Effects of Co-Transfection with Myostatin-Targeting siRNA and ActRIIB-Fc Fusion Protein on Skeletal Muscle Growth

Od BAYARSAIKHAN\(^1\), Nobuhiko KAWAI\(^1\), Hiroyo MORI\(^1\), Nao KINOUCHI\(^1\), Takeshi NIKAWA\(^2\), Eiji TANAKA\(^1\)

キーワード：myostatin, small-interfering RNAs, activin type IIB receptor, muscle hypertrophy

Abstract：**Background:** Myostatin (Mstn) is a secreted TGF-β family member that controls skeletal muscle growth, 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 combinative effects of myostatin-targeting siRNA (Mstn-siRNA) and ActRIIB-Fc on murine myoblast *in vitro* and *in vivo*.

**Materials and Methods:** C2C12 cells were treated by Mstn-siRNA with or without ActRIIB-Fc at 0 and 48 h after differentiation. Myotube size was measured, and gene expression of *Mstn, MuRF-1, MyoD* and *myogenin* were analyzed. Furthermore, 11-week-old, male C57BL/6 mice were injected with atelocollagen (ATCOL)-mediated Mstn-siRNA and Mstn-siRNA/ActRIIB-Fc locally into the masseter muscle twice a week. Histological and biochemical analyses were performed using the dissected muscles.

**Results:** Transfection of Mstn-siRNA and Mstn-siRNA/ActRIIB-Fc resulted in significant increases in the myotube diameter of the C2C12 cells compared with untreated control. Also, treatment with Mstn-siRNA and Mstn-siRNA/ActRIIB-Fc could lead to an upregulation of *MyoD* and *myogenin* gene expression and downregulation of *Mstn* and *MuRF-1*. *In vivo*, muscle fibril hypertrophy was observed in both Mstn-siRNA and Mstn-siRNA/ActRIIB-Fc treated groups. Moreover, western blotting analysis showed that the p-Smad2/3 expression level was decreased by treatment of Mstn-siRNA/ActRIIB-Fc. In contrast, MyoD and myogenin protein levels were increased by combined treatment, compared with the other groups.

**Conclusions:** These suggest that double inhibition of myostatin is potentially useful for myogenesis and muscle growth promotion. This may be a good as new treatment remedy for patients with various muscle atrophies, including muscular dystrophy.

\(^1\) Department of Orthodontics and Dentofacial Orthopedics, Institute of Biomedical Sciences, Tokushima University Graduate School
\(^2\) Department of Nutritional Physiology, Institute of Biomedical Sciences, Tokushima University Graduate School
I. Introduction

Recent advances in the field of muscle biology have led to new interest in the pharmacological treatment of muscle wasting. Myostatin (Mstn) is a member of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors. The expression pattern of Mstn suggests that it plays a role in regulating muscle development and function⁴. A number of studies have examined the effects of Mstn on C2C12 myoblasts either by treated cells with purified recombinant Mstn protein or by transfected cells with sense or antisense Mstn expression construct. These studies have demonstrated that Mstn is capable blocking both proliferation and differentiation of muscle cells²⁻⁵.

Furthermore, two studies showed that Mstn 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 muscles¹,².

In recent years, RNA interference (RNAi) has become more and more important in gene silencing and drug development because of its high specificity, significant effect, minor side effects and ease of synthesis. 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 homology⁶⁻⁷. Research efforts are currently underway to develop siRNAs as therapies for various diseases. 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.

Atelectocollagen (ATCOL) is a highly purified pepsin-treated type I collagen from the calf dermis⁸. Collagen is a fibrous protein in the connective tissue and plays an important role in the maintenance of the morphology of tissues and organs⁹. ATCOL-based delivery of siRNA resulted in efficient inhibition of metastatic tumors in vivo¹⁰,¹¹. We have also reported that 2-week treatment with ATCOL-based myostatin-targeting siRNA (Mstn-siRNA/ATCOL) complex increased muscle mass and enhanced muscle activity¹²⁻¹⁴. These findings suggest that the delivery of Mstn-siRNA/ATCOL complex 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. Mstn binds to the ActRIIB, which regulates the Smad signaling pathway to inhibit MyoD and myogenin expression and to decrease the movement of myogenic stem cells from G to S phase²⁻⁳,¹⁵,¹⁶. Interestingly, 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 dramatic muscle growth both in vitro and in vivo¹⁷⁻²⁰.

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 force¹³. 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 challenge. Thus, increased muscle mass and enhanced muscle force is the primary goals of any new treatment with clinically satisfactory outcome.

The aim of this study was to evaluate the effectiveness of double Mstn inhibition by co-delivery of Mstn-siRNA and ActRIIB-Fc on murine myoblasts and masseter muscle.

II. Materials and methods

1. Mstn-siRNA and ActRIIB-Fc treatment

We were used two different blocks presenting to affect on Mstn molecular signaling pathway by the Mstn-targeting siRNA (Koken, Tokyo, Japan) and potent cell surface receptor ActRIIB (R&D Systems, Inc., Minneapolis, MN, USA). Synthetic 21-nucleotide RNAs sequences used to knock down mouse Mstn were 5'-AAGAUGACGAUUAUCAGCUAUA-3' and 5'-UAGCGUGAUAAUCGUCAUUU-3'.

2. Cell culture

Murine myoblasts, C2C12 cells with stable overexpression of Mstn, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) consisting of 20% fetal bovine serum (FBS) and 50 U/ml penicillin in a humidified at 37°C, 5% CO₂ atmosphere. At 95% confluence, the cells were fused by replacing the medium with DMEM containing 2% horse serum (differentiation medium). At 0 and 48 h, cells were co-transfected with 10 μM Mstn-siRNA via Lipofectamine™ RNAiMAX (Life Technologies, Gaithersburg, MD, USA) and 1 μM recombinant human ActRIIB-Fc Chimera according to the manufacturer’s instruction.

3. Measurement of myotube diameter

Myotubes were photographed at ×10 magnification after 4 days of co-transfection using BIOREVO BZ-9000 fluorescent microscope (KEYENCE, Osaka, Japan). At least 100 myotubes were measured per group using BIOREVO
4. RNA analysis

The total RNA was extracted using a Pure Link® RNA Mini Kit (Life Technologies, MD, USA). cDNA synthesis was performed using a PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan). RT-PCR was carried out using a Step One Plus™ with SYBR Premix Ex Taq™ II (Takara Bio) to examine the mRNA expression level of *Mstn*, *MuRF-1* and myogenic regulatory factors (*MyoD* and *myogenin*). The primer pairs for the mouse genes are listed in Table. Expression levels of all genes were normalized to the expression level of β-actin within the same sample.

5. Animal experiments

Eleven-week-old C57BL/6 male mice were kept at constant ambient temperature of 22°C under a constant day/night rhythm, and fed on a solid diet ad libitum. At 0 and 4 days, 10 μM Mstn-siRNA/ATCOL complex with or without 1 μM ActRIIB-Fc were injected into the left masseter muscle. The control mice received injection of sterilized phosphate-buffered saline (PBS) into the right masseter muscle.

6. Histological analysis

The masseter muscles were dissected 1 week after the administration of Mstn-siRNA/ATCOL with or without ActRIIB-Fc. The harvested muscles were placed in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen-cooled isopentane. The frozen samples were sectioned transversely (6 μm) at the center of the masseter muscle using a cryostat (Leica Microsystems, Tokyo, Japan) and stained with hematoxylin and eosin (H&E) to examine the muscle morphology.

7. Immunoblot analysis

Muscle tissue was homogenized in lysis buffer. The samples were centrifuged, and protein concentration of each supernatant was measured using bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The proteins were transferred to a polyvinylidene difluoride membrane and probed with the primary antibody according to the instructions provided by the manufacturer; the antibodies used were rabbit polyclonal anti-p-Smad2/3 (1:500), anti-Smad2/3 (1:500), anti-MyoD (1:1000), anti-myogenin (1:1000) and anti-β-actin (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were anti-rabbit (1:5000) (Cell Signaling Technology, Danvers, MA, USA). Membranes were incubated for 1 h with the appropriate secondary anti-rabbit (Cell Signaling Technology) antibodies conjugated to horseradish peroxidase. Bound antibodies were visualized using the LumiGLO reagent (Cell Signaling Technology).

8. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by the Bonferroni/ Dunn test as a post hoc test to examine mean difference at the 5% level of significance.

### III. Results

Transfection of Mstn-siRNA or Mstn-siRNA/ActRIIB-Fc resulted in significantly (p < 0.01) increases in the myotube diameter of the C2C12 cells compared with untreated control. Furthermore, co-transfected myotube was significantly (p < 0.01) greater in size (Figure 1A) and diameter (Figure 1B) than those treated with Mstn-siRNA alone. In C2C12 cells co-transfected with Mstn-siRNA and ActRIIB-Fc, *Mstn* and

<table>
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<tr>
<th>Target gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td><em>Myostatin</em></td>
<td>5'-CAGCCTGAATCCAACCTAGG-3'</td>
<td>5'-TCGAGTCGCTACCAGCTTCTGCA-3'</td>
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<tr>
<td><em>myogenin</em></td>
<td>5'-CATGGTGCCCAGGGCACTTAC-3'</td>
<td>5'-TATCCTCCACGGTAGCCTGCA-3'</td>
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<tr>
<td><em>MyoD</em></td>
<td>5'-GCCTGCAAGCGCAAGGCACAC-3'</td>
<td>5'-GCAGCGGTCACGGTAG-3'</td>
</tr>
<tr>
<td><em>MuRF-1</em></td>
<td>5'-AGAGGAGAAGAGAGCGGCCTG-3'</td>
<td>5'-CTGGCACCTGAGAGAGAG-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CCCTCAGCCTCCTGCGTC-3'</td>
<td>5'-CGGAGGTGACATCTCCTGC-3'</td>
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Fig. 1 *In vitro* effects of co-transfection with Mstn-siRNA and ActRIIB-Fc on C2C12 myotube.
(A) Microphotograph of the myotube of treated with Mstn-siRNA and Mstn-siRNA plus ActRIIB-Fc. Scale bar, 100 μm. (B) Myotube diameters treated with Mstn-siRNA and co-Mstn-siRNA/ActRIIB-Fc were significantly greater than those of untreated control. Data are mean ± SD. **p < 0.01.

![Graph A](image)

![Graph B](image)

Fig. 2 *In vitro* effects of co-transfection with Mstn-siRNA and ActRIIB-Fc on myogenic and muscle atrophic gene expression.
(A and B) Mstn and *MuRF-1* mRNA expression level was significantly lower in Mstn-siRNA treated and Mstn-siRNA/ActRIIB-Fc treated cells than in untreated control. (C) *MyoD* gene expression level in the co-transfected cells was significantly higher than that in untreated control. (D) *Myogenin* gene expression level was increased in Mstn-siRNA treated and co-transfected cells when compared untreated control. *p < 0.05, **p < 0.01.

*MuRF-1* gene expression were significantly (*p < 0.05) lower than untreated control (Figure 2A and B). *MyoD* and *myogenin* gene expression levels were significantly (*p < 0.05 or *p < 0.01) higher in both Mstn-siRNA and co-transfected groups than untreated control (Figure 2C and D). Furthermore, *MyoD* gene expression level was significantly (*p < 0.05) increased in co-transfected group compared with Mstn-siRNA treated group (Figure 2C). We confirmed that ActRIIB-Fc alone could significantly upregulate the mRNA expression of *MyoD* and *myogenin* compared with untreated control in C2C12 cells (Supplementary Figure S). These results indicate that the diameters of the Mstn-siRNA and Mstn-siRNA/ActRIIB-Fc treated muscle fibril are increased by their hypertrophy.

Histological analysis showed that injection of Mstn-siRNA alone markedly increased the size of the myofibrils of the masseter muscles compared with the untreated control.
muscle. Furthermore, the results were more significant when the combination of Mstn-siRNA and ActRIIB-Fc was used, compared with the individual treatment (Figure 3A).

Western blot analysis showed that combined administration markedly reduced p-Smad2/3 expression level. Moreover, higher levels of MyoD and myogenin were detected in the treated muscles with Mstn-siRNA and ActRIIB-Fc, compared with untreated control and Mstn-siRNA-treated muscles (Figure 3B). In vivo data demonstrate that the attenuation of Smad2/3 phosphorylation is required for double inhibition mediated muscle hypertrophy, as well as for the potentiation of MyoD and myogenin protein synthesis that contributes to this hypertrophic process.

IV. Discussion

Previous studies demonstrated that gene translation by Mstn-targeting siRNA leads to marked increase in muscle mass within a few weeks of application. It is also reported that the potent cell surface receptor ActRIIB binds and inhibits Mstn, leading to dramatic muscle growth. To the best of our knowledge, the effect of the combination of Mstn-siRNA and ActRIIB-Fc on muscle growth has not been investigated previously. Thus, the present study has firstly described the effectiveness of double inhibition of Mstn gene translation by Mstn-targeting siRNA and ActRIIB-Fc in vitro and in vivo.

Mstn decreases the proliferation and differentiation of myoblasts by repressing the expression of MyoD family of basic helix-loop-helix transcription factors, which include MyoD, myogenin, Myf5 and MRF-4. To confirm the effectiveness of ActRIIB-Fc on muscle growth and differentiation, we transfected into mouse myoblast cell line. As the result, our supplementary data showed that ActRIIB-Fc increases the mRNA expression of MyoD and myogenin.

Our study indicated that co-Mstn-siRNA/ActRIIB-Fc treatment increased the murine myotube size (Fig. 1A) through an upregulation of regulatory genes responsible for myogenesis, such as MyoD and myogenin leading to myoblast fusion and myotube maturation. We also found that the Mstn and MuRF-1 (atrophy-related gene) mRNA expression levels were equally downregulated by Mstn-siRNA alone and Mstn-siRNA plus ActRIIB-Fc combination. ActRIIB-Fc does not affect the expression of Mstn and MuRF-1 in the myoblast differentiation. Previous study reported that Mstn upregulates atrophy-related gene through FOXO, leading to muscle atrophy. In addition, Mstn inhibits myogenic differentiation by downregulating MyoD and myogenin expression. These results suggest that the combination of Mstn-siRNA and ActRIIB-Fc effectively increases the number and size of myotube by enhancing the expression of various genes.

Active Mstn binds to ActRIIB with greater affinity than to activin type IIA receptor (ActRIIA) and engages the signaling cascade leading to inhibition of myoblast growth. ActRIIB-Fc is a fusion protein of the receptor extracellular domain with immunoglobulin Fc that acts as a decoy receptor for Mstn.

In our in vivo study, the combined administration of Mstn-siRNA and ActRIIB-Fc significantly increased muscle fibril size, compared with those treated with Mstn-siRNA alone and untreated control. Our results also showed that the increased muscle fibril size was due to the inhibition of Mstn signal.
by the combined administration. The two components of this combination with different mechanisms of suppression of Mstn signaling seem to produce synergistic effects. The p-Smad2/3 protein expression was equally downregulated by Mstn-siRNA alone and Mstn-siRNA plus ActRIIB-Fc combination. These results suggest that ActRIIB-Fc does not affect the expression of Mstn 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. Moreover, MyoD and myogenin protein levels were increased in masseter muscles with Mstn-siRNA and ActRIIB-Fc administration compared with the untreated control and individual treatment. The double inhibition of Mstn with different suppression mechanisms seems to produce synergistic effects.

Taken together, the present results demonstrated that the combination of Mstn-siRNA plus ActRIIB-Fc may be a promising therapeutic modality for muscle atrophic diseases.

V. Acknowledgment

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VI. Conflicts of Interest

The authors declare no conflict of interest.

VII. References

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