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SHORT COMMUNICATION

Atelocollagen-mediated local and systemic applications of myostatin-targeting siRNA increase skeletal muscle mass

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RNA interference (RNAi) offers a novel therapeutic strategy based on the highly specific and efficient silencing of a target gene. Since it relies on small interfering RNAs (siRNAs), a major issue is the delivery of therapeutically active siRNAs into the target tissue/target cells in vivo. *For safety reasons, strategies based on vector delivery may be of only limited clinical use. The more desirable approach is to directly apply active siRNAs* in vivo. *Here, we report the effectiveness of* in vivo *siRNA delivery into skeletal muscles of normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen (ATCOL). ATCOL-mediated local application of siRNA targeting myostatin, a negative regulator of skeletal muscle growth, in mouse skeletal muscles or intravenously, caused a marked increase in the muscle mass within a few weeks after application. These results imply that ATCOL-mediated application of siRNAs is a powerful tool for future therapeutic use for diseases including muscular atrophy.*
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Keywords: *myostatin; RNA interference; atelocollagen; muscle; mouse; muscular dystrophy*

RNA interference (RNAi) is the process of sequence-specific, posttranscriptional gene silencing in plants and animals from flatworms to human,[1] which is mediated by ~22-nucleotide small interfering RNAs (siRNAs) generated from longer double-stranded RNA. Since it was demonstrated that siRNAs can intervene gene silencing in mammalian cells without induction of interferon synthesis or nonspecific gene suppression,[2] an increasing number of remedies utilizing highly specific siRNAs targeted against disease-causing or disease-promoting genes have been developed.[3] Effective delivery of active siRNAs to target organs or tissues is therefore the key to the development of RNAi as a broad therapeutic platform. For this purpose, different strategies have been used to deliver and achieve RNAi-mediated gene silencing *in vivo*;[3] for example, polymers represent a class of materials that meet the needs of a particular siRNA delivery system, condensing siRNAs into nano-sized particles taken up by cells.[4] However, some of the synthetic polymers, which have been used for delivery of nucleic acids, may trigger cell death in a variety of cell lines and thus suffer from limitations for its application in siRNA delivery *in vivo*.[4] On the other hand, atelocollagen (ATCOL), a pepsin-treated type I collagen lacking in telopeptides in N and C terminals that confer its antigenicity, has been shown to elicit an efficient delivery of chemically unmodified siRNAs to metastatic tumors *in vivo*.[5–7] In this study, we sought to examine the effectiveness of siRNA-ATCOL therapy for a nontumorous systemic disease, targeted against myostatin (growth/differentiation factor 8, GDF8), a negative regulator of skeletal muscle growth.[8]

Skeletal muscles are the crucial morphofunctional organs, and their atrophy causes severe conditions for life such as muscular dystrophies. Duchenne muscular dystrophy (DMD), for instance, is a severe muscle wasting disorder affecting 1 out of 3500 male birth.[9] There is currently no effective treatment, but gene therapy approaches are offering viable avenues for treatment development.[10] As one of therapeutic approaches, inhibition of myostatin by using anti-myostatin-blocking antibodies has been employed to increase muscle mass.[11] However, generating antibodies against recombinant target proteins is time consuming and requires a lot of efforts. Recently, we demonstrated that inhibition of myostatin by overexpression of the myostatin prodomain[12] prevented muscular atrophy and

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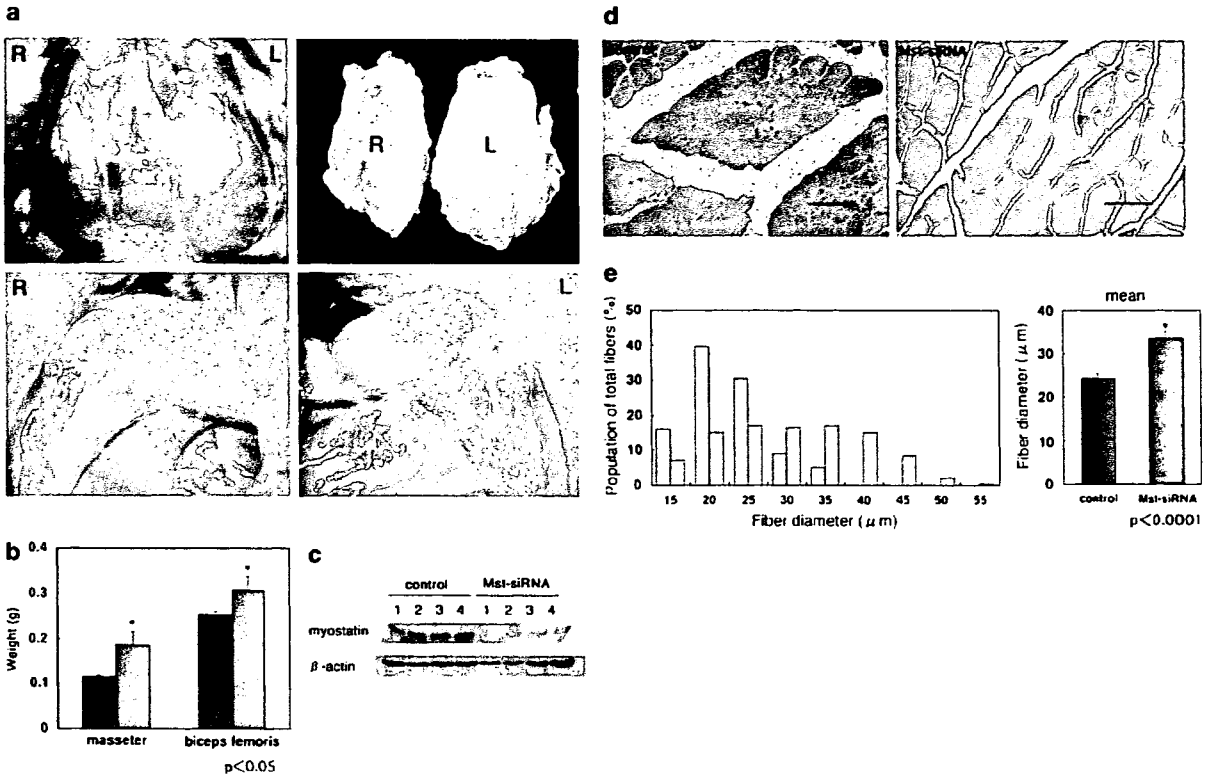

Figure 1 Local administration of the Mst-siRNA/atelocollagen (ATCOL) complex increases skeletal muscle mass and fiber size in wild-type mice through inhibition of myostatin expression. For the experiments depicted in (**a**–**e**) Mst-siRNAs (final concentration, 10 μM) were mixed with ATCOL (final concentration for local administration, 0.5%.[5]) (AteloGene, Kohken, Tokyo, Japan) according to the manufacturer's instructions. After anesthesia of mice (20-week-old male C57BL/6) by Nembutal (25 mg/kg, i.p.), the Mst-siRNA/ATCOL complex was injected into the masseter and biceps femoris muscles on the left side. As a control, scrambled siRNA/ATCOL complex was injected into the contralateral (right) muscles. After 2 weeks, the muscles on both sides were harvested and processed for analysis. (**a**) Photographs of muscles. Increased muscle mass were observed in the Mst-siRNA/ATCOL-treated (L) masseter (upper panels) and biceps femoris (lower panels), but not in the contralateral muscles (R). (**b**) Muscle weight. Mst-siRNA/ATCOL-treated muscles had an increased weight significantly compared to those with control siRNA/ATCOL (masseter, 0.185 ± 0.041 versus 0.115 ± 0.019 g; biceps, 0.307 ± 0.040 versus 0.232 ± 0.039 g; $n = 4$; $P < 0.05$). Student's *t*-test was used for determining statistical significance. Graphical representation of data uses the following convention: mean ± s.d.; treated muscles or mice in red; control muscles or mice in blue. (**c**) Western blot analysis of myostatin (52 kDa) in the control and Mst-siRNA/ATCOL-treated masseter muscles, assessed at 2 weeks after single injection. Total 80 μg of masseter muscle homogenates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes for immunoblotting. After a blocking reaction (5% nonfat milk/1% bovine serum albumin in phosphate-buffered saline (PBS) and 0.05% Triton X-100), the blots were incubated for 1 h at room temperature with mouse monoclonal anti-myostatin antibody (1:500; R&D Systems, Minneapolis, MN, USA) or anti-β-actin. After incubation with a secondary antibody (1:10000; horseradish peroxidase-conjugated anti-rat antibody; Biosource International, Camarillo, CA, USA), the blots were developed using the ECL Plus kit (Amersham, Buckinghamshire, UK). We used a purified myostatin protein and proteins extracted from cells transformed with a myostatin cDNA to confirm that the bands are due to 52 kDa myostatin. (**d**) Hematoxylin and eosin staining of the control and Mst-siRNA/ATCOL-treated masseter muscle. Muscles were fixed in 4% paraformaldehyde/PBS at 4 °C overnight, dehydrated and paraffin-embedded. Serial sections (5 μm thickness) were cut at mid-belly of muscle and stained. Scale bar, 50 μm. (**e**) Distribution of myofibril sizes of the control (blue bars) and Mst-siRNA/ATCOL-treated (red bars) muscles. The right panel shows the average myofibril size (33.6 ± 1.5 versus 24.4 ± 1.1 μm; $n = 200$; $P < 0.0001$). NIH Image (NIH, USA) software was used for morphometric measurements.

normalized intracellular myostatin signaling in the model mice for limb-girdle muscular dystrophy 1C.[13] On the other hand, Magee *et al.*[14] demonstrated that downregulation of myostatin expression by transduction of a plasmid expressing a short-hairpin interfering RNA (shRNA) against myostatin using electroporation can increase local skeletal muscle mass. For safety reasons, however, strategies based on vector delivery may be of only limited clinical use. The more desirable approach is to directly apply active siRNAs *in vivo*. As one of the practical platforms for siRNA delivery, we sought to employ an ATCOL-mediated oligonucleotide delivery system to apply myostatin-targeting siRNA into muscles.

We utilized the siRNA sequences reported previously[14] (GDF8 siRNA26, 5′-AAGATGACGATTAT CACGCTA-3′, position 426–446). It has been noted that this sequence can target myostatin mRNA not only of mouse but also human, rat, rabbit, cow, macaque and baboon, based on Blast search (National Center for Biotechnology Information).[14] To confirm the silencing effect of this siRNA, we constructed a plasmid of pSilencer 2.1-U6 neo containing the target sequence and transfected the plasmid into a mouse myoblast cell line, C2C12 cells, which had been made forced to stably express myostatin. We confirmed that the RNAi construct could effectively downregulate the expression

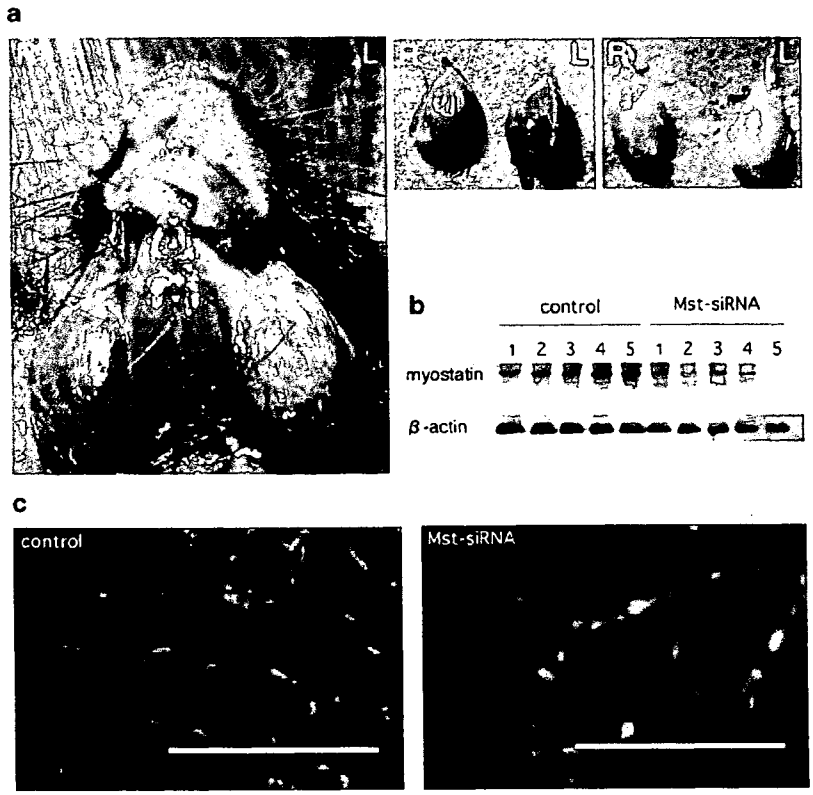

Figure 2 Mst-siRNA/atelocollagen (ATCOL) treatment improves myofibril size in *mdx* mice. (**a**) Photographs of muscles. The leftward masseter (left and middle panels) and tibial (right panel) muscles injected with the Mst-siRNA/ATCOL complex intramuscularly show a marked increased muscle mass in 20-week-old *mdx* male mice. (**b**) Western blot analysis of the control and Mst-siRNA/ATCOL-treated masseter muscles, assessed at 2 weeks after single injection. Myostatin protein levels in the muscles injected with the Mst-siRNA/ATCOL complex are markedly decreased, but not in the contralateral muscles injected with the control-siRNA/ATCOL. (**c**) Immunohistochemical analysis of the cross-sectional myofiber area of the masseter muscle, with the anti-laminin α2 antibody (4H8-2, Sigma, St Louis, MO, USA), showing increased fiber size in the Mst-siRNA/ATCOL-treated (right panel) muscle, compared to that of control (left panel). Alexafluor 594-conjugated anti-rat immunoglobulin G antibodies (A-11007, Invitrogen, Carlsbad, CA, USA) were used for immunohistochemistry. Scale bar, 100 μm.

of myostatin in the C2C12 cells[15] (Supplementary Figure S1).

We prepared the nanoparticle complex containing the GDF8 siRNA26 (10 μM) and ATCOL. Then, we injected the GDF8 siRNA26-ATCOL (Mst-siRNA/ATCOL) complex into the masseter and biceps femoris muscles of 20-week-old C57BL/6 mice. As a control, we injected control-scrambled siRNAs/ATCOL complex in the contralateral muscles. We observed gross morphology of the muscles and dissected the muscle tissues 2 weeks after injection. After injection of the Mst-siRNA/ATCOL complex, both muscles (on the left side) were enlarged, while no significant change was observed on the contralateral side (Figure 1a). We also measured the muscle weight, finding that the Mst-siRNA/ATCOL-treated muscles weighed significantly more than those on the control side (Figure 1b). The Mst-siRNA/ATCOL-treated muscles were further examined by a western blot analysis for myostatin (52 kDa), showing the decreased expression of myostatin on the treated side (Figure 1c). We quantified each result as a ratio to the internal control and statistically analyzed a difference between control (average ratio 0.90 ± 0.07) and treated (average ratio 0.44 ± 0.22) muscles. This difference is significant ($P < 0.01$, Student's t-test, $n = 4$). Histological analysis showed that the myofibril sizes of the masseter muscles treated with the Mst-siRNA/ATCOL complex were larger than those of the control (Figure 1d). Examining the sizes of 200 myofibers per group, the population of myofibril sizes indicated a shift from smaller to larger fibers in the Mst-siRNA/ATCOL-treated muscle (Figure 1e). The average myofibril size of the muscle treated with Mst-siRNA/ATCOL gained approximately 1.3 times more than that of control (Figure 1e). No obvious morphological change was observed in other tissues than the treated masseter muscles. In the meanwhile, we did not observe any general sign of ill health and deaths during the period of experiment. These results indicate that the increase of the Mst-siRNA/ATCOL-treated muscle mass is caused by their hypertrophy and that the siRNA complex gives no obvious adverse effects.

We next questioned whether this effect of hypertrophy after local injection of the Mst-siRNA/ATCOL complex observed in normal mice was relevant to dystrophin-deficient *mdx* mouse, an animal model for DMD.[16] We intramuscularly injected the same Mst-siRNA/ATCOL complex into the masseter and tibial muscles on the left side of 20-week-old *mdx* male mice. Within 2 weeks after the single injection, a dramatically increased muscle

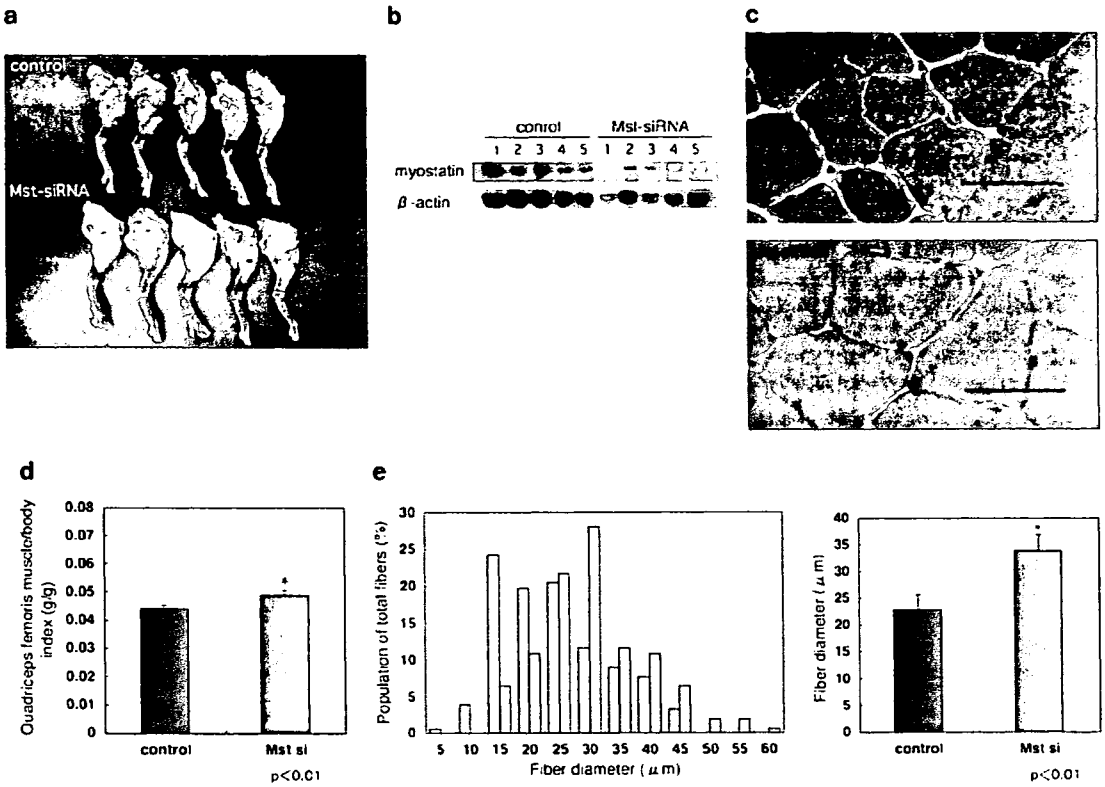

Figure 3 Systemic administration of the Mst-siRNA/atelocollagen (ATCOL) complex induces muscle enlargement in the mouse through inhibition of myostatin expression. For systemic administration, the siRNA (final concentration, 40 μM)/ATCOL (final concentration, 0.05%[6] complex, 200 μl) was introduced intravenously via orbital veins at 4, 7 and 14 days after the first application ($n = 5$). As a control, control-scrambled siRNAs were injected into wild-type male mice (20 weeks, $n = 5$). After 3 weeks, the quadriceps muscles on both sides were harvested and processed for analysis. (**a**) Photographs of lower limbs from control (upper panel) and Mst-siRNA/ATCOL-treated (lower panel) mice. (**b**) Western blot analysis of the control and Mst-siRNA/ATCOL-treated muscles (quadriceps femoris), assessed at 3 weeks after triple injection. (**c**) Hematoxylin and eosin staining of the control (upper panel) and Mst-siRNA/ATCOL-treated quadriceps muscle (lower panel). Scale bar, 50 μm. (**d**) Comparison of muscle weight/body weight index between the Mst-siRNA/ATCOL and control-siRNA/ATCOL-treated mice (0.048 ± 0.002 versus 0.043 ± 0.001 $n = 5$; $P < 0.01$). (**e**) Distribution of myofibril sizes of the control and Mst-siRNA/ATCOL-treated quadriceps muscles. The right panel shows the average myofibril size (33.92 ± 2.91 versus 22.95 ± 1.54 μm, $n = 156$; $P < 0.01$).

mass was observed in the Mst-siRNA/ATCOL-treated muscle (Figure 2a). Western blot analysis showed that the protein levels of myostatin in the muscles treated with the Mst-siRNA/ATCOL complex were significantly decreased (average ratio 0.55 ± 0.03), but not in the contralateral muscles treated with control siRNAs/ATCOL complex (average ratio 0.83 ± 0.01) (Figure 2b; $P < 0.05$, $n = 5$). Furthermore, immunohistochemical analysis on the masseter using an anti-laminin α2 antibody showed increase in the mean myofiber size of the Mst-siRNA/ATCOL-treated muscle (Figure 2c), as is the case for the wild-type (not shown). On the basis of these results, it seems that myostatin maintains satellite cells or muscle stem cells in a quiescent state. Reduced myostatin activity would lead to activation of these cells and fusion into existing fibers (Supplementary Figure S1e and f), resulting in fiber hypertrophy as proposed previously.[14]

We further examined whether systemic administration of the Mst-siRNA/ATCOL complex would have an effect on silencing the myostatin expression and lead to muscle enlargement. The Mst- or control siRNA/ATCOL complex was applied intravenously into normal mice four times in 3 weeks. Strikingly, we observed an obvious enlargement of skeletal muscles of lower limbs (Figure 3a), masseters and other muscles. Since change in the muscles of lower limbs is much larger than others, we used them for further analyses. We confirmed reduction of myostatin proteins in the muscles treated with the Mst-siRNA/ATCOL complex (average ratio 0.67 ± 0.11) (Figure 3b; $P < 0.01$, $n = 5$; average ratio for control 0.87 ± 0.03). We observed that the treated lower limbs are much larger than the controls, although the average body weights were 26.7 ± 0.7 and 25.8 ± 0.4 g for controls and treated mice, respectively. No increase in the body weight of the treated mouse was observed, probably because increase in the muscle weight compensated for reduction of fat accumulation.[17] To show increase in muscle weights, we used the muscle weight/body weight ratio (Figure 3d), in case the body weight exhibited variation. Significant increase in muscle fiber size (Figures 3c and e) was also observed after 3 weeks. These results indicate that siRNAs targeting against myostatin, intravenously administered with ATCOL, can specifically repress the expression of myostatin, inducing muscle hypertrophy in normal mice.

We present evidence that local and systemic applications of siRNA against myostatin coupled with ATCOL markedly stimulate muscle growth *in vivo* within a few

weeks. Local application of siRNA/ATCOL complex was shown to be effective to target the vascular endothelial growth factor gene in a xenografted tumor,[18] while ATCOL was used for systemic siRNA delivery into tumor-bearing mouse models and proved to be effective for silencing exogenous genes as luciferase and metastasis-associated genes as EZH2.[6] However, it has not been elucidated until this study whether the siRNA complex could have an effect of muscle growth on normal tissues by repression of muscle-specific genes. It has been thought that the enhanced permeability and retention (EPR) effect in tumor tissues could facilitate selective targeting of siRNA/polymer complex.[6] In spite of the significance of the EPR effect in tumor therapies, it is noticeable that normal and nontumor diseased tissues can be targets for siRNA-based drugs applied systemically. It was reported that nuclease activity to siRNA could be prevented[18] and cellular uptake of siRNAs was elevated by ATCOL.[5] Although the precise mechanisms by which ATCOL achieves these effects have not been elucidated to date, ATCOL complexed with DNA molecules was demonstrated to be efficiently transduced into mammalian cells.[19] Thus, similarly siRNA/ATCOL complexes may be transduced into cells probably by the same mechanisms as observed for DNA molecules. As a simple administration of myostatin-siRNA/ATCOL complex has a muscle growth effect, this novel method for fighting against muscle atrophy would be of considerable value for clinical applications. In tumor-bearing mice, it was reported that ATCOL could distribute siRNAs against luciferase to normal liver, lung, spleen and kidney tissues as well as bone-metastatic lesions.[6] ATCOL was also reported to display low toxicity and low immunogenicity when it is transplanted *in vivo*.[20,21] Taken together with our results, application of siRNAs with ATCOL would be promising for a therapeutic remedy against various diseases not only of muscles, but also of these organs.

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