Enhanced Myogenesis in Adult Muscle by Transgenic Expression of Myostatin Propeptide

Myostatin, a member of transforming growth factors-β, is a negative regulator of muscle growth in mammals. Previous studies revealed that transgenic mice expressing myostatin propeptide had enhanced muscle mass. The transgenic mice had no significant gain of adipose tissue, and more importantly, did not display insulin resistance after undergoing a high-fat diet during adulthood. The objective of this study was to investigate the mechanisms by which myostatin propeptide depressed myostin activity. We employed immunohistochemistry procedures to study myostatin expression in muscle tissue using the transgenic mice and their wild-type littermate controls. Our experiments illustrate that even with a normal diet, the transgenic mice with enlarged muscle mass showed less adipose tissue accumulation compared with that of the wild type mice. The immunohistochemistry staining of the muscles located the target sites for myostatin and myostatin propeptide expression, showing that propeptide upregulations does not interfere with myostatin protein levels, while the effects of such myostatin disruption in transgenic mice was further demonstrated with hematoxylin and eosin staining of the muscles and adipose tissues. Our nuclei count suggested that adult transgenic mice are still in active myogenesis to promote faster muscle growth. This research provides molecular and cellular evidence for enhanced muscle growth through depressing myostatin by its propeptide, which may help establish physical activity as an effective means for preventing obesity and type 2 diabetes.

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Introduction

Muscles are essential for human health. Studying muscle growth has many clinical applications, as it can be used to treat serious muscle-related diseases such as muscular dystrophy, in which patients lose their abilities to contract muscles, or muscle wasting, which is predominant during the later stages in HIV/AIDS patients. Recent studies have revealed that without sufficient muscle growth during adolescence, one will be less equipped to prevent obesity and diabetes in adulthood. For our research, we targeted a key negative regulator of muscle growth called myostatin. Myostatin makes a precursor protein digested by a protease into two peptides: C-terminal that is the matured form of myostatin, and an N-terminal called myostatin propeptide which inhibits the C-terminal. Thus, overexpression of myostatin propeptide will allow for muscle growth. In transgenic mice produced by our lab, myostatin propeptide was overexpressed, as this led to enhanced muscle mass. In addition, the transgenic mice also had reduced fat deposits after undergoing a high fat-diet and improved glucose tolerance and insulin sensitivity.

The mystery behind the mechanisms of muscle growth in transgenic mice and what implications such growth could have on the accumulations of adipose tissue will be our principal investigation. Possible methods of muscle growth could be explained in one of two ways: prenatal and postnatal. In the prenatal stages myogenesis occurs when mononucleated cells called myoblasts fuse together to form myotubes in which the nuclei are centrally aligned. These myotubes will grow and mature into myofibers with the nuclei aligned along the edge or the basil lamina of the fiber. This growth occurs by an increase of fiber; through the location of the nuclei, it can be determined if the muscle is still developing or matured. In the postnatal stages, satellite cells simply adhere to the existing muscle fibers, thus only increasing fiber size. We studied which growth method the transgenic mice undergo to enhance muscle mass and the possible implications it has on fat buildup.

Materials and Methods

IACUC approval was obtained prior to all tissue sampling.

Method for myostatin protein localization

Immunohistochemistry on the C-terminal using rabbit antibody and ABC kit

WT and TG gastrocnemius and bicep muscle samples were extracted and fixed in pharmalyn, then rehydrated with 100%–70% alcohol and embedded with paraffin. They were then cut and made into glass slides. To stain them, they had to be deparafinized by heating in an oven at 60°C for 20 min. Xylene was added, followed by 100% ethanol. Sections were treated with 1% hydrogen peroxide in methanol for 30 min, washed in PBS, and placed in a moist chamber and covered with 5% normal goat serum in PBS for 30 min. Primary antibody (GDF-8 BL 891 Affinity Purified) was diluted to 1:250

in 1% NGS and incubated overnight at 4°C. Samples were then washed in TPBS containing 1% TritonX-100, covered with biotinylated goat anti-rabbit for 30 min at room temperature. After three TPBS washings of 5 min each, ABC working solution was added for 30 min. Samples were washed in TPBS before being incubated in 0.05% DAB containing 0.01% hydrogen peroxide for 3 min. They were washed in tap water, and counterstained with dilution (1:2 H_2O) hematoxylin for 1–3 min before being de-hydrated in ethanol, cleared in xylene, and mounted in permount.

Immuno florescence double labeling of myostatin propeptide (N-terminal).

WT and TG muscle samples were extracted, fixed, embedded, cut, deparaffinized, rehydrated in the same manner as described previously. They underwent three PBS washings, a 30 min incubation with 3% NGS in PBS in the moist chamber, and 1:50 dilution of primary antibody (GDF-8 N19) in 3% NRD for 2 hours. They were washed in TPBS and covered with Rabbit Anti-Goat with 1:300 dilution for 30 min at room temperature. After washing in PBS, coverslips with P2 counterstain mounting medium was applied.

Mechanisms of Muscle Growth in TG

Hematoxylin and Eosin Staining

WT and TG muscle samples were prepared as mentioned in the previous procedures. After re-hydration, they were placed in distilled water for 5 min followed by 16 dips in hematoxylin and were washed for 30 sec. 1% Eosin was added for 1 min, then 1% Glacial acetic acid for another 1 min. The samples were washed in tap water until the red stain turned light red. Dehydration using absolute alcohol was followed by a 5 min xylene rinse before mounting with pertex.

Nuclei Count and Statistical Analysis

Ten random microscopic fields under 40× magnification were selected for both WT and TG. We counted the number of nuclei in the center and the basal lamina for each muscle fiber and took the average. We also proceeded to count the average number of nuclei per 100 um^2 for WT and TG gastrocnemius and bicep muscles. Analysis of both recordings was used to provide explanations for TG muscle growth.

Effects of Muscle Growth on Adipose Tissue

Ten randomly selected microscopic fields under 10× magnification were chosen for WT and TG fat tissues. Using computer software, we were able to measure the diameter of each adipose cell, and take the average.

RESULTS AND DISCUSSION

We found that myostatin propeptide over expression does not alter myostatin protein levels and only inhibits its function to limit muscle growth. When myostatin propeptide is upregulated, it forms a cystine knot around myostatin, prohibiting it from escaping as would normally happen if the propeptide was not over expressed. This known structure appears to keep the protein levels intact but prohibits it from functioning. The significance of transgenic propeptide leading to muscle growth was seen in our nuclei count. Myogenesis begins during the prenatal stages, so the fact that there are still numerous nuclei in the center of the TG fiber indicates that myogenesis is still active during adult stages, leading to continuous muscle growth. Secondly, there are more nuclei aligned along the basal lamina of the TG fiber, suggesting that myogenesis is accelerated through faster myofiber generation. In both incidences, the average number of nuclei in the transgenic mice exceed far beyond that of the wild type, thus emphasizing the phenomenon of myogenesis activity. Lastly, increased muscle mass can lead to decreased fat accumulation; however, the exact mechanisms involved remains unclear, and we will aim to address this in our future studies.

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