MicroRNA-351 inhibits denervation-induced muscle atrophy by targeting TRAF6

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Abstract. MicroRNAs (miRs) have been observed to be involved in the modulation of various physiopathological processes. However, the impacts of miRNAs on muscle atrophy have not been fully investigated. In the present study, the results demonstrated that miR-351 was differentially expressed in the tibialis anterior (TA) muscle at various times following sciatic nerve transection, and the time-dependent expression profile of miR-351 was inversely correlated with that of tumor necrosis factor receptor-associated factor 6 (TRAF6) at the mRNA and protein levels. The dual luciferase reporter assay indicated that miR-351 was able to significantly downregulate the expression levels of TRAF6 by directly targeting the 3'-untranslated region of TRAF6. Overexpression of miR-351 inhibited a significant decrease in the wet weight ratio or cross-sectional area of the TA muscle following sciatic nerve transection. Western blot analysis indicated that the protein expression levels of TRAF6, muscle ring-finger protein 1 (MuRF1) and muscle atrophy F-box (MAFBx) in denervated TA muscles were suppressed by overexpression of miR-351. These results demonstrate that miR-351 inhibits denervation-induced atrophy of TA muscles following sciatic nerve transection at least partially through negative regulation of TRAF6 as well as MuRF1 and MAFBx, the two downstream signaling molecules of TRAF6.

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Introduction

Skeletal muscle atrophy occurs under various pathophysiological conditions, such as aging, starvation, disuse, severe injury, cancer and other cachectic diseases (1-3). Previous studies have been concerned with denervation-induced skeletal muscle atrophy following peripheral nerve injury (4,5). Therefore, isobaric tags for relative and absolute quantification coupled with two-dimensional liquid chromatography-tandem mass spectrometry were performed in order to identify the differentially expressed proteins in the tibialis anterior (TA) muscle 1 and 4 weeks following sciatic nerve transection in rats (4). The results demonstrated that tumor necrosis factor receptor-associated factor 6 (TRAF6) was upregulated in denervated TA muscle (4). Conversely, the results also demonstrated that knockdown of TRAF6 significantly attenuated glucocorticoid-induced myotube atrophy *in vitro* and *in vivo* (5).

As is well known, TRAF6 is a unique E3 ubiquitin ligase and adaptor protein. It is involved in activation of various signaling cascades including mitogen-activated protein kinase, nuclear factor-κB, and phosphoinositide 3-kinase/protein kinase B in response to various cytokines (6-8), and also important for the interaction with multiple components of the ubiquitin-proteasome system (UPS) in some cell types (9-11). Due to its biological traits, TRAF6 regulates skeletal muscle mass and UPS activation in denervation or starvation-induced muscle atrophy (6,12), and catalyzes lysine 63-linked polyubiquitination of several target proteins through its association with the dimeric ubiquitin-conjugating enzyme Ubc13/Uev1A (13).

MicroRNAs (miRNAs, miRs) are a novel class of small, non-coding RNAs of ~22 nucleotides in length. miRNAs negatively regulate the expression of target genes through interaction with the 3'-untranslated regions (3'-UTR) of the target mRNAs in many biological processes (14). There have been extensive studies of the functions of miRNAs in normal physiological phenomena and numerous diseases (15,16). For instance, previous studies have demonstrated that targeting miR-122 in patients with chronic HCV genotype 1 infection caused prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance, which indicted that targeting miR-122 may be an effective and safe treatment

strategy for HCV infection (17,18). However, the involvement of miRNAs in muscle-associated biological processes has not been fully investigated. To our knowledge, it has only been reported that miR-1 and miR-133 are able to promote differentiation and proliferation of the skeletal and cardiac muscle cells (19), and the expression of miR-351 in differentiated C2C12 cells is upregulated following treatment with dexamethasone (20). The underlying mechanisms, however, are still to be further explored. The present study aimed to examine whether certain miRNAs, such as miR-351, are able to regulate denervation-induced skeletal atrophy in a model of rat sciatic nerve injury and TA muscle denervation, providing a potential novel molecular target for the clinical treatment of denervation-induced muscle atrophy.

Materials and methods

Animal surgery and treatments. A total of 126 adult male Sprague-Dawley rats (weight, 200 g; age, 2 months) were supplied by the Experimental Animal Center of Nantong University (Nantong, China) with routine provision of food and water, and were maintained at 24˚C with a 12:12 h light/dark cycle. All animal experiments were carried out in accordance with the institutional animal care guidelines of Nantong University and approved by the Administration Committee of Experimental Animals, Jiangsu Province.

A total of 72 rats underwent surgery for sciatic nerve transection as described previously (21). The TA muscle was harvested from a cohort of rats at 0, 3, 7 and 14 days post-surgery. Another cohort of 54 rats was randomly divided into two groups to receive a multi-point injection of miR-351 agomir and negative control agomir (both at 2.5 nmol in 50 μl vehicle; Guangzhou RiboBio Co., Ltd., Guangzhou, China) into the TA muscle. The injection was administered a total of 5 times at a regular interval of 2 days, the rats were sacrificed by cervical dislocation and the TA muscle was dissected.

Dual luciferase reporter assay. HEK-293T cells were cultured in growth medium consisting of Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml of streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 100 U/ml of penicillin (Sigma-Aldrich) in a 37˚C, 5% CO_2 incubator. The cells were co-transfected with 600 ng of the wild-type or mutant 3'-UTR luciferase reporter (Guangzhou RiboBio Co., Ltd., Guangzhou, China) and 20 μM of the miR-351 agomir or the negative control duplexes using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in 24-well plates. The sequences of these reporters were as follows: TRAF6 3'-UTR: 5'-ACGCTCATC AATTTGTCAGGGAA; TRAF6 3'-UTR mutant: 5'-ACG CTCATCAATTTGAGTCCCTA. Following transfection for 24 h, the cells were harvested by adding 100 μl of lysis buffer (Promega Corporation, Madison, WI, USA). The activity of firefly and Renilla luciferases was measured from the cell lysates using the dual luciferase reporter assay system (Promega Corporation). TargetScan prediction (www.targetscan.org/vert_71) was then used to deduce the biological targets of miRNAs.

Muscle morphological observation. At 14 days post surgery, the animals in the two groups that had been injected with miR-351 agomir and negative control agomir were anesthetized with 30 mg/kg pentobarbital (Merck Millipore, Darmstadt, Germany) and perfused through the left ventricle sequentially with saline and 4% paraformaldehyde. The TA muscle injected with either miR-351 agomir or negative control agomir was harvested and weighed, in order to calculate the wet weight ratio of the muscle.

For Masson's trichrome staining, the harvested TA muscle were post-fixed in 4% paraformaldehyde at 4˚C for 24 h, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin, and cut into 5 μm-thick transverse sections using a freezing microtome. Hematoxylin and eosin staining was applied to every section, and images were captured under a light microscope (magnification, x40). The average cross-sectional area (CSA) of the TA muscle were then determined using a Leica Q5501 W image analysis system (Leica Mycrosystems GmbH, Wetzlar, Germany) as described previously (22).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the TA muscle (100 mg/group), harvested as described previously, using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RT to cDNA was carried out with a SuperScript First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.). All primers were purchased from Generay Biotech Co., Ltd. (Shanghai, China). The primers used in this study were as follows: TRAF6 forward, 5'-GCAGAGGAATCACTTGGCACG-3' and reverse, 5'-CAC GGACGCAAAGCAAGGTT-3'; miR-351 forward, 5'-ACA CTCCAGCTGGGTCCCTGAGGAGCCCTTGG-3' and reverse, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTC AGTTGAGTCAGGCTC-3'; U6 forward, 5'-CTCGCTTCG GCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGCGT-3'; and GAPDH forward, 5'-CAACGGGAAACC CATCACCA-3' and reverse, 5'-ACGCCAGTAGACTCCACG ACAT-3'. RT-qPCR was conducted in a StepOne™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using FastStart® SYBR Green qPCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The reaction mixture contained 1 μl cDNA from each sample that was mixed with 10 μl 2X Fast Start® SYBR Green qPCR Master Mix, 2 μl 10X ROX of the assays-on-demand kit (Biotium, Fremont, CA, USA), 1 μl primer, and 6 μl of RNase/DNase-free water (Biotium). A three-step fast cycle protocol was used, with cycling conditions as follows: Denaturation at 95˚C, 10 sec; annealing at 60˚C, 30 sec; extension at 70˚C, 10 sec. The relative mRNA expression was measured through the $2^{-\Delta\Delta Cq}$ method (23). U6 snRNA and GAPDH served as an internal control.

Western blot analysis. The total protein of the TA muscles, harvested as previously described, was extracted using a T-PER tissue protein extraction reagent (Pierce Biotechnology; Thermo Fisher Scientific, Inc.) and quantified using a Coomassie Plus Bradford assay kit (Pierce Biotechnology; Thermo Fisher Scientific, Inc.). Samples containing 15 μg total protein were separated by 12% (w/v) SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD

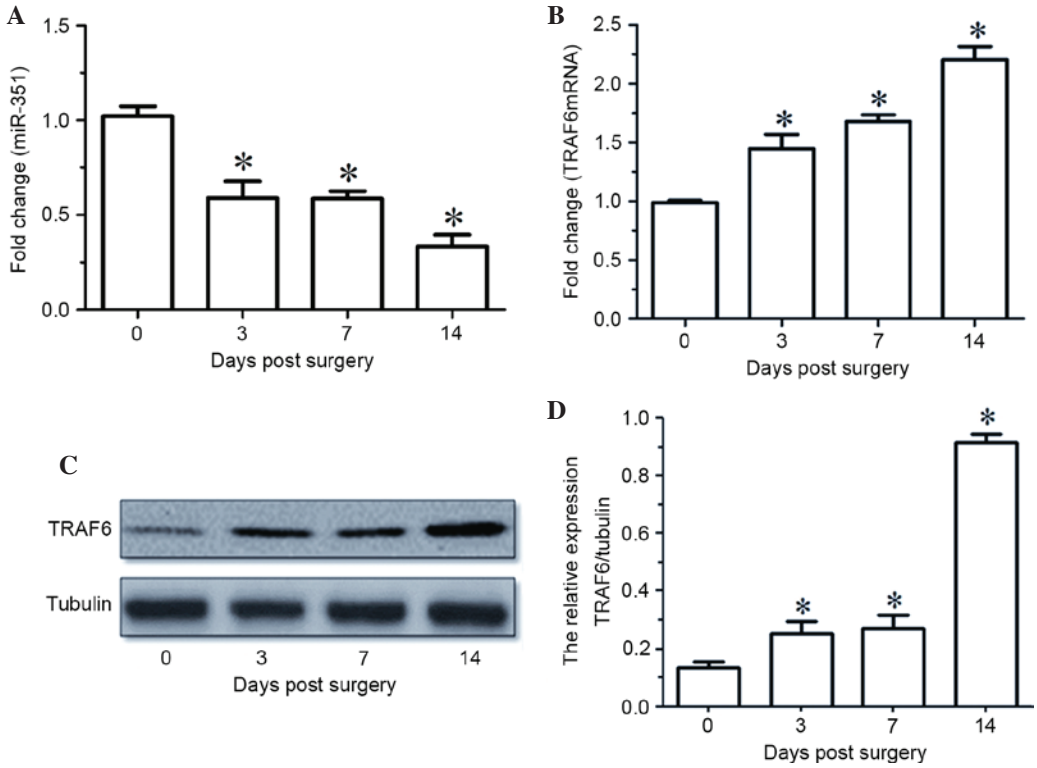

Figure 1. (A and B) miR-351 and TRAF6 expression levels in TA muscles at various time points (0, 3, 7, 14 days) following sciatic nerve transection, as determined by reverse transcription-quantitative polymerase chain reaction. (C) Western blot analysis and (D) quantified results demonstrating the protein expression levels of TRAF6 in the TA muscles at various time points (0, 3, 7, 14 days) following sciatic nerve transection. Data are presented as means ± standard deviation, n=9 per animal group. *P<0.05, vs. 0 days post sciatic nerve transection. Tubulin was used as a loading control in the western blot analysis. miR, microRNA; TRAF6, tumor necrosis factor receptor-associated factor 6; TA, tibialis anterior.

Millipore, Bedford, MA, USA). Following incubation for 1 h with 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween-20 [TBS-T; 0.05% (v/v) Tween-20], the membrane was probed with the following primary antibodies diluted in blocking buffer overnight at 4˚C: Rabbit anti-TRAF6 polyclonal antibody (1:1,000; Abgent, Inc., San Diego, CA, USA), goat anti-muscle ring finger 1 (MuRF1) polyclonal antibody (1:1,000; R&D System, Minneapolis, MN, USA), rabbit anti-muscle atrophy F-box (MAFBx) polyclonal antibody (1:1,000; LifeSpan BioSciences, Inc., Seattle, WA, USA), and rabbit anti-β-tubulin polyclonal antibody (1:2,000; Abcam, Cambridge, MA, USA). Following washing with TBS-T, the membrane was incubated with secondary antibodies diluted in blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 1 h. These were anti-rabbit (dilution, 1:5,000; cat. no. 611-701-127; Rockland Immunochemicals, Inc., Pottstown, PA, USA) or anti-goat IgG (dilution, 1:6,000; cat. no. 605-706-125, Rockland Immunochemicals, Inc.). The images were scanned with the Odyssey infrared image system (LI-COR Biosciences), and the absorbance data were analyzed using PDQuest 7.2.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analyses. All data are presented as means ± standard error of the mean. Comparison between groups was assessed by unpaired Student's *t* test using SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). Unless otherwise specified, all assays were performed in triplicate. P<0.05 was considered to indicate a statistically significant result.

Results

Time-dependent expression profile of miR-351 and TRAF6 in TA muscle following sciatic nerve transection. RT-qPCR indicated that the expression levels of miR-351 in the TA muscle were gradually decreased with time following sciatic nerve transection (Fig. 1A). The mRNA and protein expression levels of TRAF6 in the TA muscle were upregulated at various times following sciatic nerve transaction, as determined by RT-qPCR and western blot analysis (Fig. 1B-D). Notably, the temporal expression profile of miR-351 in the TA muscle was inversely correlated with that of TRAF6 during the period of post-sciatic nerve injury.

Post-transcriptional downregulation of TRAF6 expression by miR-351. TargetScan prediction determined that the TRAF6 3'-UTR and miR-351 had a conservative matching area. To confirm that TRAF6 was negatively regulated by miR-351 through direct binding to the 3'-UTR of TRAF6, the wild-type 3'-UTR and mutant 3'-UTR of TRAF6 were constructed and inserted into the downstream region of the luciferase reporter gene, respectively. In the luciferase activity assay, miR-351 agomir or negative control agomir with p-Luc-UTR luciferase reporter were co-transfected into HEK 293T cells. Due to the presence of the wild-type 3'-UTR of TRAF6, miR-351 was able to significantly decrease the relative luciferase activity by >50%, whereas the relative luciferase activity of mutant 3'-UTR was not decreased by miR-353, suggesting that miR-353-induced decrease in the relative luciferase activity

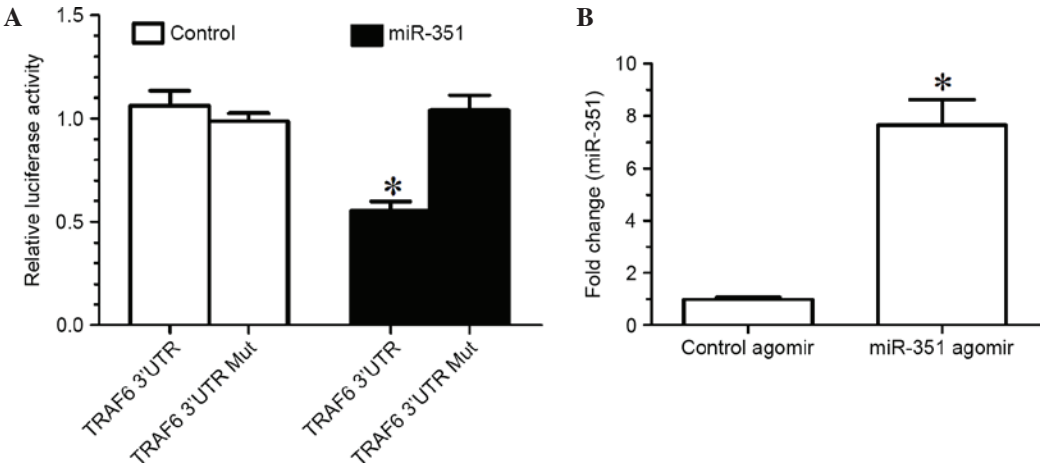

Figure 2. (A) Relative luciferase activity of HEK-293T cells that were co-transfected with miR-351 agomir (miR-351) or negative control agomir (control) with p-Luc-UTR vectors. The Renilla luciferase vector was used as an internal control. (B) Expression levels of miR-351 in the TA muscle of rats treated with miR-351 agomir (2.5 nmol in 50 μl) was significantly increased compared with those of the rats treated with control agomir (2.5 nmol in 50 μl). *P<0.05, vs. the control. UTR, untranslated region; miR, microRNA; Mut, mutant.

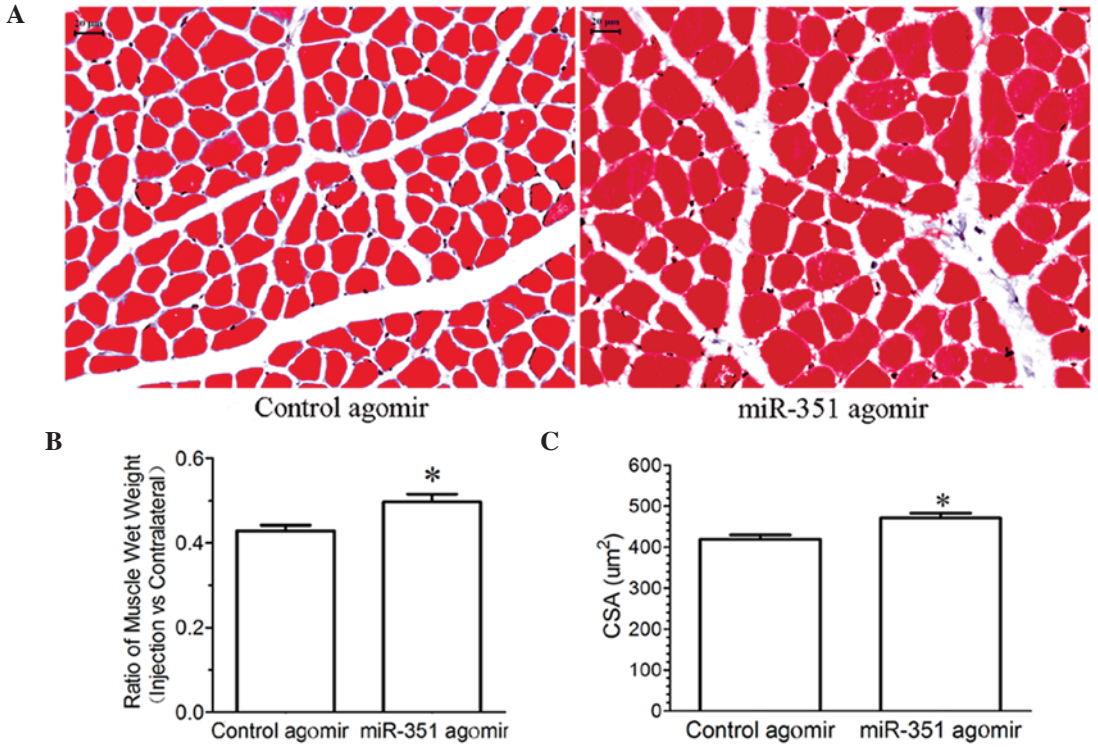

Figure 3. (A) Masson's trichrome staining for determining the CSA of the TA muscle of rats injected with miR-351 agamir or control agamir. Scale bar = 20 μm; (B and C) Bar graphs showing the wet weight ratio and CSA of the TA muscle of rats injected with miR-351 agomir or control agomir. Data are presented as means ± standard deviation, n=8 per animal group. *P<0.05, vs. the control. CSA, cross-sectional area; TA, tibialis anterior.

was sequence-specific (Fig. 2A). In other words, miR-351 is able to downregulate TRAF6 expression by directly targeting the 3'-UTR of TRAF6.

Inhibition of denervation-induced TA muscle atrophy by over-expression of miR-351. The expression levels of miR-351 in the TA muscle of rats treated with miR-351 agomir were significantly increased compared with those treated with control agomir (Fig. 2B), suggesting that injection of miR-351 agomir into the TA muscle was an effective method for increasing the expression levels of miR-351 in the muscle.

Morphological observation showed that treatment with miR-351 agomir inhibited a significant decrease in either the wet weight ratio or CSA of the TA muscle compared with control agomir (Fig. 3), suggesting that overexpression of miR-351 may inhibit denervation-induced muscle atrophy.

Suppression of the expression of TRAF6, MuRF1 and MAFBx in denervated TA muscle by miR-351. Western blot analysis indicated that the protein expression levels of TRAF6, MuRF1 or MAFBx in the TA muscle were inhibited following treatment with miR-351 agomir compared with those following treat-

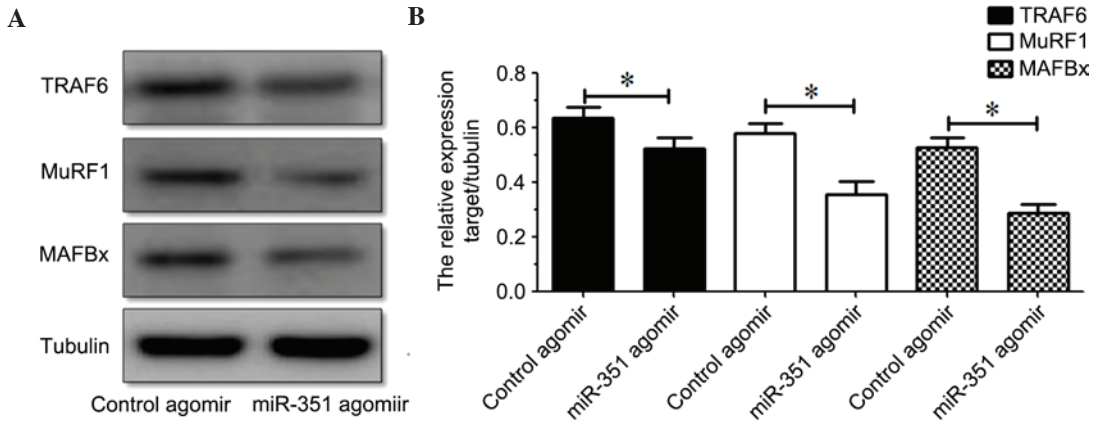

Figure 4. (A) Western blot and (B) quantification showing the protein expression levels of TRAF6, MuRF1, and MAFBx in the TA muscle of rats injected with miR-351 agamir or control agamir. Data are presented as means ± standard deviation, n=9 per animal group. *P<0.05, vs. the control. Tubulin was used as a loading control in western blot analysis. miR, microRNA; TA, tibialis anterior; TRAF6, tumor necrosis factor receptor-associated factor 6.

ment with control agomir (Fig. 4). The results indicated that overexpression of miR-351 is able to suppress the protein expression of TRAF6, MuRF1 or MAFBx in denervated TA muscles.

Discussion

Recent evidence suggests that miRNAs are involved in modulation of atrophy, proliferation and differentiation of skeletal muscles (20,24,25). A previous study observed that miR-351 is transiently increased in early days of muscle regeneration (26). miR-351 inhibits the expression of E2f3, a key regulator of cell cycle progression and proliferation, promotes myogenic progenitor cell proliferation and protects early differentiating myogenic progenitor cell from apoptosis (26). These findings revealed that miR-351 is involved in muscle atrophy. The results of the present study identified miR-351 as a novel regulator of denervation-induced muscle atrophy. miR-351 was differentially expressed in the TA muscle at various times following sciatic nerve transection, and the temporal expression profile of miR-351 in the TA muscle was inversely correlated with that of TRAF6 at the mRNA and protein levels. The dual luciferase reporter assay indicated that miR-351 was able to significantly downregulate TRAF6 expression by directly targeting the 3'-UTR of TRAF6. The results further demonstrated that overexpression of miR-351 inhibited a significant decrease in the wet weight ratio or CSA of the TA muscle following sciatic nerve transection.

Studies have determined that TRAF6 expression is significantly upregulated in denervation- or starvation-induced muscle atrophy and TRAF6 affects denervation- or starvation-induced muscle atrophy through regulation of muscle-specific ubiquitin ligases MuRF1 and MAFBx (5,27). In addition, the E3 ubiquitin ligase activity of TRAF6 has proven essential for starvation-induced muscle atrophy (6,13). In this study, therefore, it was also determined whether MuRF1 and MAFBx, as two downstream signaling molecules of TRAF6, were regulated by miR-351. Western blot analysis indicated that the protein expression levels of MuRF1 and MAFBx as well as TRAF6 were all inhibited by overexpression of miR-351.

In conclusion, the results of the present study demonstrated that miR-351 has an inhibitory role in denervation-induced muscle atrophy through, at least in part, negative regulation of TRAF6 and MuRF1 and MAFBx (two muscle-specific ubiquitin ligases). Further research, however, is required in order to provide a good understanding of the detailed mechanism underlying miR-351 regulation in muscle atrophy.

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