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Co-Administration of Myostatin-Targeting siRNA and ActRIIB-Fc Fusion Protein Increases Masseter Muscle Mass and Fiber Size

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Summary Myostatin, a member of the TGF-β superfamily, is a negative regulator of skeletal muscle cell growth and differentiation, and binds with high affinity to the activin type IIB receptor (ActRIIB). The soluble ligand-binding domain of ActRIIB fused to the Fc domain of IgG (ActRIIB-Fc) potently binds and inhibits TGF-β family members in muscle, leading to rapid and marked muscle growth. The present study was designed to assess the effectiveness of the co-delivery of myostatin-targeting siRNA (Mstn-siRNA) and ActRIIB-Fc into skeletal muscle as a potential treatment of atrophic myopathies. Eleven-week-old, male C57BL/6 mice were injected with atelocollagen (ATCOL)-mediated Mstn-siRNA with/without ActRIIB-Fc locally into the masseter muscle twice a week. Inhibition of myostatin function by the combination of Mstn-siRNA and ActRIIB-Fc increased muscle weight and myofibril size in murine masseter muscle. Real-time RT-PCR analysis revealed significant downregulation of myostatin mRNA expression in both the Mstn-siRNA-treated and the combination treatment group. Furthermore, myogenin mRNA expression was upregulated in the combination treatment group, while MuRF-1 and Atrogin-1 mRNA expression was downregulated compared to administration of each compound alone. These findings suggest that double inhibition of myostatin is a potentially useful treatment strategy to increase muscle mass and fiber size and could be a useful treatment of patients with various muscle atrophies, including muscular dystrophy.

Key Words myostatin, small-interfering RNAs, activin type IIB receptor, muscle hypertrophy

Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily and plays a critical role in the regulation of skeletal muscle mass. Several strategies have been developed in the last decade for the treatment of muscular dystrophy and muscle wasting based on myostatin inhibition, myostatin-specific antibodies (*1*, *2*), a decoy myostatin receptor (*3*, *4*), and myostatin propeptide (*5*, *6*). Furthermore, two studies showed that myostatin knockout in mice (e.g., the mdx mouse model of Duchenne muscular dystrophy) resulted in a significant increase in skeletal muscle mass and functional improvement in dystrophic muscles (*7*, *8*).

RNA interference (RNAi) is a high sequence-specific gene silencing technique, in which short pieces of double-stranded RNA, small interfering RNA (siRNA), suppress the expression of the genes exhibiting sequence homology (*9*, *10*). Research efforts are currently underway to develop siRNAs as therapies for various diseases. However, in daily clinical practice, the limited stability in vivo of such siRNAs and the absence of a reliable delivery method hamper the use of siRNA for treatment. Nonetheless, evidence suggests that cationic liposomes (*11*, *12*), polymer nanoparticles (*13*), and lipid conjugation (*14*) are potentially useful delivery systems for siRNA applications.

Atelocollagen (ATCOL) is a highly purified pepsin-treated type I collagen from the calf dermis. Collagen is a fibrous protein in the connective tissue and plays an important role in the maintenance of the morphology of tissues and organs. ATCOL-based delivery of siRNA resulted in efficient inhibition of metastatic tumors in vivo (*15*, *16*). We have also reported that 2-wk treatment with ATCOL-based myostatin-targeting siRNA (Mstn-siRNA/ATCOL) increased muscle mass and enhanced muscle activity (*17–19*). These findings suggest that the delivery of Mstn-siRNA/ATCOL into skeletal muscle is safe, efficient, and effective for augmentation of muscle structure and function.

Activin type IIB receptor (ActRIIB) is a type II TGF-β superfamily receptor known as a key player in the regulation of muscle size and strength. Ligands, including myostatin and growth differentiation factor-11 (GDF-11), bind to the ActRIIB, leading to phosphorylation and nuclear translocation of Smad2/3, which mediates muscle atrophy (*20*). Interestingly, the soluble ligand-binding domain of ActRIIB fused to the Fc domain of IgG (ActRIIB-Fc) potently binds and inhibits TGF-β

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Table 1. Primers used for real-time RT-PCR.

Target gene	Forward primer	Reverse primer
Myostatin	5′-CAGCCTGAATCCAACTTAGG-3′	5′-TCGCAGTCAAGCCCAAAGTC-3′
Myogenin	5′-CATGGTGCCCAGTGAATGCAACTC-3′	5′-TATCCTCCACCGTGATGCTGTCCA-3′
MuRF-1	5′-ACGAGAAGAAGAGCGAGCTG-3′	5′-CTTGGCACTTGAGAGAGGAAGG-3′
Atrogin-1	5′-GGCGGACGGCTGGAA-3′	5′-CAGATTCTCCTTACTGTATACCTCCTTGT-3′
β-Actin	5′-CCCTCACGCCATCCTGCGTC-3′	5′-CGGCAGTGGCCATCTCCTGC-3′

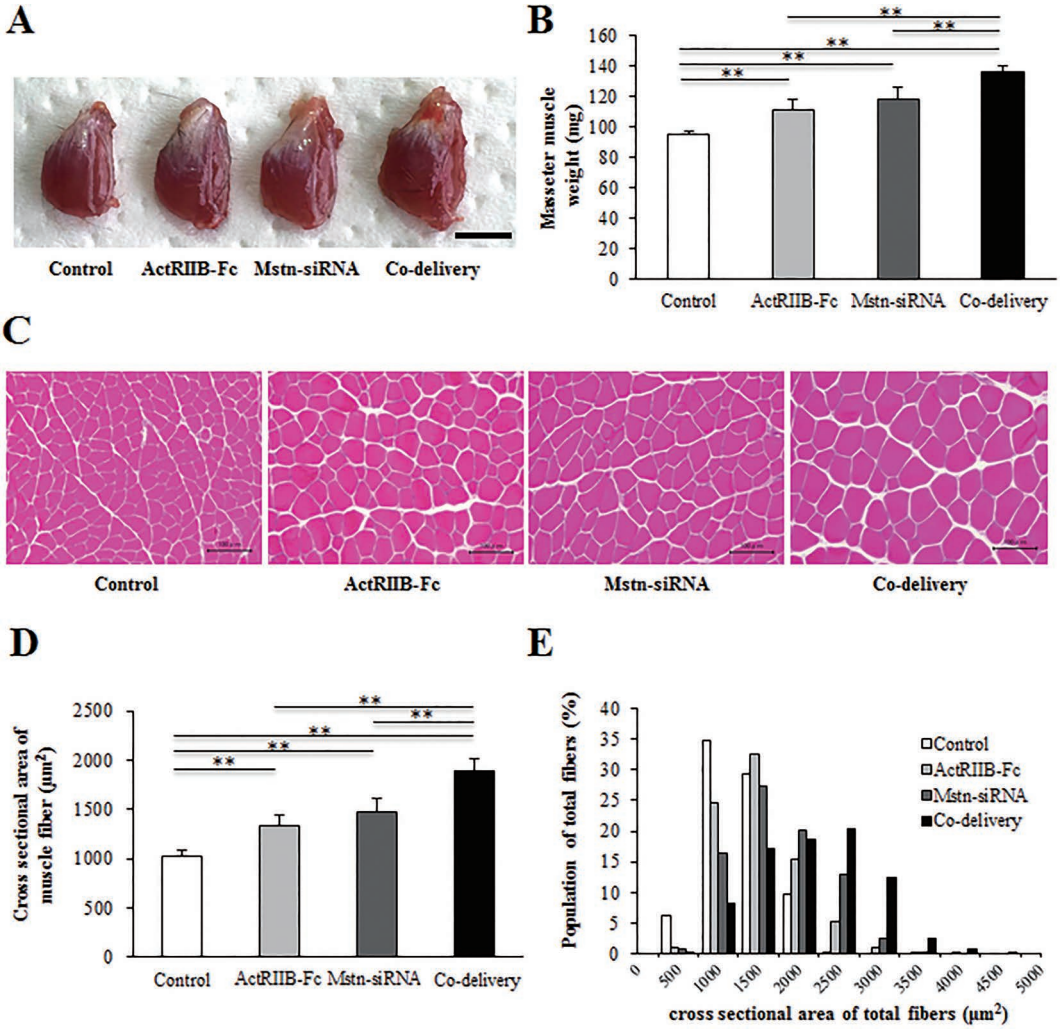

Fig. 1. Administration of Mstn-siRNA/ATCOL plus ActRIIB-Fc increased masseter muscle weight and fiber size. (A) Photograph of the masseter muscle of animals treated with Mstn-siRNA with and without ActRIIB-Fc. (B) Masseter muscle weight of the placebo, Mstn-siRNA, ActRIIB-Fc and Mstn-siRNA plus ActRIIB-Fc groups. (C) Photomicrographs of hematoxylin-eosin stained sections of the masseter muscles of representative animals of the placebo, Mstn-siRNA, ActRIIB-Fc and Mstn-siRNA plus ActRIIB-Fc groups. Scale bar, 100 μm. (D) Cross-sectional area of the muscle fibers of the masseter muscles of the placebo, Mstn-siRNA, ActRIIB-Fc and Mstn-siRNA plus ActRIIB-Fc groups. (E) Distribution histogram of the cross-sectional areas of the masseter muscle fibers. Data are mean±SD (n=4). ** p<0.01.

family members in muscle, leading to rapid and dramatic muscle growth both in vitro and in vivo (*21–24*).

We hypothesized that inhibition of myostatin by the Mstn-siRNA and ActRIIB-Fc can increase skeletal muscle mass. To test the hypothesis, the Mstn-siRNA/ATCOL/ActRIIB-Fc was delivered into the skeletal muscle, and its therapeutic effect on atrophic myopathy (e.g., muscular dystrophy) was examined.

MATERIALS AND METHODS

Small interfering RNA. Synthetic 21-nucleotide RNAs were purchased from Koken (Tokyo, Japan). The siRNAs sequences used to knockdown mouse myostatin were 5′-AAGAUGACGAUUAUCACGCUA-3′ and 5′-UAGCGUGAUAAUCGUCAUCUU-3′.

Experimental animals and local administration of Mstn-

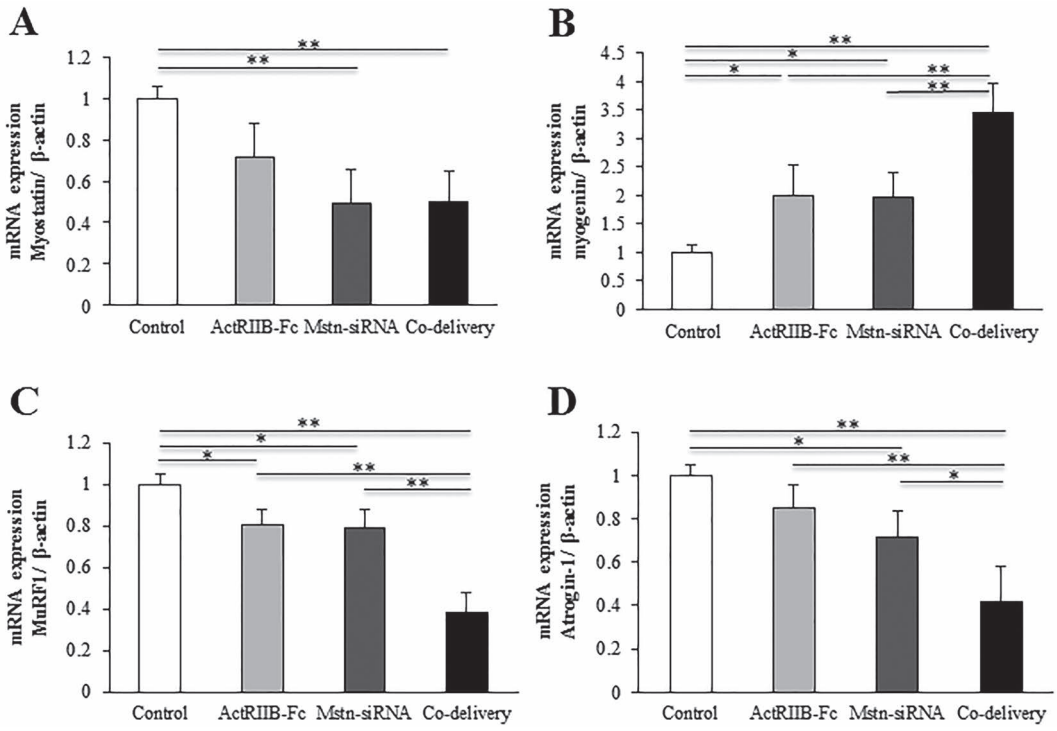

Fig. 2. Effects of administration of Mstn-siRNA/ATCOL plus ActRIIB-Fc on the expression of massetter muscle mRNA levels. Changes in the mRNA expression levels of myostatin (A), myogenin (B), MuRF-1 (C), and Atrogin-1 (D) in the masseter muscles of the placebo, Mstn-siRNA, ActRIIB-Fc and Mstn-siRNA plus ActRIIB-Fc groups. Data are mean±SD (n=4). $^{*}p<0.05$, $^{**}p<0.01$.

siRNA/ATCOL with ActRIIB-Fc fusion protein. Eleven-week-old C57BL/6 male mice were housed under a 12/12 h light/dark cycle and ambient temperature of 22˚C, and were provided with food and water ad libitum.

On the basis of previous reports (*12*, *17–19*, *21*), 10 μM of Mstn-siRNA/ATCOL and 1 μM of human ActRIIB-Fc fusion protein, purchased from Koken and R&D Systems Inc. (Minneapolis, MN), were used in this study. They were injected together into the left masseter muscles of the mice at 0 and 4 d. The control mice received injections of sterilized phosphate-buffered saline (PBS) into the left masseter muscles. The left masseter muscles were dissected 1 wk after the first injection. The Animal Care and Use Committee of Tokushima University approved all of the protocols involved in animal protection.

RNA analysis. The total RNA was extracted from masseter muscles using ISOGEN II (Nippon Gene, Tokyo, Japan). cDNA was synthesized using the PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using the Step One Plus™ with SYBR Premix Ex Taq™ II (Takara Bio) to determine the mRNA expression levels of myostatin, atrophy-related genes (MuRF-1, Atrogin-1) and myogenic regulatory factor (Myogenin). The specific primers used are listed in Table 1.

Morphometric analysis. The harvested muscles were placed in optimal cutting temperature (OCT) compound and snap-frozen in liquid nitrogen-cooled isopentane. The frozen samples were sectioned transversely (6 μm thickness) at the center of the masseter muscle using a cryostat (Leica Microsystems, Tokyo) and stained with hematoxylin and eosin (H&E) to examine the muscle morphology. Furthermore, on the frozen sections, we determined the fiber size by measuring the area of each myofiber in a fixed area. Approximately 200 myofibers were randomly selected from 6–8 fields in each tissue sample.

Statistical analysis. Data are expressed as mean± standard deviation. Differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by the Bonferroni/Dunn test. A p value of 0.05 was considered to denote a statistically significant difference.

RESULTS

Injection of Mstn-siRNA/ATCOL and ActRIIB-Fc alone resulted in significant increases in the size and weight of the masseter muscle compared with the untreated control muscle (Fig. 1A and B). The results were more pronounced following the injection of the combination of the two (i.e., both Mstn-siRNA/ATCOL and ActRIIB-Fc) (Fig. 1B), compared with the injection of each component.

Histological analysis showed that injection of Mstn-siRNA alone or with ActRIIB-Fc markedly increased the size of the myofibrils of the masseter muscles compared with the untreated control muscle (Fig. 1C). Furthermore, the results were more significant when the combination of Mstn-siRNA and ActRIIB-Fc was used, compared with the individual treatment. The mean

cross-sectional area of muscle fibers treated with Mstn-siRNA with or without ActRIIB-Fc was about 2- and 1.5-fold that of the control, respectively ($p<0.01$, Fig. 1D). Furthermore, the population of fibers within the cross-sectional area shifted from smaller to larger fibers in the Mstn-siRNA and/or ActRIIB-treated muscles (Fig. 1E).

Injection of the Mstn-siRNA/ATCOL with and without ActRIIB-Fc significantly reduced the myostatin mRNA expression level ($p<0.01$), although administration of ActRIIB-Fc alone had no effect on myostatin mRNA expression (Fig. 2A). Injection with Mstn-siRNA/ATCOL plus ActRIIB-Fc resulted in significant upregulation of the myogenin mRNA expression level (Fig. 2B), in contrast to downregulation of MuRF-1 and Atrogin-1 mRNA expression levels, compared with the injection of an individual component ($p<0.01$ and $p<0.05$, respectively, Fig. 2C and D).

DISCUSSION

We demonstrated previously that ATCOL-mediated administration of Mstn-siRNA into the caveolin-3-deficient mouse (Cav-3 Tg), which is a model of limb-girdle muscular dystrophy 1C (LGMD1C), induced a marked increase in muscle mass and significant recovery of contractile force (*18*). However, functional analysis demonstrated that the specific force of skeletal muscles treated with Mstn-siRNA in Cav-3 Tg mice was lower than that of untreated muscles in wild type mice. The result suggested that ATCOL-mediated administration of Mstn-siRNAs does not seem an ideal treatment for severe muscular diseases, such as muscular dystrophy. Since adequate improvement in muscular atrophy and dystrophy is essential, the management and augmentation of skeletal muscle metabolism remain difficult clinical challenges. Thus, increased muscle mass and enhanced muscle force is the primary goals of any new treatment with a clinically satisfactory outcome.

Previous studies demonstrated that gene translation by Mstn-targeting siRNA leads to a marked increase in muscle mass within a few weeks of application (*12*, *17*, *18*). It is also reported that the potent cell surface receptor ActRIIB binds and inhibits myostatin, leading to dramatic muscle growth (*24–26*). Based on this background, we hypothesized that the combination of these two agents would have a therapeutic advantage. Our results showed that injection of the combination of Mstn-siRNA and ActRIIB-Fc locally into the masseter muscle has a synergistic effect on the expression of muscle atrophy-related genes, myogenesis-related genes, and masseter muscle mass. These findings suggest that the beneficial effect of Mstn-siRNA seems to be enhanced by ActRIIB-Fc injected into the masseter muscle. To the best of our knowledge, the effect of the co-administration of Mstn-siRNA and ActRIIB-Fc on skeletal muscles has not been investigated yet. Therefore, first of all, we attempted to examine the effect of co-treatment on skeletal muscles of wild type mice.

Our results showed that inhibition of myostatin function by the combination of Mstn-siRNA and ActRIIB-Fc resulted in increase in the weight of the masseter muscle as well as in the size of muscle fibers, compared with the untreated control and treatment with one of the two components. Our results also showed that the increased skeletal muscle mass was due to the inhibition of myostatin signal by the combination treatment. Myostatin mRNA expression was equally downregulated by Mstn-siRNA alone and the Mstn-siRNA plus ActRIIB-Fc combination. Therefore, it was expected the phosphorylation of Smad2/3 protein expression was downregulated by co-administration of Mstn-siRNA and ActRIIB-Fc on skeletal muscles and that expectation has been currently under examination. These results suggest that ActRIIB-Fc does not affect the myostatin pathway in the masseter muscle and that the mechanism of action of the combination of Mstn-siRNA and ActRIIB-Fc is different from that of ActRIIB-Fc. Our results showed that the injection of Mstn-siRNA plus ActRIIB-Fc significantly downregulated the mRNA level of Atrogin-1 and MuRF-1 (atrophy-related genes) and upregulated the mRNA level of myogenin (a member of myogenic regulatory factors). A previous study reported that myostatin upregulates atrophy-related genes through FOXO, leading to muscle atrophy (*27*). In addition, myostatin inhibits myogenic differentiation by downregulating the muscle regulatory factors, such as MyoD and myogenin (*28*). These results suggest that the combination of Mstn-siRNA and ActRIIB-Fc effectively increases muscle mass by enhancing the expression of anabolic factors while suppressing the expression of catabolic ones.

Active myostatin binds to the ActRIIB with greater affinity than to ActRIIA and engages the signaling cascade leading to inhibition of myoblast growth (*29*). ActRIIB-Fc is a fusion protein of the receptor extracellular domain with immunoglobulin Fc that acts as a decoy receptor for myostatin (*30*). The combination of Mstn-siRNA and ActRIIB-Fc increased muscle mass, compared with each compound alone. The two components of this combination with different mechanisms of suppression of myostatin signaling seem to produce synergistic effects. Taken together, the results of the present study demonstrated that the combination of Mstn-siRNA plus ActRIIB-Fc is a promising therapeutic modality for muscular diseases.

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