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siRNAs Targeting Mouse Myostatin

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Abstract—Eight different mouse myostatin small interfering RNA (siRNAs) were synthesized and tested. Five siRNAs showed a pronounced biological effect reducing myostatin mRNA content. For two of them, the myostatin mRNA level was reduced 3- and 4-fold, respectively. The obtained siRNAs can be used for study of biological effects of myostatin, both *in vitro* and *in vivo*.

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Small (or short) interfering RNAs (siRNAs) are short double-stranded RNA molecules, 21-23 bp in length whose nucleotide sequence is identical or homologous to corresponding mRNA sequences. siRNAs demonstrate the effect of target gene inhibition, so-called RNA interference [1].

RNA interference is observed in all eukaryotic species: protozoa, fungi, plants, and animals including mammals [2-4]. Several mechanisms of siRNA action on the target protein synthesis are known to date. First, siRNAs activate a special system of RNases, the enzymatic complex RISC, which specifically cleaves the mRNA molecule complementary to siRNA [5]. Second, siRNAs can also activate a specific isoform of the RISC complex, which inhibits translation of the given mRNA [6]. Finally, siRNAs use the complex RITS for suppression of target gene transcription via histone H3 and/or DNA methylation [7]. Unlike the effect of interferons retarding expression of all cellular mRNAs, the effect of siRNAs is very specific: RNA interference virtually disappears with decrease in number of complementary nucleotides in 21-bp RNA to 14 or 15.

At the present time siRNAs are utilized not only in research practice. They are considered as promising therapeutics and successfully used for suppression of various mammalian genes (artificially introduced into the cells of gene-engineering constructs and natural cellular genes).

Using siRNAs on cell cultures, the effect of RNA interference was evoked for RNAs of some oncogenes [8, 9], inducers of apoptosis [10, 11], protein inducers of neurodegenerative disorders [12], and viral proteins [13, 14]. The given approach can also be applied in a system *in vivo*. Encouraging results were obtained on administration of siRNAs for protection from virus-induced autoimmune response [10], from development of inflammatory reaction and fibrosis of the eye [15], and from progression of transplanted malignant glioma [16]. It is proven that RNA interference can occur in myoblasts and differentiated myotubules, both on exogenous constructs and natural cellular mRNAs [17]. However, systems of siRNA delivery *in vivo* are not sufficiently studied, which retards development of the given approach.

Far from all sites in the mRNA molecule are suitable for preparation of siRNA on its basis: a random sampling of nucleotide sequences only yields 20-25% biologically active siRNAs. A completely dependable algorithm for searching for nucleotide sequences that are applicable for siRNA synthesis has not yet been designed. Regression analysis performed in [18] determined the significance of factors, such as presence of distinct nucleotides in distinct positions, tendency to the secondary structure formation in both siRNA strands, and so on. A particular importance of adenine in position $3n + 1$ was reported in [19]. Nonetheless, noncommercial software for this kind of search does not guarantee selection of successful sequences, and nucleotide sequence selection algorithms are not often published.

The goal of this study was the selection of effective myostatin expression inhibitors using RNA interference.

Abbreviations: bp) base pair; GAPDH) glyceraldehyde-3-phosphate dehydrogenase; QRT-PCR) (quantitative) real-time polymerase chain reaction; siRNA) small interfering RNA.

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Myostatin is a member of the protein family TGF-β, which limits muscle mass via inhibition of myoblast proliferation [20] and differentiation into myotubules [21]. Mutations in the myostatin gene lead to a sharp growth of muscle mass [22]. Sustained injections of neutralizing antibodies also stimulate muscle growth [23]. Recently, myostatin has been considered as a promising target in therapy of various muscular dystrophies [24, 25]. RNA interference was successfully used for inhibition of myostatin expression in fishes (zebrafish) [26] and mammals (rat) [27].

Thus, the use of siRNA for decrease of myostatin concentration may be of very practical importance. The goal of this study was to compare efficacies of different siRNAs and to choose those optimal for further experiments *in vivo*.

MATERIALS AND METHODS

To choose nucleotide sequences for synthesis of siRNAs targeting mouse myostatin, the mRNA NM_010834 sequence was analyzed with siRNA selection software packages available on the sites of the companies Dharmacon (USA), InvivoGen (USA), and Ambion (USA). Homology of chosen siRNA sequences to other mouse genome sites was checked using the BLAST software. The siRNA synthesis with the chosen sequences was performed in the company Syntol (Russia).

The lipofection and electroporation techniques were used for siRNA transfection in culture of murine myoblasts isolated from the tibialis anterior muscle [28]. Lipofection was performed using lipofectamine and oligofectamine (Invitrogen, USA) and Unifectin-56 (Unifect Group, Russia) according to the manufacturers' protocols. Electroporation was carried out on a GenePulser electroporation system (BioRad, USA) according to the protocol recommended by the manufacturer. A double-stranded oligonucleotide labeled with rhodamine (Syntol) was used in testing of transfection efficacy. The transfection efficacy was estimated by counting of fluorescent cells (in percent) under a DMLB microscope (Leica, Germany).

RNA from murine myoblast culture was isolated with Trizol (Invitrogen). Reverse transcription was carried out with an RNA isolation kit (Sileks, Russia).

The real-time polymerase chain reaction (QRT-PCR) method was used for comparison of myostatin mRNA expression in control myoblasts and myoblasts treated with siRNA. To perform it, a reagent kit (Syntol) was used containing the fluorescence dye SYBR Green and the reference dye ROX. The following primers were used [29]: direct 5′-CAGCCTGAATCCAACTTAGG-3′ and reverse 5′-TCGCAGTCAAGCCCAAAGTC-3′ targeting the myostatin cDNA (the product size 167 bp) and direct 5′-ATCACTGCCACCCAGAAGACT-3′ and reverse 5′-CATGCCAGTGAGCTTCCCGTT-3′ targeting the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA (the product size 153 bp). Amplification was carried out according to the following protocol: activation of HotTaq-polymerase at 94°C for 5 min; denaturation at 94°C for 15 sec, annealing and elongation at 60°C for 1 min, 40 cycles. Both amplification and detection were performed on a 7300/7500 real-time PCR system (Applied Biosystems, USA).

The method of difference of threshold cycles (comparative $C_T - \Delta\Delta C_T$) was used to calculate the inhibition of myostatin mRNA expression (in percentages). The difference of threshold amplification cycles (C_T) of myostatin and GAPDH ($\Delta C_T = C_{T\ myostatin} - C_{T\ GAPDH}$) was determined for each sample. Then the difference was calculated between ΔC_T of control treated with oligofectamine only and samples also treated with siRNA ($\Delta\Delta C_T = \Delta C_{T\ control} - \Delta C_{T\ experiment}$). The comparative expression level of myostatin was calculated from the equation $2^{-\Delta\Delta C_T}$ (in accordance with the recommendation of Applied Biosystems Company).

RESULTS AND DISCUSSION

The nucleotide sequence of murine myostatin mRNA was analyzed using three software products with the aim of search for target sites of RNA interference. Two, 13, and 218 sequences were found using Dharmacon siDESIGN Center, InvivoGen siRNA Wizard, and Ambion, respectively.

One sequence of two found using Dharmacon siDESIGN Center and four sequences of 13 found using InvivoGen siRNA Wizard were chosen after the verification of homology between the found sequences and other regions of murine genome using the BLAST software. Two oligonucleotide sequences identical in human and murine myostatin mRNAs were also chosen, although they were not selected by any of the three software products used. Of all sequences proposed by the Ambion software, the sequence with proven capability to induce RNA interference [27] was taken for a positive control. The selection of five siRNAs from many sequences proposed by this software was carried out in this work, and the chosen siRNA was demonstrated to be the most effective (>80%).

Neither of the sequences chosen in the present work had more than 14-nucleotide homology with other expressed murine RNAs. siRNA produced by Qiagen (The Netherlands), which does not induce interference effect, was used as a negative control; its sequence was borrowed from work [30].

Thus, eight different siRNA were studied in further experiments: uccgcauacucaucuugugca (found by Dharmacon software), acgcuaccacggaaacaauca (InvivoGen), aacccaugaaagacgguacaa (InvivoGen), augaaagacggua-

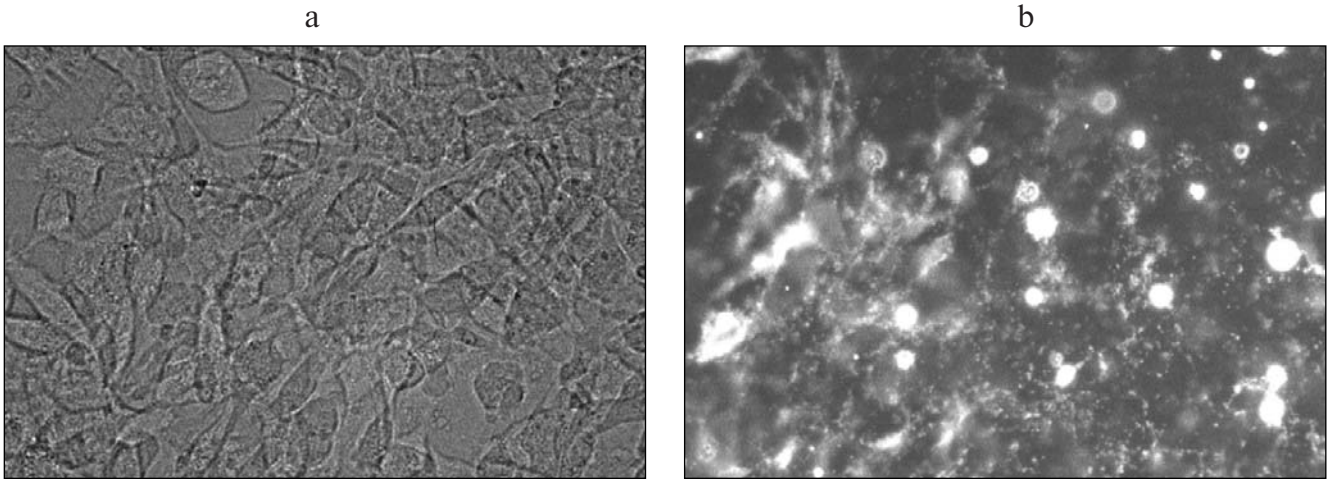

Fig. 1. Photographs of murine myoblasts of C2C12 cell line transfected by rhodamine-labeled double-stranded oligonucleotide by lipofection with oligofectamine (×400). a) Phase contrast; b) fluorescence microscopy.

caagguau (InvivoGen), aaagacgguacaagguauacu (InvivoGen), aagaacaaauaauauauggga (common for mouse and human), uauaaggccaauuacugcucu (common for mouse and human), aagaugacgauuaucacgcua (Ambion, proven efficacy, positive control). siRNA carrying the sequence uucuccgaacgugucacgu was used as a negative control.

To increase the efficacy of RNA interference, both sense and anti-sense sequences had two additional uridine residues at their 3′-termini.

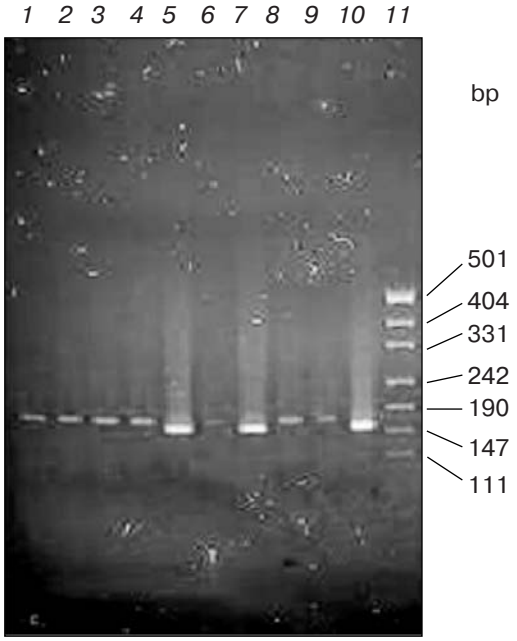

Fig. 2. Electrophoresis in 2% agarose gel of amplification products of cDNA reverse transcribed from the RNA isolated from primary myoblasts. Lanes: *1-4*, *6*, *8*, *9*) amplification on myostatin primers; *5*, *7*, *10*) amplification on GAPDH primers; *11*) standards.

The transfection efficacies in myoblast culture were compared by various methods. The fluorescent probe was incorporated only into 20% of murine myoblasts when double-stranded oligonucleotide was lipofected by lipofectamine, and the percentage of fluorescent cells did not exceed 5% with Unifectin-56. The lipofection with oligofectamine, as well as electroporation, enabled the label inclusion into more than 90% of the cells. However, under conditions providing high transfection efficacy, the electroporation procedure had a significant cytotoxic effect (~50%), so further siRNA transfection was carried out by the lipofection method using 400 pg of oligofectamine per 60-mm Petri dish (Fig. 1).

RNA isolated from myoblasts was pure enough ($D_{260}/D_{280} \geq 1.95$). PCR with cDNA synthesized from this RNA using either myostatin or GAPDH primers resulted in a single product: only one band was observed after electrophoresis (Fig. 2), and only one peak was on the melting curve. Twofold and fourfold dilutions of a template have shown a linear dependence of the threshold cycle on dilution. The amplification efficacy was near 100%, both for GAPDH and myostatin, so the application of $\Delta\Delta C_T$ method was appropriate.

Among all tested siRNAs, specimen 8 possessed the greatest activity (~75% decrease in amount of myostatin mRNA) (Fig. 3), whose nucleotide sequence was described earlier [27]. High capability of interference with the myostatin mRNA was found in specimens 3-5 (70-60%). Not so high, but significant activity in reduction of myostatin mRNA level was found in specimen 6 (~30%). The siRNA specimens 1, 2, and 7 possessed insignificant capability of induction of myostatin mRNA interference.

So, among eight selected siRNAs four demonstrated marked (75-60%) inhibitory activity. This high rate (50%) of molecules possessing biological activity gives evidence for the efficiency of the used software algorithms.

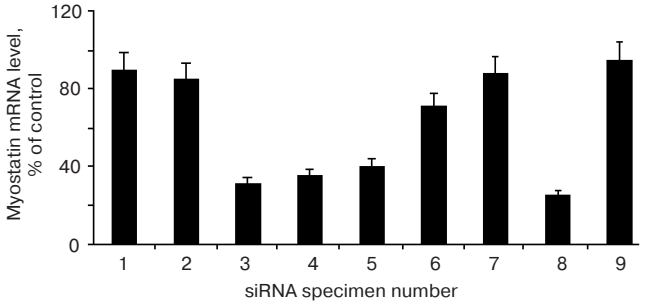

Fig. 3. Myostatin mRNA interference under the action of siRNAs. Primary murine myoblasts were transfected with siRNA using oligofectamine (400 pg per a 60-mm Petri dish; $n = 4$, $p < 0.05$). The relative amount of the myostatin mRNA was determined by QRT-PCR by the method of threshold cycle difference (the reference gene was GAPDH). siRNA not causing interference effect was taken as a negative control (9), and siRNA possessing a confirmed inhibitory effect was taken as a positive control (8).

Addition of specimen 6 (30% inhibition) to the given sampling increases the prediction efficiency to >60%.

Results of this study have shown that the previously described siRNA (specimen 8) has the highest activity in induction of RNA interference. Its sequence was found by the software of the Ambion Company. It is noteworthy that too many potentially active sites in the mRNA molecule identified by the given algorithm significantly hampers further investigations. Three of four siRNAs synthesized in accordance with sequences selected by the InvivoGen software also possessed significant capability of decreasing in amount the myostatin mRNA, which is comparable with that of siRNA of specimen 8. Other molecules including those selected by the Dharmacon software possessed significantly less, if any, capability of evoking myostatin mRNA interference.

Thus, we have demonstrated by the example of myostatin that utilization of available software allows—with comparatively low labor costs—a selection of sequences for synthesis of siRNA, which is significantly active (>60%) in induction of target gene RNA interference. Using the given approach, we selected several siRNAs that are significantly active in decrease of the myostatin mRNA level and can be used for studying physiological functions of myostatin, both *in vitro* and *in vivo*, and for evaluation of RNA interference potentialities in therapy of myodystrophies and senile dysfunctions of muscular activity.

REFERENCES

1. Fire, A., Xu, S., Montgomery, M. K., Kostos, S. A., Driver, S. E., and Mello, C. C. (1998) *Nature*, **391**, 806-811.
2. Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 14687-14692.
3. Waterhouse, P. M., Graham, M. W., and Wang, M. B. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 13959-13964.
4. Wianny, F., and Zernicka-Goetz, M. (2000) *Nat. Cell. Biol.*, **2**, 70-75.
5. Sontheimer, E. J. (2005) *Nat. Rev. Mol. Cell. Biol.*, **6**, 127-138.
6. Tang, G. (2005) *Trends Biochem. Sci.*, **30**, 106-114.
7. Morris, K. V. (2005) *Cell. Mol. Life Sci.*, **62**, 3057-3066.
8. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) *Cancer Cell*, **2**, 243-247.
9. Cioca, D. P., Aoki, Y., and Kiyosawa, K. (2003) *Cancer Gene Ther.*, **10**, 125-133.
10. Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar P., and Lieberman, J. (2003) *Nat. Med.*, **9**, 347-351.
11. Zender, L., Hutker, S., Liedtke, C., Tillmann, H. L., Zender, S., Mundt, B., Waltemathe, M., Gosling, T., Flemming, P., Malek, N. P., Trautwein, C., Manns, M. P., Kuhnel, F., and Kubicka, S. (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 7797-7802.
12. Xia, H., Mao, Q., Paulson, H. L., and Davidson, B. L. (2002) *Nat. Biotechnol.*, **20**, 1006-1010.
13. Lee, N. S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Salvaterra, P., and Rossi, J. **(**2002) *Nat. Biotechnol.*, **20**, 500-505.
14. Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K., Watanabe, M., and Mizusawa, H. (2003) *EMBO Rep.*, **4**, 602-608.
15. Nakamura, H., Siddiqui, S. S., Shen, X., Malik, A. B., Pulido, J. S., Kumar, N. M., and Yue, B. Y. (2004) *Mol. Vis.*, **10**, 703-711.
16. Uchida, H., Tanaka, T., Sasaki, K., Kato, K., Dehari, H., Ito, Y., Kobune, M., Miyagishi, M., Taira, K., Tahara, H., and Hamada, H. (2004) *Mol. Ther.*, **10**, 162-171.
17. Yi, C. E., Bekker, J. M., Miller, G., Hill, K. L., and Crosbie, R. H. (2003) *J. Biol. Chem.*, **278**, 934-939.
18. Peek, A. S. (2007) *BMC Bioinform.*, **8**, 182.
19. Katoh, T., and Suzuki, T. (2007) *Nucleic Acids Res.*, **35**, 27.
20. Thomas, M., Langley, B., Berry, C., Sharma, M., Kirk, S., Bass, J., and Kambadur, R. (2000) *J. Biol. Chem.*, **275**, 40235-40243.
21. Langley, B., Thomas, M., Bishop, A., Sharma, M., Gilmour, S., and Kambadur, R. (2002) *J. Biol. Chem.*, **277**, 49831-49840.
22. Bass, J., Oldham, J., Sharma, M., and Kambadur, R. (1999) *Domest. Anim. Endocrinol.*, **17**, 191-197.
23. Whittemore, L. A., Song, K., Li, X., Aghajanian, J., Davies, M., Girgenrath, S., Hill, J. J., Jalenak, M., Kelley, P., Knight, A., Maylor, R., O'Hara, D., Pearson, A., Quazi, A., Ryerson, S., Tan, X. Y., Tomkinson, K. N., Veldman, G. M., Widom, A., Wright, J. F., Wudyka, S., Zhao, L., and Wolfman, N. M. (2003) *Biochem. Biophys. Res. Commun.*, **300**, 965-971.
24. Tsuchida, K. (2006) *Expert Opin. Biol. Ther.*, **6**, 147-154.
25. Wagner, K. R. (2005) *Curr. Opin. Rheumatol.*, **17**, 720-724.
26. Acosta, J., Carpio, Y., Borroto, I., Gonzalez, O., and Estrada, M. P. (2005) *J. Biotechnol.*, **119**, 324-331.
27. Magee, T. R., Artaza, J. N., Ferrini, M. G., Vernet, D., Zuniga, F. I., Cantini, L., Reisz-Porszasz, S., Rajfer, J., and Gonzalez-Cadavid, N. F. (2006) *J. Gene Med.*, **8**, 1171-1181.
28. Rando, T. A., and Blau, H. M. (1994) *J. Cell Biol.*, **125**, 1275-1287.
29. Artaza, J. N., Bhasin, S., Magee, T. R., Reisz-Porszasz, S., Shen, R., Groome, N. P., Fareez, M. M., and Gonzalez-Cadavid, N. F. (2005) *Endocrinology*, **146**, 3547-3557.
30. Hassan, M. Q., Tare, R. S., Lee, S. H., Mandeville, M., Morasso, M. I., Javed, A., vav Wijnen, A. J., Stein, J. L., Stain, G. S., and Lian, J. B. (2006) *J. Biol. Chem.*, **281**, 40515-40526.