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

Od Bavarsaikhan, Nobuhiko Kawai, Hiroyo Mori, Nao Kinouchi, Takeshi Niki and Eiji Tanaka

1Department of Orthodontics and Dentofacial Orthopedics, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima 770–8504, Japan
2Department of Nutritional Physiology, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima 770–8503, Japan

(Received December 27, 2016)

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 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

*To whom correspondence should be addressed.
E-mail: etanaka@tokushima-u.ac.jp
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′-AAGAUGAGUAAUACGCUA-3′ and 5′-UAGCGUAAUACGCUAUU-3′.

Experimental animals and local administration of Mstn-
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
Our results showed that injection of the combination of Mstn-siRNA and ActRIIB-Fc into the masseter muscle has a synergistic effect on the expression of Mstn-siRNA and ActRIIB-Fc resulting 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.

Acknowledgments

This research was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology (No. 26463095 and No. 26293436) and from the Japanese Ministry of Health, Labor and Welfare.

REFERENCES

1) LeBrasseur NK, Schelhorn TM, Bernardo BL, Cosgrove PG, Loria PM, Brown TA. 2009. Myostatin inhibition enhances the effects of exercise on performance and metabolic outcomes in aged mice. J Gerontol A Biol Sci Med Sci 64: 940–948.

2) Murphy KT, Ryal JG, Snell SM, Nair L, Koopman R, Krasney PA, Ibebujo C, Holden KS, Loria PM, Salatto CT, Lynch GS. 2010. Antibody-directed myostatin inhibition
improves diaphragm pathology in young but not adult dystrophic mdx mice. Am J Pathol 176: 2425–2434.

3) Lee SL, Reed LA, Davies MV, Girgenrath S, Goaf ME, Tomkinson KN, Wright JF, Barker C, Ehrentraut G, Holmstrom J, Trowell B, Gertz B, Jiang MS, Sebald SM, Matzuk M, Li E, Liang LF, Quattlebaum E, Stotish RL, Wolfman NM. 2005. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. Proc Natl Acad Sci USA 102: 18117–18122.

4) Chiu SC, Peekhau N, Weber H, Adamski S, Murray EM, Zhang HZ, Zhao JZ, Ernst R, Lineberger J, Huang L, Hampton R, Arnold BA, Vitelli S, Hamuro L, Wang WR, Wei N, Dillon GM, Miao J, Alves SE, Glantschnig H, Fang W, Wilkinson HA. 2013. Increased muscle force production and bone mineral density in ActRlB-Fc-treated mature rodents. J Gerontology 68: 1181–1192.

5) Bogdanovich S, Perkins KJ, Krug TOB, Whittemore LA, Khurana TS. 2005. Myostatin propeptide-mediated amelioration of dystrophic pathophysiology. FASEB J 19: 543–549.

6) Hamrick MW, Arounleut F, Kellum E, Cun M, Immel D, Liang LF. 2010. Recombinant myostatin (GDF-8) propeptide enhances the repair and regeneration of both muscle and bone in a model of a deep penetrating muscleoskeletal injury. J Trauma 69: 579–583.

7) McPherson AC, Lawler AM, Lee SJ. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387: 83–90.

8) Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, Kambadur R. 2000. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem 275: 40235–40243.

9) Fire A, Xu SQ, Montgomery MK, Costas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806–811.

10) Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tusche T. 2001. Duplexes of 21-nucleotide RNA mediate RNA interference in cultured mammalian cells. Nature 411: 494–498.

11) Santel A, Aleku M, Keil O, Endruschat J, Esche V, Fisch G, Dames S, Löffler K, Giese K, Klippel A, Kaufmann J. 2006. A novel siRNA-ligand technology for RNA interference in the mouse vascular endothelium. Gene Ther 13: 1222–1234.

12) Mori H, Kawai N, Kinouchi N, Hichijo N, Ishida T, Kawakami E, Noji S, Tanaka E. 2014. Effectiveness of cationic liposome-mediated local delivery of myostatin-targeting small interfering RNA in vivo. Dev Growth Differ 56: 223–232.

13) Aigner A. 2006. Gene silencing through RNA interference (RNAi) in vivo: Strategies based on the direct application of siRNAs. J Biotechnol 124: 12–25.

14) Zhang C, Tang N, Liu X, Liang W, Xu W, Torchilin VP. 2006. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. J Control Release 112: 229–239.

15) Takeshita F, Minakuchi Y, Nagahara S, Homma K, Sasaki H, Hirai K, Teratani T, Namatame N, Yamamoto Y, Hanai K, Kato T, Sano A, Ochiya T. 2005. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo. Proc Natl Acad Sci USA 102: 12177–12182.

16) Takeshita F, Ochiya T. 2006. Therapeutic potential of RNA interference against cancer. Cancer Sci 97: 689–696.