin (Gamer et al. 1999; McPherron et al. 1999), and follistatin knockout mice have been shown to have reduced muscle mass at birth (Matzuk et al. 1995), which would be consistent with over-activity of myostatin.

To determine whether these molecules are also capable of blocking myostatin activity *in vivo*, transgenic mice were generated in which the myosin light chain promoter/enhancer

was used to drive expression of either the myostatin propeptide or follistatin. From pronuclear injections of the propeptide construct, were obtained three transgenic mouse lines (two of these, represented independently segregating transgene insertion sites in one original founder animal) that showed increased muscling. The muscle weights of animals from each line were increased by 20-110% compared to those of nontransgenic control animals (Lee and McPherron 2001).

The mechanism of the skeletal muscle growth

The most dramatic effects on skeletal muscle were obtained by using the follistatin construct. Two founder animals (F3 and F66) that showed increased muscling were obtained. In one of these animals, muscle weights were increased by 194-327% relative to control animals, resulting from a combination of hyperplasia (66% increase in fiber number to 13,051 in *musculus gastrocnemius/plantaris*) and hypertrophy (28% increase in fiber diameter to 55 μm).

These results suggest that at least part of the effect of follistatin may result from inhibition of another ligand besides myostatin. Clearly, analysis of additional follistatin transgenic lines will be essential in determining whether other ligands may also be involved in negatively regulating muscle growth.

On the basis of the *in vitro* and transgenic mouse data presented here, the following working model for the regulation of myostatin activity was proposed. After proteolytic processing, the myostatin C-terminal dimer is maintained in a latent complex with its propeptide and perhaps other proteins as well.

Myostatin is a TGF-ß family member that acts as a negative regulator of muscle growth. In the above description is shown that mice lacking myostatin have a dramatic and widespread increase in skeletal muscle growth (McPherron and Lee 1997). Here we have shown that deletion of myostatin affects adipose tissue mass in addition to skeletal muscle mass. Specifically, myostatin-deficient mice have a significant reduction in fat accumulation with advancing age despite the fact that they have normal food intake, normal body temperature, and a slightly reduced metabolic rate.

Future tasks

Additional experiments will be required to elucidate the precise mechanism by which myostatin regulates fat metabolism. One possibility is that myostatin acts directly on adipose tissue. In support of a direct mechanism for myostatin action is the recent report that myostatin can inhibit differentiation of adipocytes *in vitro* (Kim et al. 2001). If myostatin is acting directly on adipocytes *in vivo*, myostatin could be acting either systemically or locally. Myostatin mRNA is known to be expressed in fat, although the expression levels are substantially lower in adipose tissue than in skeletal muscle (McPherron and Lee 1997).

A second possibility is that the effects of the myostatin mutation in adipose tissue are an indirect effect of the lack of myostatin signaling in skeletal muscle. It is possible, for example, that the anabolic effects of the myostatin mutation on skeletal muscle tissue per se may shift energy metabolites in such a manner as to prevent fat accumulation elsewhere in the body. Another possibility is that lack of myostatin signaling in muscle affects the activity of hypothetical second messengers (Mauvais-Jarvis 2000) released by muscle that act on adipose tissue. Also, we cannot rule out the possibility that myostatin acts, directly or indirectly, on other tissues such as the CNS that then regulate adipose tissue.

In support of an indirect mechanism, similar effects on fat accumulation have been reported in other genetically altered mice that have increases in muscle mass. For example, transgenic mice over-expressing either IGF-1 Musarò et al. (2001) or ski (Sutrave et al.

1990) in skeletal muscle have been described as being virtually devoid of fat, although quantitative analyses were not reported. The opposite effect, i.e., an increase in fat accumulation, has been reported in mice having decreased muscle mass as a result of a muscle-specific knockout of the insulin receptor gene (Brüning et al. 1998).

An elucidation of the mechanism by which myostatin regulates fat metabolism *in vivo* ultimately will require the analysis of genetically manipulated animals in which components of the myostatin signaling pathway have been blocked specifically in either skeletal muscle or adipose tissue. In this regard, we have shown that myostatin can bind to the activin type II receptors, Act RIIA and Act RIIB, in vitro and that transgenic mice expressing a dominant negative form of Act RIIB in skeletal muscle have dramatic increases in skeletal muscle mass comparable to those seen in myostatin knockout mice (Lee and McPherron 2001).

Preliminary analysis of fat pads has shown that these transgenic mice also have decreased fat accumulation, which would be consistent with an indirect effect of myostatin on adipose tissue. However, the interpretation of these data is complicated by the fact that although a skeletal muscle–specific myosin light chain promoter/enhancer was used to drive expression of the mutant receptor; expression of the transgene was also detected in adipose tissue. Although the expression level of the transgene in adipose tissue was extremely low compared with the level in skeletal muscle, it is possible that this low-level expression was sufficient to block myostatin signaling in fat (Tesfaye et al. 2003).

In our work, we have tested the inhibiting capacity of heparin on rabbits *Oryctolagus cuniculus* (18-60-day-old). The dosage of heparin was increased step by step based on the boby mass increase of the animals. The observed body mass increase is compared with control groups not treated with heparin (Table 1).

Table 1. Average body mass of rabbits of the experimental and control groups

	Day 1 g	Day 20 g	Day 40 g	Day 60 g
Experiment n = 9	196.37 ± 22.18	471.28 ± 32.95	574.79 ± 220.97	1570.00 ± 88.67
Control n = 9	190.24 ± 25.98	411.41 ± 34.6	467.99 ± 83.24	1352.88 ± 86.45
Differences	6.13	59.87	106.80	218.12

The aim of this experiment was to analyze the influence of heparin as a possible agent to inhibit the myostatin protein.

We observed that heparin behaves as a good marker of the hyperplasia of skeletal muscle fibers. So far we were not able to determine any hypertrophy fibers different to the control groups.

Conclusion

Whatever the mechanism by which myostatin regulates the skeletal muscle development and fat metabolism, it was demonstrated that loss of myostatin activity can have beneficial metabolic effects in the hypertrophy of skeletal muscles. Specifically, it was shown that the myostatin mutation could partially suppress both fat accumulation and the development of hyperglycemia.

Although the role of myostatin in humans has yet to be clarified, the findings raise the possibility that myostatin inhibitors may be useful agents for the prevention or treatment of metabolic disorders such as obesity and type II diabetes.

However, myostatin is the only secreted protein that has been demonstrated to play a negative role in regulating muscle mass *in vivo,* additional experiments will be required to prove aspects of this overall model and to identify the other signaling components. Several data suggest that, myostatin inhibitors such as follistatin and the myostatin propeptide, or activin type II receptor inhibitors may be effective muscle-enhancing agents for both human and agricultural applications.

Based on these and other facts we recommend to do further investigation especially, looking for other possible myostatin inhibiting substances and their effect on farm animals due to the production of lean and bulky muscle.

Niektoré faktory ovplyvňujúce morfológiu kostrového svalstva

Myostatin je členom TGF-β rodiny. Zohráva negatívnu úlohu v regulácii rastu kostrového svalstva. Účinky mutácií v tukových tkanivách sú nepriamymi účinkami nedostatku myostatinu na ktoré poukazuje kostrové svalstvo.

Pre pochopenie mechanizmu regulácie tukového metabolizmu myostatinu *in vivo* vyžaduje genetickú manipuláciu zvierat, kde zložky myostatinovej signalizačnej cesty sú zablokované, buď v kostrových svalstvách alebo tukových tkanivách. Hoci hladina expresie transgénu v tukových tkanivách je nízka v porovnávaní s hladinou v kostrovom svalstve, je to možné že táto nízka hladina je dostatočná k zablokovaniu pôsobenia myostatinu v tukoch. Niektoré údaje poukazujú na to, že inhibítory myostatinu (ako napr. follistatin a myostatinové peptidy alebo aktivin typu II. receptor inhibítory), môžu byť účinnými zložkami pre regeneráciu svaloviny u človeka a pre získanie kvalitného chudého mäsa u zvierat.

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