

miR-340-5p: A potential direct regulator of Nrf2 expression in the post-exercise skeletal muscle of mice

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Abstract. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that serves a critical role in protecting against cellular stress induced by exercise. The effects of exercise training on Nrf2 expression have been widely studied; however, the post-transcriptional/translational regulation of Nrf2 is poorly understood. The aim of the present study was to identify the exercise-induced microRNAs (miRNAs/miRs) targeting Nrf2. A total of 20 C57BL/6J mice were divided into the control (n=10) and exercise (n=10) groups. Following eight weeks of aerobic exercise training, Nrf2 mRNA expression in the hind-limb muscles was significantly increased in the exercise group, while that of Nrf2 protein remained unchanged. In addition, 58 differentially expressed miRNAs have been detected; among them, miR-101a-3p and miR-340-5p were predicted to target the 3' untranslated region of Nrf2 mRNA by analysis with three bioinformatics tools. The binding affinity of the two miRNAs were verified via a dual luciferase reporter assay; only miR-340-5p was determined to bind directly to Nrf2 mRNA. Additionally, miR-340-5p mimics and inhibitors were transfected into C2C12 cells to investigate the biological effects of endogenous miR-340-5p on the expression of Nrf2. The results revealed that the expression levels of Nrf2 protein in C2C12 cells were significantly decreased in the miR-340-5p mimics group and significantly increased in the miR-340-5p inhibitors group, while Nrf2 mRNA expression levels were unchanged. The results indicated that miR-340-5p serves a role in the post-transcriptional regulation of Nrf2 expression. In conclusion, the novel findings of the present study were that miR-340-5p may be a potential direct regulator of Nrf2 gene expression and might be involved in the regulation of Nrf2 protein expression in mouse skeletal muscles following

aerobic exercise. These results may provide insight into the underlying regulatory mechanisms of Nrf2 protein expression in skeletal muscle during aerobic exercise.

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that serves an important role in protecting cells from oxidative stress (1). Nrf2 protein contains the Kelch-like ECH-associated protein 1 (Keap1) binding domain. Under normal conditions, Nrf2 forms a complex with Keap1 and is of a low concentration in the cytoplasm (2). Researchers have reported that reactive oxygen species (ROS), key factors that activates Nrf2, induces the dissociation of Keap1 from Nrf2, permitting the translocation of Nrf2 into the nucleus (3). Once in the nucleus, Nrf2 heterodimerizes with small muscle aponeurotic fibrosarcoma (Maf) protein and then binds to the antioxidant response element (ARE) of downstream antioxidant genes, activating their transcription (4). An increasing body evidence indicates that exercise upregulates the expression of Nrf2 in various tissues, which protects cells from ROS-induced damage (5-9). Our recent pilot study using mouse skeletal muscle revealed no significant changes in the expression levels of Nrf2 protein following eight weeks of aerobic exercise training; however, this may be due to relatively low exercise intensities. Notably, the expression levels of Nrf2 mRNA increased significantly in the experiment. This discrepancy may suggest that post-transcriptional control and/or post-translational modification are involved in the regulation of Nrf2 expression in mouse skeletal muscle post-exercise. Among the multiple factors that may affect Nrf2 protein expression, microRNAs (miRNAs/miRs) have been proposed as key post-transcriptional regulators of gene expression (10).

miRNAs are short non-coding RNAs of ~21 nucleotides in length and serve a central role in many aspects of cell biology (11,12). Typically, by binding to the 3' untranslated region (3'UTR) of a complementary mRNA sequence, miRNAs can directly degrade the transcript or inhibit translation (11-13). Previous studies of normal and cancer cells have reported that few miRNAs could directly or indirectly regulate Nrf2 (14-19). It has been suggested that miR-144 (15), miR-28 (14,20), miR-153, miR-27a and miR-142-5p could

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directly regulate Nrf2 (18); miR-200a (16), miR-23a (17) and miR-141 (18,19) targeted Keap1 (a negative regulator of Nrf2) to indirectly promote the degradation of Nrf2 protein. In addition, miR-155 disrupted the Nrf2-dependent signaling pathway in hepatic cells; however, whether this occurs in a direct or indirect manner remains unknown (21). Nevertheless, miRNAs modulating the expression of Nrf2 induced by aerobic exercise training have not been well studied. However, accumulating evidence suggests that the expression levels of some miRNAs, including miR-1, miR-107, miR-161, miR-23, miR-133 and miR-486, known as the skeletal muscle-specific microRNAs (myomiRs), tend to be regulated in response to exercise training (22,23).

On this basis, the aim of the present study was to screen and identify the potential miRNAs that directly regulate Nrf2 expression within the skeletal muscle of mice following exercise. The present study first evaluated the effects of an 8-week aerobic exercise training program on the expression of Nrf2 mRNA and protein, and miRNAs in mouse skeletal muscle. Secondly, the profile of differentially expressed miRNAs in the exercise and non-exercise control groups was analyzed and filtered to determine putative miRNAs that target the 3'UTR of Nrf2. Finally, *in vitro* analysis was conducted to verify the selected candidate miRNAs. The present study aimed to improve understanding of the regulatory mechanism of miRNAs in the effects of aerobic exercise training on the expression Nrf2 protein in skeletal muscle.

Materials and methods

Animal and exercise program. The present study was approved by the Animal Care and Use Committee of Beijing Sport University (Beijing, China). A total of 20 male C57BL/6J mice (20±2 g, 8-weeks-old) were purchased from Charles River Development, Inc. (Beijing, China) with a body weight of 18±2 g. The animals were housed indoors under a temperature of 20-25°C, humidity of 50-70%, 12-h light/dark cycles, and had *ad libitum* access to deionized water and standard chow. They were randomly divided into the control (C; n=10) and exercise (E; n=10) groups. Mice in the C group were housed without exercise training. Mice in the E group were trained according to an aerobic exercise protocol, which was adopted from a previous study with some modifications (24). Briefly, two days prior to the formal exercise protocol, the animals were familiarized to running on a treadmill at a low intensity for 10 min/day, following which the animals were made to run on the treadmill for 1 h at 12 m/min. Mice of the E group were trained 6 days per week for a total of 8 weeks. To avoid acute effects from the last exercise session, the trained mice were able to recover for at least 48 h with free access to food and water prior to tissue collection. The mice were euthanized by cervical dislocation and whole hind limb muscle samples (a mixture of muscles) were excised, cleaned of blood and connective tissue, rapidly frozen in liquid nitrogen, and stored at -80°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of Nrf2 mRNA. Total RNA was extracted from C2C12 cells (China Agricultural University, Beijing, China) or 50 mg of whole hind-limb muscles (a mixture of muscles) with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific,

Inc., Waltham, MA, USA) following the manufacturer's instructions. RT-PCR was carried out using the ReverTra Ace Qpcr RT kit (Toyobo Life Science, Osaka, Japan), and was performed in a reaction volume of 20 µl containing 2 µl total RNA, 1 µl primer mix, 1 µl RT enzyme mix, 4 µl 5X RT buffer and 6 µl nuclease-free water. For the synthesis of cDNA, the reaction mixtures were incubated at 65°C for 5 min, 37°C for 15 min and 98°C for 5 min. qPCR was performed on an ABI 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR Green Real-time PCR Master Mix kit (Toyobo Life Science) with the previously synthesized cDNA template (FSQ-101 Toyobo Life Science) in a reaction volume of 20 µl. The primers obtained from Qiagen GmbH (Hilden, Germany) were as follows: Nrf2 (cat. no. QT00095270) and 18S gene (cat. no. QT010036875) as a reference gene, which were confirmed with software (ABI 7500RT PCR). qPCR was performed with a final volume of 20 µl containing 10 µl SYBR Green Real-time PCR Master Mix, 2 µl primers, 2 µl cDNA and 6 µl nuclease-free water. The thermocycling conditions were as follows: 95°C for 10 min, then 40 cycles of 95°C for 15 sec, 60°C for 60 sec and 72°C for 30 sec. The difference in expression between the E and C groups was calculated using the $2^{-\Delta\Delta C_q}$ method, as described previously (25). To assess the specificity of the amplified PCR products, melting curve analysis was conducted following the last cycle.

Western blotting analysis. Once total protein (20 µg) from 50 mg mouse skeletal muscle samples or total protein (10 µg) from C2C12 cells (2.5×10⁵ per well) was extracted using radioimmuno-precipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China), the protein concentration was determined with a bicinchoninic acid kit (Thermo Fisher Scientific, Inc.). Proteins were separated on a Bolt Bis-Tris plus 4-12% gel (Thermo Fisher Scientific, Inc.) by electrophoresis at 200 V for 35 min; the fractionated proteins were then transferred to a nitrocellulose transfer membrane using the iBlot Gel Transfer System (Invitrogen; Thermo Fisher Scientific, Inc.) at 20 V for 7 min and 30 sec. The membrane was blocked for 60 min at the room temperature in Tris-buffered saline with 0.01% Tween-20 (TBST) containing 5% nonfat milk. The samples were then incubated overnight at 4°C using the following primary antibodies: Anti-Nrf2 antibodies (1:200, cat. no. sc-722; and 1:2,000, cat. no. sc-365949; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and β-actin antibody (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). Of the primary antibodies against NRF2, the sc-722 antibody was used in animal experiments and the sc-365949 antibody was used in cell experiments. The blots were washed 3 times with 1X TBST, then incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. zb-2301; OriGene Technologies, Inc., Beijing, China) at 37°C for 1 h. Following washing with 1X TBST, the blots were visualized with an ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). The density of the protein bands was analyzed using the Molecular Imager® ChemiDoc™ XRS + with Image Lab™ Software version 6.0.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein expression levels were normalized to that of β-actin and then expressed as the fold change of the control group.

miScript miRNA PCR array. Total RNA of skeletal muscle from 50 mg muscle samples was extracted using an miRNeasy

Mini kit (Qiagen GmbH). The total RNA was assessed with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and its quality fulfilled the requirements of total RNA absorbance ratios: >2.0 (260/280 nm) and >1.8 (260/230 nm). Then, 1.5 ng RNA was employed to conduct RT-PCR to produce cDNA with the miScript II RT kit (Qiagen GmbH). RT-PCR was performed in a reaction volume of 20 μ l containing 2 μ l total RNA, 4 μ l 5X miScript HiSpec Buffer, 2 μ l 10X miScript Nucleics Mix, 2 μ l miScript Reverse Transcriptase Mix and 10 μ l nuclease-free water. The reaction mixtures were incubated at 37°C for 60 min and 95°C for 5 min. miRNA expression was analyzed using a miScript miRNA PCR Array (Qiagen GmbH) according to the manufacturer's instructions. Prior to analysis, the miScript miRNA quality control PCR Array was employed to determine the quality of the extracted cDNA template, which met the criterion for conducting miScript miRNA PCR array analysis. In addition, 96-well plates were used to determine the expression profile of 940 miRNAs from one mouse muscle sample via array analysis. A reaction mix with a final volume of 25 μ l containing 12.5 μ l 2X QuantiTect SYBR Green Master Mix, 2.5 μ l 10X miScript Universal Primer, 1 μ l cDNA and 9 μ l nuclease-free water was employed. The thermocycling conditions were as follows: 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. The Cq values were obtained using an ABI 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and were analyzed using online data analysis tools based on the $\Delta\Delta Cq$ method (relative gene expression = $2^{-(\Delta Cq_{\text{sample}} - \Delta Cq_{\text{control}})}$) (25). The P-value and log2 fold change (log2 FC) between the C and E groups were calculated; $P < 0.05$ and $|\log_2 \text{FC}| \geq 1$ were regarded to indicate differentially expressed miRNAs.

Bioinformatics prediction. TargetScan v7.1 (www.targetscan.org/) (26-28), miRanda (released August 2010, www.microrna.org/) (29,30) and DIANA v4.0 (diana.imis.athena-innovation.gr/DianaTools/index.php?r=miroT_CDS/index) (31,32) were used to predict the putative miRNAs that target the 3'UTR of Nrf2. To reduce the number of false positives and increase fidelity, the intersection of the prediction results from these three tools was considered to indicate the putative miRNAs. In addition, by analyzing the selected differentially expressed miRNAs with the miScript miRNA PCR array, miR-340-5p and miR-101a-3p were proposed to be the miRNAs that may target Nrf2 mRNA following 8 weeks of aerobic exercise.

miRNA mimics and plasmid construction. miRNA mimics are innovative molecules designed for gene silencing approaches that contain non-natural or artificial double stranded miRNA-like RNA fragments (33). These RNA fragments are constructed to contain a sequence motif on its 5'-end that is partially complementary to the target sequence in the 3'UTR of the transcript (33). On this basis, miRNA mimics and a control were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences were as follows: miR-101a-3p mimic, 5'-UACAGUACUGUGAUAACU GAA-3'; miR-340-5p mimic, 5'-UUAUAAAGCAAUGAG ACUGAUU-3'; and mimic control, 5'-UUUGUACUACAC AAAAGUACUG-3'.

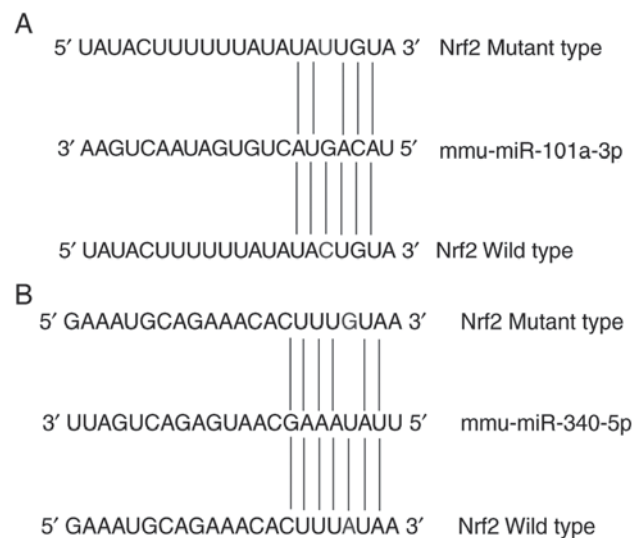


Figure 1. Mutations in the putative binding site within the 3'UTR of Nrf2. (A) The mutation in the putative binding site of miR-101a-3p within the 3'UTR of Nrf2. (B) The mutation in the putative binding site of miR-340-5p within the 3'UTR of Nrf2. Nrf2, nuclear factor erythroid 2-related factor 2; miR, microRNA; UTR, untranslated region.

The pMIR-Report luciferase and the control reporter pRL-TK plasmids were purchased from Ambion (Thermo Fisher Scientific, Inc.). The full length 3'UTR sequence of Nrf2 containing the putative target site for miR-101a-3p or miR-340-5p was synthesized, and then inserted into the XhoI and HindIII sites of the pMIR-Report luciferase vector by GENEWIZ Beijing (Beijing, China); the construct was named pMIR-Nrf2. A mutation of Nrf2 3'UTR in the putative binding site of miR-101a-3p or miR-340-5p was introduced into the putative seed-matching sequence of pMIR-Nrf2 (Fig. 1A and B); these constructs were named pMIR-Nrf2-Mut1 for miR-101a-3p and pMIR-Nrf2-Mut2 for miR-340-5p. As determined from the miRanda database (www.microrna.org/microrna/home.do), miR-340-5p was predicted to target two binding sites within the 3'UTR of Nrf2 at 84-90 (mirSVR score = -0.9843) and 366-380 (mirSVR score = -0.1789), while miR-101a-3p had two binding sites located at 258-263 (mirSVR score = -0.3732) and 350-355 (mirSVR score = -0.3981). MirSVR scoring is a machine learning method for ranking miRNA target sites, in which a lower mirSVR score indicates a stronger association between a miRNA and a target gene (34). Thus, the miRNA with the lowest mirSVR score was chosen as the target miRNA in the present study. A base in the target site was changed to generate a mutation in the 3'UTR of Nrf2 (Fig. 1).

Cell culture, transfection and dual luciferase reporter assay. 293T cells were obtained from China Agricultural University (Beijing, China), and were cultured in complete growth medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA)], 100 μ g/ml penicillin and 100 μ g/ml streptomycin (HyClone; GE Healthcare Life Sciences) at 37°C in a humidified incubator containing 5% CO₂.

A total of $\sim 2 \times 10^5$ 293T cells were seeded in a 24-well plate in DMEM containing 10% FBS 24 h prior to transfection. The experiment was set for four

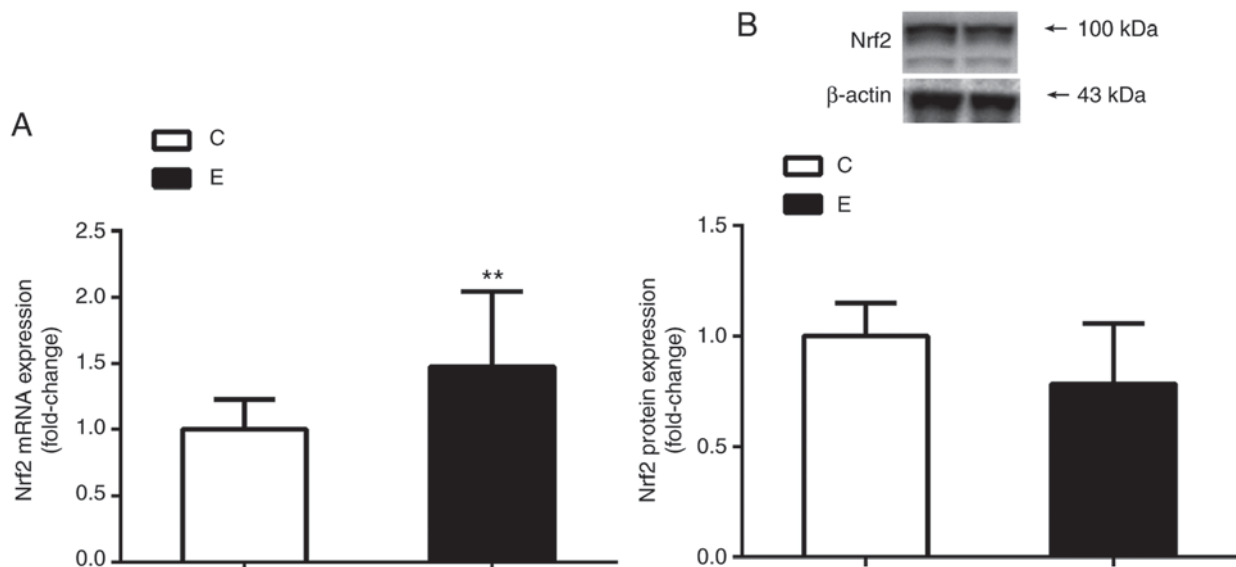


Figure 2. Expression of Nrf2 mRNA and protein expression in the C and E groups. Mouse skeletal muscle was collected following 8 weeks of aerobic exercise training (n=9-10 mice/group). Nrf2 (A) mRNA and (B) protein expression levels in the skeletal muscle were measured by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The values are expressed as the means \pm standard error of the mean. ** $P < 0.01$ vs. C group. Nrf2, nuclear factor erythroid 2-related factor 2; C, control; E, exercise.

groups as follows: pMIR-Nrf2+miRNA mimics control, pMIR-Nrf2+miRNA mimics (miR-101a-3p or miR-340-5p), pMIR-Nrf2-Mutation+miRNA mimics control, and pMIR-Nrf2-Mutation+miRNA mimics (Mutation1/Mutation2 corresponds to mutations in the putative binding sequence of miR-101a-3a and miR-340-5p, respectively). Cell transfection was performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Briefly, the culture medium was replaced with Opti-minimum essential medium (MEM) from the kit. The pMIR-Report or the mutant luciferase constructs were mixed with pRL-TK at a ratio of 20:1, and miRNA mimics or the control were diluted with 50 μ l Opti-MEM to a final concentration of 50 nM. These diluted components were combined and incubated for 20 min at room temperature. The mixture (100 μ l) was then added to each well and the medium was replaced with DMEM containing 10% FBS following culture for 6 h; transfection was carried out in triplicate. Following a 48 h incubation, the cells were harvested and lysed in the passive lysis buffer included in the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). The luciferase activity of firefly and *Renilla* were measured using the Dual-Luciferase[®] Reporter Assay System in a GloMax[®] 20/20 luminometer (Promega Corporation); the firefly luciferase activity was normalized to that of *Renilla*. The relative luciferase activity was used to validate whether miR-340-5p and miR-101a-3p regulate the expression of Nrf2 by binding to the 3'UTR.

Overexpression and knockdown of miR-340-5p in C2C12 cells. miRNA inhibitors are chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNA molecules (35). In the present study, C2C12 cells (1.5×10^5 per well) were cultured in DMEM under the same conditions as those used aforementioned for 293T cells. The cells were seeded in 6-well plates upon reaching

70% confluence the day prior to transfection. miR-340-5p mimics (5'-UUAUAAAGCAAUGAGACUGAUU-3'), inhibitors (5'-AAUCAGUCUCAUUGCUUUAUAA-3'), mimics control (5'-UUUGUACUACACAAAAGUACUG-3') and inhibitor control (5'-UCACAACCUCCUAGAAAGAGUAGA-3') (Guangzhou RiboBio Co., Ltd.) were transfected into C2C12 cells at a concentration of 150 nM using Lipofectamine 3000[™] (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The medium was changed 24 h post-transfection; C2C12 cells were harvested following 48 h to determine the mRNA and protein expression levels of Nrf2.

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Cell experiments were performed in triplicate, and independently repeated at least three times. Statistical calculations were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Data were analyzed by an Independent Samples t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of exercise training on Nrf2 mRNA and protein expression. The results of RT-qPCR analysis revealed that the mRNA expression levels of Nrf2 in the E group were significantly increased when compared with the C group ($P < 0.01$). Additionally, no significant difference was observed in the levels of Nrf2 protein expression between the two groups following 8 weeks of aerobic exercise training as determined by western blotting (Fig. 2).

Effects of exercise training on miRNAs expression. According to the standards of $P < 0.05$ and $|\log_2 \text{FC}| \geq 1$, 58 significantly differentially expressed miRNAs between the C and E groups were identified in the present study. Among

Table I. Differential expression of microRNAs between two groups by miScript microRNA polymerase chain reaction array analysis.

miRNA ID	t-test	Fold change
miR-142-3p	0.005	38.83
miR-126a-3p	0.020	19.90
miR-15a-5p	0.004	12.84
miR-29b-3p	0.016	22.48
miR-27a-3p	0.020	8.19
miR-30e-5p	0.033	10.50
miR-22-3p	0.024	10.43
miR-30a-5p	0.045	7.22
miR-140-5p	0.001	9.02
miR-17-5p	0.020	7.27
miR-29a-3p	0.025	7.48
miR-19b-3p	0.010	49.43
miR-20a-5p	0.016	11.05
miR-106b-5p	0.016	12.10
miR-99a-5p	0.011	15.27
miR-19a-3p	0.012	47.25
miR-199a-5p	0.016	9.57
miR-411-5p	0.031	2.88
miR-425-5p	0.032	3.65
miR-335-5p	0.049	4.26
miR-101a-3p ^a	0.016	62.88
miR-744-5p	0.034	-2.21
miR-29c-3p	0.007	6.46
miR-30b-5p	0.014	31.18
miR-148b-3p	0.046	3.04
miR-106a-5p	0.014	6.00
miR-714	0.015	-2.35
miR-376b-5p	0.044	3.49
miR-20b-5p	0.026	9.32
miR-337-3p	0.003	6.22
miR-338-3p	0.010	13.73
miR-148a-3p	0.043	9.49
miR-497-5p	0.029	6.64
let-7i-3p	0.007	3.58
miR-1187	0.044	-3.78
miR-145a-5p	0.037	3.70
miR-190a-5p	0.031	8.56
miR-193a-5p	0.013	-2.15
miR-199b-5p	0.023	9.57
miR-24-2-5p	0.015	5.75
miR-29a-5p	0.037	6.13
miR-340-5p ^a	0.037	2.95
miR-425-3p	0.042	-3.46
miR-709	0.026	-5.14
miR-450a-5p	0.035	7.86
miR-1249-3p	0.033	-2.57
miR-3069-3p	0.028	-2.89
miR-486-3p	0.010	-2.22
miR-136-5p	0.002	13.43
miR-190a-3p	0.047	4.37

Table I. Continued.

miRNA ID	t-test	Fold change
miR-3106-5p	0.048	-2.86
miR-1a-2-5p	0.007	100.92
miR-1947-3p	0.003	-4.20
miR-667-5p	0.049	-4.05
miR-3076-5p	0.035	-2.55
miR-1a-1-5p	0.036	34.30
miR-143-3p	0.015	39.94
miR-486-3p	0.017	-3.02

^amiRNAs selected as candidate miRNAs for further study.
miR/miRNA, microRNA.

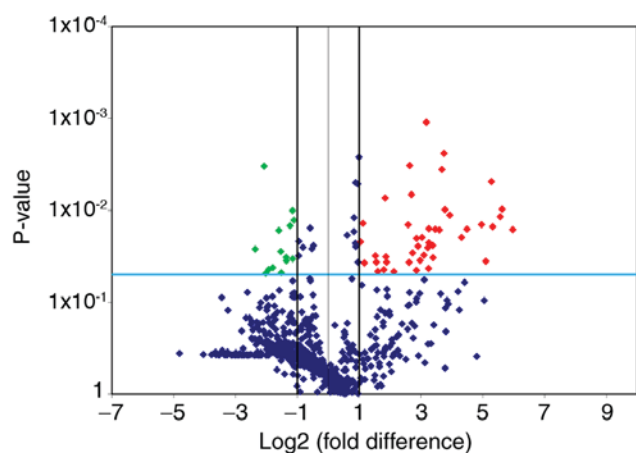


Figure 3. Volcano plot of differentially expressed miRNAs between the C and E groups. The green blocks indicate downregulated miRNAs ($\log_2 \text{FC} \leq -1$) and the red blocks indicate upregulated miRNAs ($\log_2 \text{FC} \geq 1$). The blue line indicates a P-value of 0.05. miRNA, microRNA; FC, fold change.

them, the expression levels of 14 miRNAs were downregulated ($\log_2 \text{FC} \leq -1$), whereas 44 miRNAs were upregulated ($\log_2 \text{FC} \geq 1$) following 8 weeks of aerobic exercise training (Table I; Fig. 3).

Bioinformatics prediction of the putative miRNAs that target the 3'UTR of Nrf2. DIANA, miRanda and TargetScan prediction tools identified 40, 84 and 293 miRNAs that target the 3'UTR of Nrf2, respectively; 4 miRNAs (miR-101a-3p, miR-142-5p, miR-1950 and miR-340-5p) were identified when combining the results of prediction analysis with these three tools (Fig. 4A). In addition, by combining the 58 differentially expressed miRNAs between the E and C groups (Table I), miR-101a-3p and miR-340-5p (Fig. 4B; Table I) were selected as candidate miRNAs that targeted the 3'UTR of Nrf2 and chosen for further study.

miR-340-5p may directly target 3'UTR of Nrf2 gene. As shown in Fig. 5, the relative luciferase activity in the pMIR-Nrf2+miR-340-5p mimics group was significantly lower than that of the pMIR-Nrf2+miRNA mimics control group

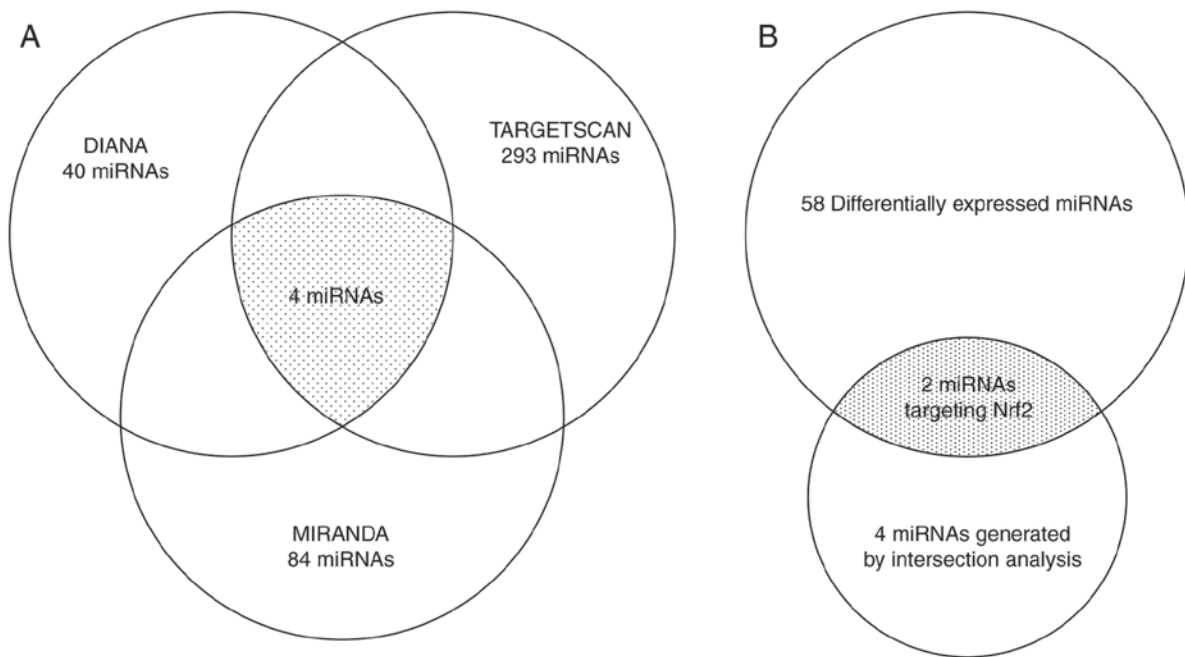


Figure 4. miRNAs targeting the 3'UTR of Nrf2 as determined by miRNA target prediction tools. (A) The miRNAs targeting the 3'UTR of Nrf2 were predicted using DIANA, miRanda and TargetScan tools; 4 miRNAs targeting NRF2 were predicted by intersection analysis. (B) Two potential miRNAs targeting the 3'UTR of Nrf2 were selected. miRNA, microRNA; Nrf2, nuclear factor erythroid 2-related factor 2; UTR, untranslated region.

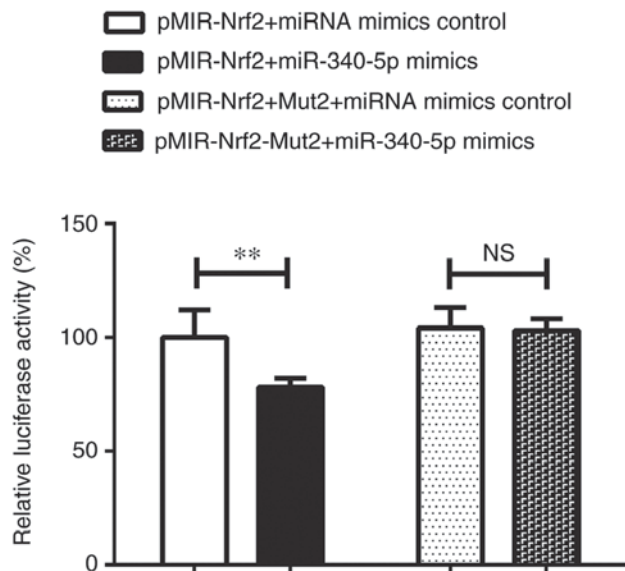


Figure 5. miR-340-5p suppresses the luciferase activity of pMIR-Nrf2 plasmid. A dual luciferase reporter assay was used to assess the associations between miR-340-5p and its target, Nrf2 mRNA. The values are expressed as the mean \pm standard error of the mean. ** $P < 0.01$, as indicated. NS, no significance; miR/miRNA, microRNA; Nrf2, nuclear factor erythroid 2-related factor 2; Mut, mutation.

($P < 0.01$). This indicated that miR-340-5p was capable of binding to the pMIR-Nrf2 plasmid and suppressed luciferase activity. Additionally, no significant difference was observed in relative luciferase activity between the pMIR-Nrf2-Mut2+miRNA mimics control and pMIR-Nrf2-Mut2+miR-340-5p mimics groups ($P > 0.05$; Fig. 5). These results further indicated that the binding site of miR-340-5p was located within the 3'UTR of Nrf2.

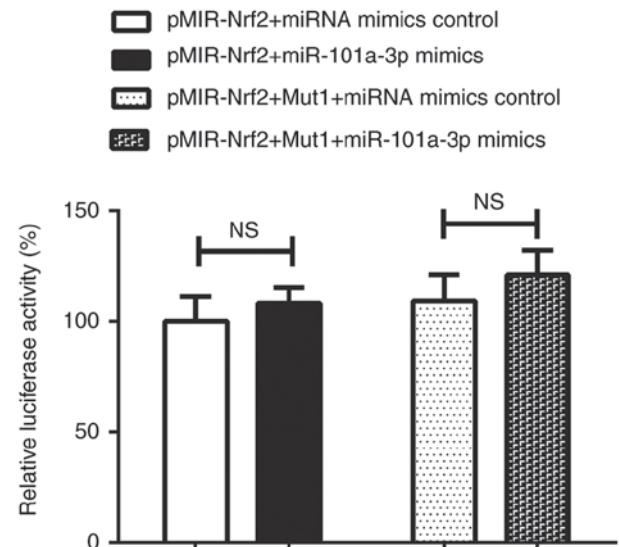


Figure 6. miR-101a-3p does not suppress the luciferase activity of the pMIR-Nrf2 plasmid. A dual luciferase reporter assay was used to assess the associations between miR-101a-3p and its target Nrf2 mRNA. The values are expressed as the mean \pm standard error of the mean. NS, no significance; miR/microRNA; Nrf2, nuclear factor erythroid 2-related factor 2; Mut, mutation.

miR-101a-3p does not directly target 3'UTR of Nrf2 gene. There was no significant difference in the relative luciferase activity between the pMIR-Nrf2+miR-101a-3p mimics and the pMIR-Nrf2+miRNA mimics control groups ($P > 0.05$). In addition, there was no significant difference in relative luciferase activity between the pMIR-Nrf2-Mut1+ miRNA mimics control and pMIR-Nrf2-Mut1+miR-101a-3p mimics groups ($P > 0.05$; Fig. 6). Therefore, these data indicated that miR-101a-3p did not suppress luciferase activity and could not bind the pMIR-Nrf2 plasmid.

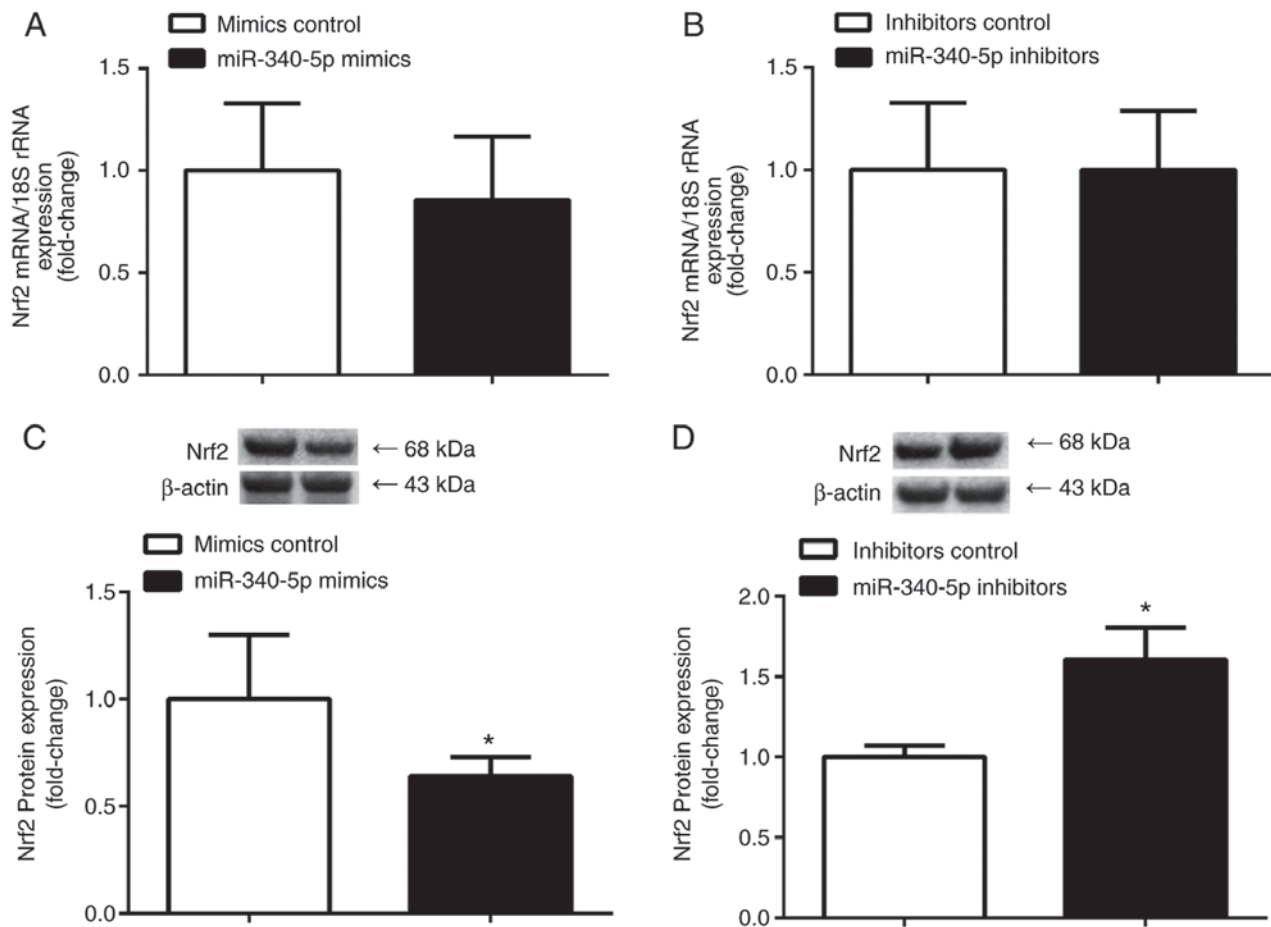


Figure 7. Expression of Nrf2 mRNA and protein in C2C12 cells. (A) RT-qPCR analysis of Nrf2 mRNA expression; no significant difference in Nrf2 mRNA expression was observed between the mimics control and miR-340-5p mimics groups. (B) RT-qPCR analysis of Nrf2 mRNA expression; no significant difference between the inhibitors control and miR-340-5p inhibitors groups was observed. (C) The protein expression levels of Nrf2 were significantly reduced following treatment with miR-340-5p mimics, as measured by western blotting. (D) The protein expression levels of Nrf2 were significantly increased following treatment with miR-340-5p inhibitors, as measured by western blotting. The values are expressed as the mean \pm standard error of the mean. * $P < 0.05$ vs. the corresponding control. miR, microRNA; Nrf2, nuclear factor erythroid 2-related factor 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Effect of miR-340-5p overexpression and knockdown on endogenous Nrf2 mRNA and protein expression levels. To demonstrate the effect of miR-340-5p on endogenous Nrf2 mRNA and protein expression levels, the present study transiently transfected C2C12 cells with miR-340-5p mimics, inhibitors or control, and the expression of Nrf2 mRNA and protein was determined. Transfection of miR-340-5p mimics or inhibitors significantly decreased and increased Nrf2 protein expression, respectively ($P < 0.05$); however, the mRNA expression levels remained unchanged ($P > 0.05$; Fig. 7).

Discussion

The main finding of the present study was that the mRNA expression levels of Nrf2 in skeletal muscle significantly increased following 8 weeks of aerobic exercise training, but the protein expression levels did not increase. One miRNA, miR-340-5p, was revealed to directly bind to the 3'UTR of Nrf2 and may be a direct post-transcriptional regulator of Nrf2 in skeletal muscle following aerobic exercise training.

It has been demonstrated that aerobic exercise training increased the expression levels of Nrf2 mRNA and protein in

the cardiomyocytes of rats following myocardial infarction, which protected against myocardial infarction-induced oxidative injury (36); physical exercise of moderate intensity also protected against experimental 6-hydroxydopamine-induced hemi-parkinsonism through the Nrf2-ARE signaling pathway (20). Some studies have confirmed that Nrf2 protein expression in whole cell or nuclear fractions was associated with exercise intensity. For example, the effects of varying intensities of treadmill exercise on hippocampal Nrf2 protein expression in adult C57B1 male mice were studied. The results revealed that the greater the intensity of exercise, the higher the protein expression levels of Nrf2 (37). Another experiment examined the association between exercise intensity and the protein expression levels of Nrf2 in blood cells, and revealed that the greater the intensity of exercise, the higher the Nrf2 protein levels in the nucleus (38). In addition, the expression of miRNAs that are affected by exercise have been investigated. miRNA expression profiling revealed that exercise significantly increased the expression of miR-181, miR-1 and miR-107, but reduced that of miR-23; no changes in the expression of miR-133 were noted (22). In the present study, the expression levels of Nrf2 mRNA were increased, while no significant changes in

Nrf2 protein expression within mouse skeletal muscle following aerobic exercise for 8 weeks were reported. It was speculated that no changes in the expression of Nrf2 protein in trained mice may be associated with lower exercise intensities. In addition, the regulation of miRNAs may be involved in targeting Nrf2 mRNA. It has been reported that miRNAs may serve important roles in post-transcriptional gene regulation by targeting mRNAs for cleavage or translational repression (39). It is well known that if a miRNA is completely complementary with an mRNA transcript, the miRNA will cleave and degrade its target; otherwise, the miRNA will bind the mRNA to execute translational inhibition (40). These results suggest that miRNA translational repression may be one of factors that lead to this discrepancy in the expression levels of Nrf2 mRNA and protein.

A total of 58 miRNAs exhibiting significant differential expression following 8 weeks of aerobic exercise intervention were studied in the present study. By combining the results of prediction analysis, and the profiles of differentially expressed miRNAs between the C and E groups, miR-340-5p and miR-101a-3p were determined to be the miRNAs that target the 3'UTR of Nrf2. To verify this, the present study measured the luciferase signals of these two miRNAs, which revealed that the luciferase activity of the pMIR-Nrf2+miR-340-5p mimics group was suppressed, while that of the pMIR-Nrf2+miR-101a-3p mimics group was unchanged, compared with their corresponding control groups. Additionally, the luciferase activity of the plasmid with mutant type Nrf2 3'UTR was not affected by miR-340-5p or miR-101a-3p mimics. These results indicated that only miR-340-5p could target the 3'UTR of Nrf2 directly, and may be involved in downregulating the protein expression of Nrf2 following 8 weeks of aerobic exercise training; miR-101a-3p was revealed to not directly regulate Nrf2. However, it has been shown that Cullin 3 is a ubiquitous ligase that degrades Nrf2 (41). miR-101a-3p was reported to bind to the 3'UTR of Cullin 3 to suppress expression and stabilize Nrf2 via the inhibition of the proteasomal degradation pathway (4,42). Thus, miR-101a-3p may also serve an important role in targeting Nrf2 expression as an indirect regulator; however, further research is required in the future to verify this.

To investigate if miR-340-5p had an effect on the endogenous mRNA and protein expression levels of Nrf2, the present study detected the expression of Nrf2 in C2C12 cells transfected with miR-340-5p mimics or inhibitors. The results revealed that miR-340-5p mimics decreased the expression levels of Nrf2 protein, whereas the expression levels increased in response to miR-340-5p inhibitors. However, miR-340-5p mimics and inhibitors had no effect on Nrf2 mRNA expression. Taken together, it could be concluded that miR-340-5p directly regulated the expression of Nrf2 by inhibiting the translation of Nrf2 mRNA. These data further supported that in the present *in vivo* study, aerobic exercise may have induced the expression of miR-340-5p, inhibiting the expression of Nrf2 protein, which may explain the discrepancy in the mRNA and protein expression levels of Nrf2.

In addition, Keap1 (43), Cullin-associated and neddylation-dissociated 1 (CAND1) (44), and small Maf proteins (45) have also been reported to be important regulators of Nrf2 expression. miRNAs may indirectly affect the expression of Nrf2 by targeting Keap1, CAND1 and small Maf proteins.

In conclusion, the results of the present study provide evidence that miR-340-5p may be a miRNA that directly targets the 3'UTR of Nrf2. Therefore, miR-340-5p may be regarded as a potential direct regulator of Nrf2 expression in the post-exercise skeletal muscle of mice. These observations may provide insight into the miRNA-associated mechanisms underlying the effects of aerobic exercise training on Nrf2 protein expression in skeletal muscle.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TM and YZ conceived and designed the experiments. TM and YL performed the experiments and analyzed the data. YZ and JW contributed to the reagents/materials/analysis. TM, YZ and JW gave final approval of the version to be published.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Beijing Sport University (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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