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ORIGINAL ARTICLE

Effect of RNA oligonucleotide targeting Foxo-1 on muscle growth in normal and cancer cachexia mice

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Foxo-1, a member of the Foxo forkhead type transcription factors, is markedly upregulated in skeletal muscle in energy-deprived states such as fasting, cancer and severe diabetes. In this study, we target the Foxo-1 mRNA in a mouse skeletal myoblast cell line C2C12 and *in vivo* models of normal and cancer cachexia mice by a Foxo-1 specific RNA oligonucleotide. Our results demonstrate that the RNA oligonucleotide can reduce the expression of Foxo-1 in cells and in normal and cachectic mice, leading to an increase in skeletal muscle mass of the mice. In search for the possible downstream target genes of Foxo-1, we show that when Foxo-1 expression is blocked both in cells and in mice, the level of MyoD, a myogenic factor, is increased while a muscle negative regulator GDF-8 or myostatin is suppressed. Taken together, these results show that Foxo-1 pays a critical role in development of muscle atrophy, and suggest that Foxo-1 is a potential molecular target for treatment of muscle wasting conditions.
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Keywords: Foxo-1; muscle wasting; cancer cachexia; RNA oligonucleotides

Introduction

Muscle wasting is common in many disease states and conditions including cancer cachexia, aging, sepsis, denervation, disuse, inactivity, burns, AIDS and muscular dystrophies.[1] The recent discovery of a range of transcriptional and growth factors involved in the regulation of muscle development has advanced the understanding of the molecular mechanisms in the development and function of skeletal muscles. One of the factors, Foxo-1, which is a member of the Foxo forkhead type transcription factors, is markedly upregulated in skeletal muscle in energy-deprived states such as fasting, cancer and severe diabetes. Foxo-1 was originally cloned from a rhabdomyosarcoma because of its aberrant fusion with another transcription factor, PAX3, resulting from a chromosomal translocation.[2] Overexpression of Foxo-1 in skeletal muscle showed a reduced skeletal muscle mass.[3] In addition, Foxo-1 has been shown to stimulate fusion of primary mouse myoblasts to myotube and regulate various cellular functions, including cell cycle and apoptosis.[4–8] Another recently identified factor, GDF-8 or myostatin, has been characterized as an important and potent negative regulator of skeletal muscle growth and inhibitor of myoblast proliferation that belongs to the TGF-β family of secreted growth and differentiation factors. Mutations in the GDF-8 gene showed a marked increase in body weight and muscle mass in cattle. Disruption of the GDF-8 gene in mice induced a highly muscled phenotype due to both muscle hypertrophy and hyperplasia.[9–11] Increases in GDF-8 expression have been observed in conditions of muscle atrophy and/or dystrophy, while the GDF-8 mRNA is modified in response to muscle loading after atrophy.[12]

To explore Foxo-1 as a potential molecular target for muscle wasting conditions and gain insight of the mechanism of Foxo-1 regulation of muscle development and growth, we used a modified RNA oligonucleotide targeting the Foxo-1 mRNA in muscle cells and in normal and cachectic mice. Our studies demonstrated that the modified RNA oligonucleotide could decrease the expression of Foxo-1 in cells and *in vivo*, leading to an increase in muscle weight. Further studies show that the suppression of the Foxo-1 causes the downregulation of GDF-8 and upregulation of MyoD. This study should lay the basis for further understanding of the mechanisms of muscle wasting in various diseases and for developing nucleic acid-based therapeutics for muscle wasting conditions, such as cancer cachexia.

Materials and methods

RNA oligonucleotides

The modified RNA oligonucleotides targeted to Foxo-1 and the control were synthesized and HPLC purified at Oligos Etc Inc (Oregon).

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Cell culture and transfection

C2C12 mouse skeletal myoblast cells were routinely maintained in a growth medium consisting of DMEM supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 100 IU ml^{-1} penicillin and 100 mg ml^{-1} streptomycin and incubated at 37 °C under 5% CO_2. Differentiation was initiated by DMEM supplemented with 2% horse serum (differentiation medium). When the cells reached confluence, the growth medium was shifted to differentiation medium to induce myotube formation. After 4 days, myotubes were transfected with RNA oligonucleotides (2 μM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested for RNA analysis 48 h post transfection.

Animals

BALB/c female mice (6–8 weeks old) weighing 15–18 g were obtained from Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (CAS), Beijing. The animal experiments were undertaken within the guidelines of regulations for the use of experimental animal of CAS.

RNA oligonucleotide treatment

In all the experiments, mice were administered with RNA oligonucleotides via tail vein injection at the dose of 100 μg 0.1 ml per injection two times weekly for 4 weeks. Mice were weighed every day during the treatment period. Two days after the last injection, the leg muscle including the quadriceps (rectus femoris), the gastrocnemius, triceps and the EDL were dissected out and weighed individually. The variation of the muscle weight was less than 5%.

Establishment of S-180 tumor bearing mice

Cells 1×10^7 S-180 were inoculated in 0.2 ml of PBS i.p. into BALB/C mice. The mice were fed with water and food normally for 3 weeks. The leg muscle including the quadriceps (rectus femoris), the gastrocnemius, triceps and the EDL were dissected, weighed and freshly used for protein and mRNA extraction. The injections of RNA oligonucleotides started 3 weeks post inoculation of the tumor cells.

RNA extraction and real-time PCR analysis

Total RNA was extracted from cells or tissues using TRIzol (Invitrogen). RNA was separated electrophoretically on agarose gels under denaturing conditions to confirm the integrity of ribosomal RNA bands. Single-strand cDNA synthesis was carried out using 2 μg of total RNA by the reverse transcription (RT) reaction (Promega). Real-time fluorescence quantitative PCR was performed in an Applied Biosystems Prism 7000 instrument in the reactions containing an Applied Biosystems SYBR green master mix reagent and oligonucleotide pairs to the endogenous control β-actin gene, MyoD, GDF-8 and foxo1 cDNA. The reagents were denatured at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The primer sequences (5′–3′) were as follows:

β-actin forward GAACCCTAAGGCCAACCGTGAA; β-actin reverse, CTCAGTAACAGTCCGCCTAGAA; MyoD forward 5′-GCAAGACCACCAACGCTGAT-3′; MyoD reverse 5′-GGTTCGGGTTGCTGGACGTG-3′; GDF-8 forward 5′CAGACCCGTCAAGAC TCCTA CA-3′; GDF-8 reverse 5′-CAGTGCCTGGGCTCATGT CAAG-3′; Foxo-1 forward 5′-GTACGCCGACCTCAT CACCA-3′; Foxo-1 reverse 5′-TGCTGTCGCCCTTATC CTTG-3′.

Average C_T values for MyoD, GDF-8 and foxo1 were calculated and normalized to C_T values for β-actin, and the normalized values were subjected to a $2^{-\Delta C_t}$ formula to calculate the fold change between the control and experiment groups. The formula and its derivations were obtained from the ABI Prism 7000 Sequence Detection System user guide. All reactions were performed in triplicates.

Western blotting

Protein was extracted from cell and the muscle tissue of different groups with lysis buffer containing100 mM Tris–HCl, 0.05% CA-630100 μg ml^{-1} PMSF, 100 μg ml^{-1} DTT, 1 μg ml^{-1} aprotinin and 1 μg ml^{-1} leupeptin. Protein concentration of each sample was measured by the Bradford method (Bio-Rad Laboratories). Protein extracts were heat denatured at 95 °C for 5 min, electrophoretically separated on a 12.5% SDS–PAGE, and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat dry milk in TBST buffer (50 mM Tris–HCl, 100 mM NaCl and 0.1% Tween -20, pH 7.4) and incubated with monoclonal anti-MyoD diluted at 1/200 (BD, Pharmingen, CA), monoclonal anti-GDF-8 diluted at 1/100 (Santa Cruz Biotechnology, CA) and anti-β actin diluted at 1/250 (Santa Cruz Biotechnology). The membrane was further incubated for 90 min at 37 °C. The signals were visualized by subsequent chemoluminescent reaction with a horseradish peroxidase-conjugated IgG (1:3000) in the ECL system (Amersham, NJ). The image and data were analyzed with Bandscan 5.0 software.

Statistical analysis

Data were expressed as means ± s.d. and subjected to one-way AVONA with factors of treatment, disease or wild type. Comparisons between two groups were performed by unpaired Student's *t*-test. A value of $P < 0.05$ was considered significantly different.

Results

Inhibition of Foxo-1 expression by a RNA oligonucleotide targeted to Foxo-1 mRNA in C2C12 cells

To target the foxo-1 mRNA, we employed the following strategy to design antisense RNA oligonucleotides: (i) selecting an oligonucleotide that is adjacent to or overlaps a target region of the 5′-UTR, the translational start sequence, the 3′-UTR, or the translational termination site of the foxo-1 mRNA, (ii) determining the Gibbs free energy value associated with the oligonucleotide in reference to the target gene, and (iii) assessing T_m in

reference to the target gene. Totally, four antisense RNA molecules were designed based on the mouse Foxo-1 mRNA sequence (NM019739). The sequences of the antisense molecules are AGCAGAGAAGUACCGGGA (GGR5); CUUCGGCCAUGGUGC(GGR6); AUGGG GUGGGACAGAGGC(GGR7) and CUCUUAGCCUG ACACCCA(GGR8). A randomized oligonucleotide was also designed as a control sequence (AGACCTCTCATA GCAGCTGAT). To increase the stability *in vivo*, the RNA oligonucleotides were chemically modified by 2′-O-methyl and with a butanol tag at the 3′-end of each molecule.

To test the effect of the Foxo-1 RNA oligonucleotides on the Foxo1 expression *in vitro*, a mouse skeletal myoblast cell line C2C12 was used and transfected with the RNA oligonucleotide with the aid of Lipofectamine. When the transfected cells were analyzed for the transfection efficiency using a FITC-labeled oligonucleotide, an approximately 80% of cells were transfected as measured by FACS analyses (data not shown). Using quantitative RT-PCR (Q-RT-PCR), four RNA oligonucleotides were screened in C2C12 cells (data not shown). Among four RNA oligonucleotides, only GRR7 was shown to downregulate significantly the Foxo-1 mRNA expression by 81% compared with the control (Figure 1). This molecule was selected for the subsequent studies both *in vitro* and *in vivo*. This indicated that the Foxo-1 RNA oligonucleotide could efficiently lead to degradation of the target mRNA in a sequence-specific manner.

Effect of Foxo-1 RNA oligonucleotide on GDF-8 and MyoD expression in C2C12 cells

GDF-8 and MyoD are two of the most important regulators in myoblast differentiation and muscle growth. We had proposed that Foxo-1 may regulate the pathways related to the muscle growth via both positive and negative regulators, such as GDF-8 (negative) and MyoD (positive). To test this hypothesis, we examined the expression levels of GDF-8 and MyoD in C2C12 cells transfected with the Foxo-1 RNA oligonucleotide. The results showed that in the transfected cells, the GDF-8 expression at mRNA level was decreased by 57.5% while the MyoD mRNA level was increased by 83% compared to control (Figures 2a and b). These changes were further confirmed at the protein level using western blots, where GDF-8 was decreased by 48.5%, while MyoD was increased by 65.6% relative to the control (Figures 2c and e). As target controls, c-fos and NF-κBp65 were examined for their expression upon treatment by the Foxo-1 antisense RNA. As expected, there was no effect on the expression of these two transcriptional factors (Figure 2c), indicating that the action of GRR7 was target-specific. These results suggest that during the differentiation of C2C12 cells induced by horse serum, GDF-8 and MyoD could be two of the downstream target genes regulated by Foxo-1.

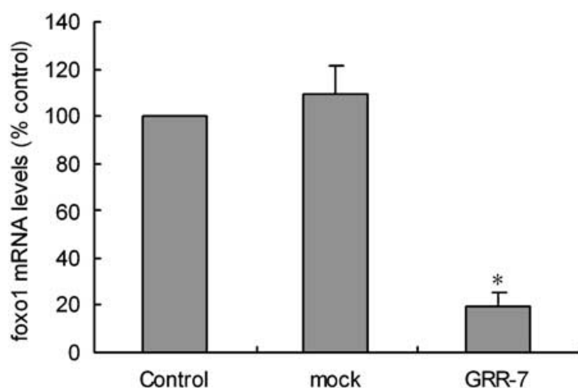

Figure 1 Quantitative reverse transcription-PCR analysis of effect of Foxo-1RNA oligonucleotide (GRR7) on mRNA expression in C2C12. *$P<0.05$. Control: treated with control oligonucleotide; mock: treated with the transfection reagent.

Effect of Foxo-1RNA oligonucleotide on target gene expression and muscle growth in normal mice

To examine if the RNA oligonucleotide could downregulate the Foxo-1 expression *in vivo* and impact muscle growth, we first used normal mice as a test system. The RNA oligonucleotide was given to mice via a tail vein injection twice weekly for 4 weeks. RNA samples were extracted from the leg muscle and subjected to Q-RT-PCR analyses. As shown in Figure 3, the Foxo-1 mRNA level in the treatment group decreased by 65.7%, compared with the control. When the muscle tissues were collected and weighed at the end of the experiments, it was shown that the muscle weight in the group treated with the Foxo-1 RNA oligonucleotide was increased by 10% relative to the control group ($P<0.05$, $n=8$) (Figure 4) with a net increase of 0.1 g. Injection of the RNA oligonucleotides did not have deleterious effect on the animal as evidenced by healthy body weight and well-being. Together, the RNA oligonucleotide targeting the Foxo-1 mRNA could inhibit the Foxo-1 expression *in vivo*, leading to an increase in the muscle weight.

Increase of GDF-8 and MyoD in Foxo-1-inhibited normal mice

As shown in C2C12 cells, the inhibition of Foxo-1 expression by the RNA oligonucleotide led to an increased level of MyoD and decreased level of GDF-8. We asked if this observation could be seen *in vivo*. To answer this question, the muscle tissues from the groups treated with the Foxo-1 RNA oligonucleotide or control oligonucleotide were harvested and used for the RNA and protein extraction. Q-RT-PCR analyses revealed that the GDF-8 mRNA expression was decreased by 49% and MyoD was increased by 58%, compared to the control group (Figures 5a and b). When western blots were performed, a similar expression profile was observed, where the GDF-8 protein level decreased by 38.5% and the MyoD level increased by 42% in the RNA oligonucleotide-treated group (Figures 5c–e). As in C2C12 cells, GRR7 had no effect on the expression of the control targets, c-fos and NF-κBp65 (Figure 5c). This

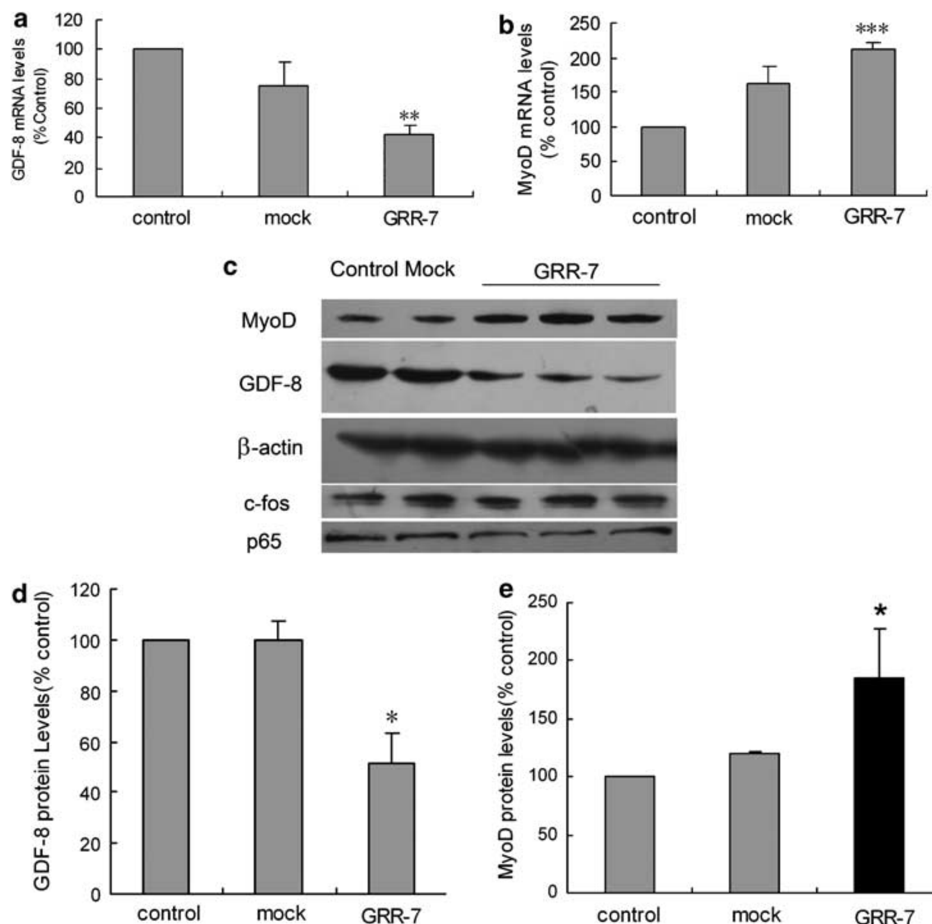

Figure 2 The effect of RNA oligo targeted to foxo1 on expression of MyoD and GDF-8 in C2C12. (**a**) GDF-8 mRNA expression; (**b**) MyoD mRNA expression; (**c**) western blot analysis of GDF-8, MyoD, β-actin, c-fos and NF-κBp65 protein expression. Lane 1: control; lane 2: mock; lanes 3–5: Foxo-1 oligonucleotide-treated cells. (**d** and **e**) Quantitative analysis of the western blots for the expression of GDF-8 and MyoD at protein level in C2C12. $*P<0.05$. Control: treated with control oligonucleotide; mock: treated with the transfection reagent.

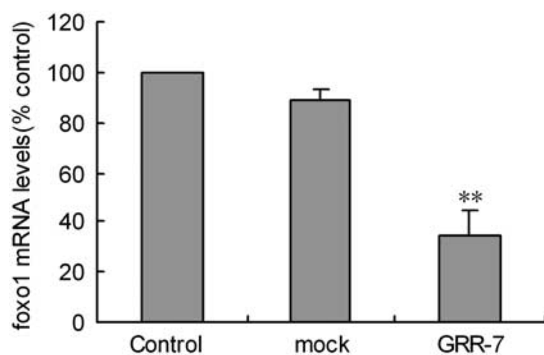

Figure 3 Quantitative reverse transcription-PCR analysis of Foxo-1 mRNA expression in normal mice treated with Foxo-1-targeted RNA oligonucleotide. Data are mean±s.d. with $n=6$ in each group. $**P<0.01$. Control: treated with control oligonucleotide; mock: treated with the transfection reagent.

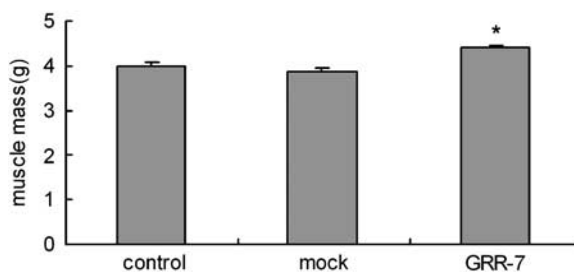

Figure 4 Muscle weight in normal mice treated with Foxo-1-targeted RNA oligonucleotide. Data are mean±s.d. with $n=6$ in each group. $*P<0.05$.

consistency of the data between *in vitro* and *in vivo* further indicated a close relationship between Foxo-1 and GDF-8, MyoD.

Improvement of muscle growth and modulation of expression of Foxo-1, MyoD and GDF-8 by Foxo1 RNA oligonucleotide in S-180 tumor-bearing mice

Skeletal muscle atrophy is a debilitating response to fasting, disuse and cancer. To further explore the involvement of Foxo-1 and its related regulators in the pathophysiology of muscles, we chose S-180 mouse tumor

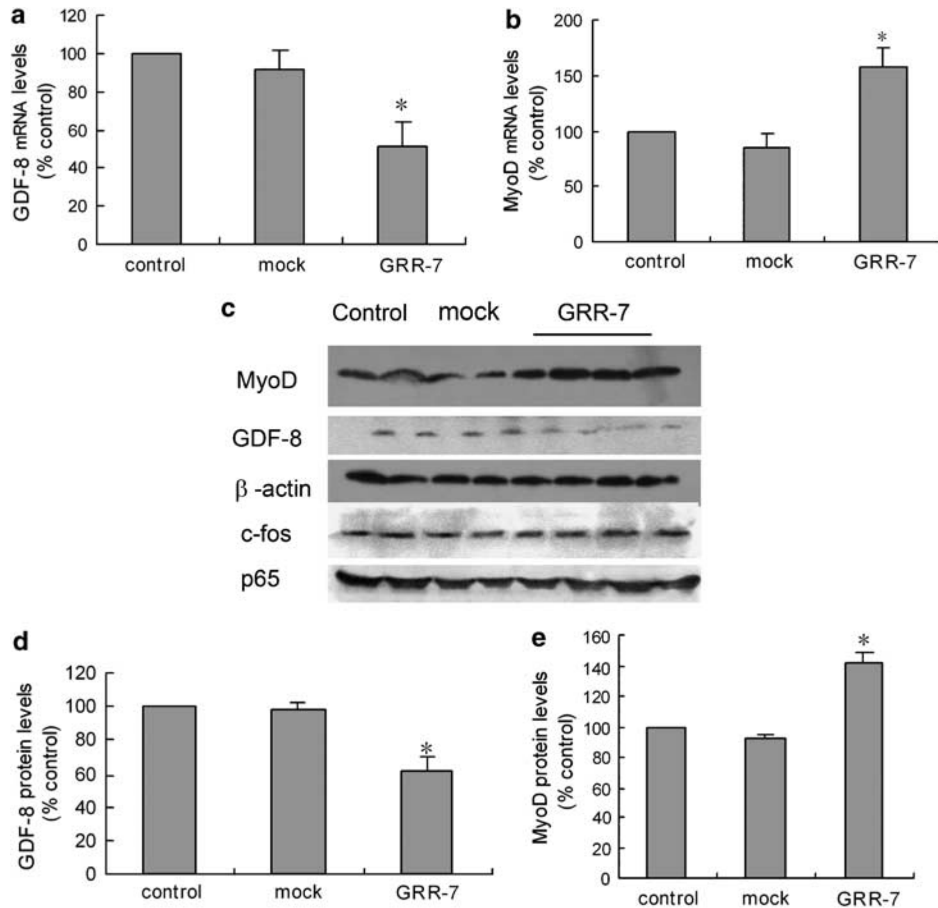

Figure 5 Expression of MyoD and GDF-8 in normal mice treated with RNA oligonucleotide targeting Foxo-1. (**a** and **b**) Quantitative reverse transcription-PCR analysis of GDF-8 and MyoD; (**c**) Western blot analysis of MyoD, GDF-8, β-actin, c-fos and NF-κBp65 expression. Lanes 1,2: control; lanes 3–4: mock; lanes 5–8: RNA oligonucleotide-treated mice. (**d** and **e**) Quantitative analyses of GDF-8 and MyoD protein expression. $*P<0.05$. Control: treated with control oligonucleotide; mock: treated with the transfection reagent.

to create a cancer cachexia model as cachectic conditions could be established by inoculating ascetic tumors.[13] We showed that after 14 days post inoculation, tumor-bearing mice significantly lost muscle weight (Figure 6b). Further molecular analyses demonstrated that in the muscle tissues of S-180 bearing mice, the Foxo-1 mRNA expression level increased by 91.2% ($P<0.01$, Figure 6a) compared with normal mice. Thus, the S-180-implanted mice showed some characteristic features of the cancer cachexia and could be used as a cachectic model in which we conducted the following experiments. First, the Foxo-1 RNA oligonucleotide was injected into the S-180 bearing mice via tail vein and the muscle tissues were collected for Q-RT-PCR analyses. The results showed that there was a 75% decrease in the expression of Foxo-1 (Figure 7a) and the muscle mass increased by 32.8% in the treatment group (Figure 7b). Second, we measured the expression of GDF-8 and MyoD in the S-180 mice where the Foxo-1 expression was inhibited. At the mRNA level, the GDF-8 expression decreased by 53% (Figure 8a) and the MyoD expression increased by 66% compared with the control group (Figure 8b). At the protein level, GDF-8 decreased by 40.4%, and MyoD increased by 41.8% (Figures 8c–e). When the control targets (c-fos and NF-κBp65) were examined, no effect of GRR7 was observed (Figure 8c). As in normal mice, the injection of the foxo-1 RNA oligonucleotide had no toxic effect on tumor-bearing mice. Taken together, these findings demonstrated that the suppression of Foxo-1 expression by a targeted RNA oligonucleotide could improve the muscle growth in cachectic mice and this action may be mediated by upregulation of MyoD and downregulation of GDF-8 expression.

Discussion

The present study demonstrates direct, sequence-specific inhibition of Foxo-1 expression in myoblast cells and increase in muscle weight in mice by a modified RNA antisense molecule targeting the Foxo-1. These properties suggest that this novel, small-molecule gene-targeting agent may be useful as both a direct and indirect therapeutic agent for muscle-wasting-associated conditions.

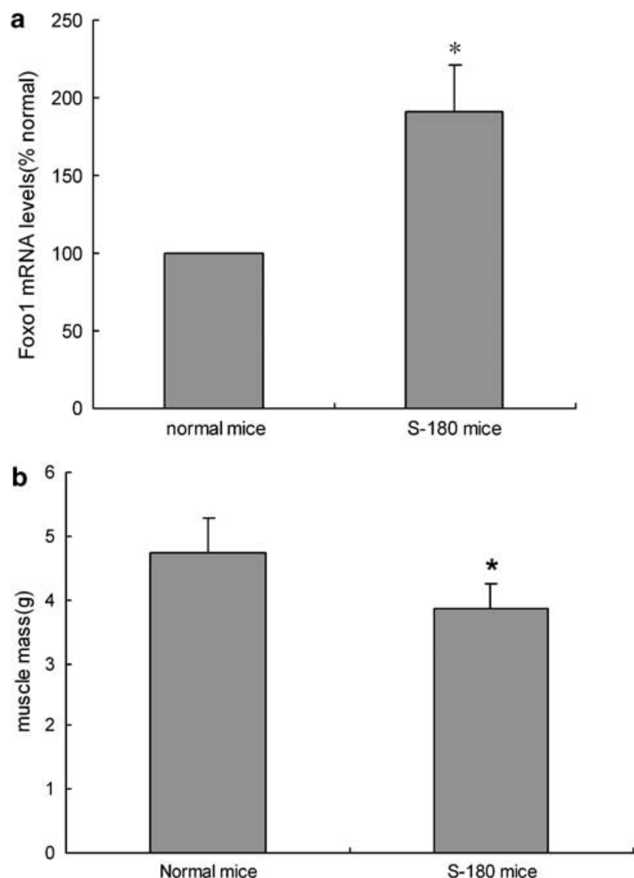

Figure 6 Characterization of S-180-bearing mice for a cancer cachexia model. (**a**) Quantitative reverse transcription-PCR analysis of FOXO1 expression at mRNA level in normal mice; (**b**) muscle weight measurement, $*P<0.05$.

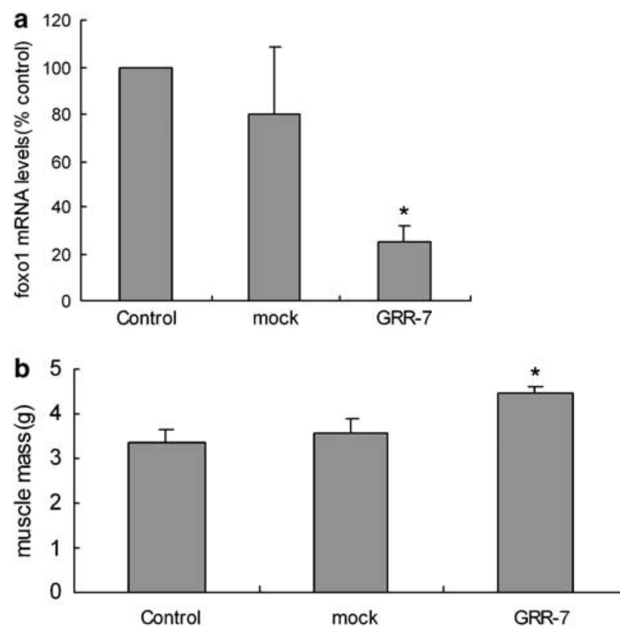

Figure 7 Effect of Foxo-1 RNA oligonucleotide in S180 mice. (**a**) Quantitative reverse transcription-PCR analysis of Foxo1 at mRNA level in S-180mice treated with RNA oligonucleotide targeted at Foxo-1. (**b**) Muscle weight measurement in S-180-bearing mice. ($*P<0.05$, $n=6$). Control: treated with control oligonucleotide; mock: treated with the transfection reagent.

Although drugs that target transcription are in wide therapeutic use, they were all identified on the basis of their effect on a specific biological process and were only subsequently shown to target transcription. The recent understanding of the mechanism of action of these drugs and mechanisms of transcriptional regulation offers hope for a new generation of drugs on the basis of their ability to modulate the synthesis of transcriptional factors. Forkhead transcription factors of the Foxo subfamily are emerging as a shared component among pathways regulating diverse cellular functions, such as differentiation, metabolism and proliferation. Constitutively, overexpression of the active Foxo factors was sufficient to induce atrophy in myotubes and in whole muscle. In the present study, we found that the suppression of FOXO1 expression by a modified RNA oligonucleotide could promote the muscle growth in both normal and cachectic mice. In this regard, our results are consistent with the finding that muscle-specific overexpression of FOXO1 was associated with marked decreases in muscle mass and fiber size in mice.[14] In the Foxo-1 overexpression studies it was proposed that the decreases in muscle mass might be caused by the change of the degradation rate of skeletal muscle proteins, such as atrogin-1 and MuRF1.[14] However, over-expression of MAFbx/atrogin did not impact the muscle growth. This suggested that in addition to MAFbx/atrogin, Foxo transcriptional factors may activate a broad spectrum of genes involved in the muscle development and growth. In our knockdown study, we show that the suppression of Foxo-1 expression by the antisense RNA oligonucleotide accompanied with a decreased level of GDF-8 and increased level of MyoD. This suggests that the effect observed both in cells and in mice may be related to other muscle-specific regulators controlled by Foxo-1, such as GDF-8 and MyoD and provides some new clues for how Foxo-1 regulates the muscle growth.

It has been reported that the blockage of the GDF-8 expression by an anti-GDF-8 antibody (JA16) was shown to increase muscle mass in healthy adult mice.[15] GDF-8 could regulate myoblast differentiation by inhibiting MyoD activity and expression via Smad 3, resulting in the failure of the myoblasts to differentiate into myotubes.[16] Based on the above and our findings, Foxo-1 modulation of the muscle differentiation and growth may be realized by a direct transcriptional regulation of GDF-8. Thus, our results not only contribute to the understanding of the molecular mechanism the Foxo-1 function in muscle cells, but also indicate that targeting a transcriptional factor at an early stage in a regulatory network could be a preferred strategy for molecular intervention of pathological process, such as muscle wasting conditions.

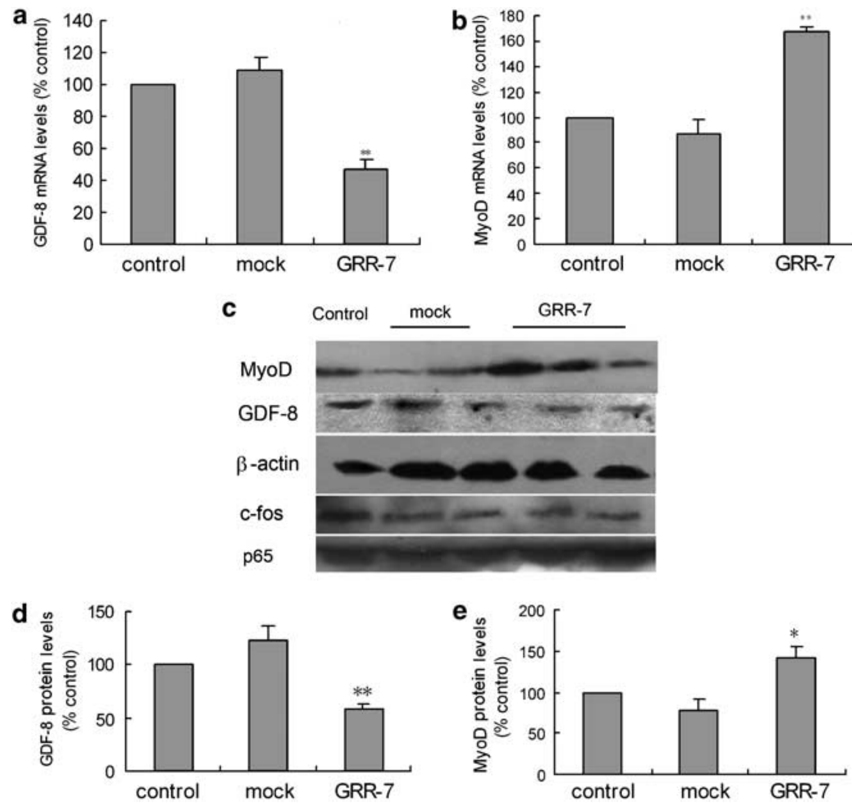

Figure 8 Expression of MyoD and GDF-8 in S-180 bearing mice treated with RNA oligonucleotide targeting Foxo-1. (**a** and **b**) Quantitative reverse transcription-PCR analysis of GDF-8 and MyoD; (**c**) western blot analysis of MyoD, GDF-8, β-actin, c-fos and NF-κBp65 expression. For MyoD expression, lane 1: control, lanes 2, 3: mock, lanes 4–6: treated mice. For GDF-8 expression, lane 1: control, lane 2: mock and lanes 3–5: treated mice. (**d** and **e**) Quantitative analyses of GDF-8 and MyoD protein expression in S-180-bearing mice. $^*P<0.05$. Control: treated with control oligonucleotide; mock: treated with the transfection reagent.

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