miR-182 attenuates atrophy-related gene expression by targeting FoxO3 in skeletal muscle

Matthew B. Hudson,1 Jill A. Rahner,1 Bin Zheng,1 Myra E. Woodworth-Hobbs,2 Harold A. Franch,1 and S. Russ Price1,3

1Department of Medicine, Renal Division, Emory University, Atlanta, Georgia; 2Nutrition and Health Sciences Ph.D. Program, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, Georgia; 3Biomedical Laboratory Research and Development Service, Atlanta VA Medical Center, Decatur, Georgia

Submitted 24 December 2013; accepted in final form 22 May 2014

SKELETAL MUSCLE ATROPHY occurs in many conditions including chronic kidney disease, diabetes, cancer, and elevated glucocorticoids. MicroRNAs (miR) may play a role in the wasting process. Activation of the forkhead box O3 (FoxO3) transcription factor causes skeletal muscle atrophy in patients, animals, and cultured cells by increasing the expression of components of the ubiquitin-proteasome and autophagy-lysosome proteolytic systems. To identify microRNAs that potentially modulate the atrophy process, an in silico target analysis was performed and miR-182 was predicted to target FoxO3 mRNA. Using a combination of immunoblot analysis, quantitative real-time RT-PCR, and FoxO3 3′-UTR luciferase reporter genes, miR-182 was confirmed to regulate FoxO3 expression in C2C12 myotubes. Transfection of miR-182 into muscle cells decreased FoxO3 mRNA 30% and FoxO3 protein 67% (P < 0.05) and also prevented a glucocorticoid-induced upregulation of multiple FoxO3 gene targets including MAFbx/atrogen-1, autophagy-related protein 12 (ATG12), cathepsin L, and microtubule-associated protein light chain 3 (LC3). Treatment of C2C12 myotubes with dexamethasone (Dex) (1 μM, 6 h) to induce muscle atrophy decreased miR-182 expression by 63% (P < 0.05). Similarly, miR-182 was decreased 44% (P < 0.05) in the gastrocnemius muscle of rats injected with streptozotocin to induce diabetes compared with controls. Finally, miR-182 was present in exosomes isolated from the media of C2C12 myotubes and Dex increased its abundance. These data identify miR-182 as an important regulator of FoxO3 expression that participates in the control of atrophy-inducing genes during catabolic diseases.

miR-182; FoxO3; muscle; glucocorticoids

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1 This article is the topic of an Editorial Focus by John J. McCarthy (18a).
Address for reprint requests and other correspondence: M. B. Hudson, Renal Division, Rm. 3327, Woodruff Memorial Research Bldg., 1639 Pierce Dr., Emory Univ., Atlanta, GA 3032 (e-mail: mbhudson@emory.edu).

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METHODS

C2C12 cell culture. Mouse C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were cultured in growth media (Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) plus 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA)); only passage 7 cells or less were studied. Myoblasts were grown to ~95% confluence in a six-well plate and then differentiated into myotubes by replacing growth media with differentiation media (DMEM supplemented with 2% fetal bovine serum and 1% penicillin and streptomycin) for 3 days. For some experiments, myotubes were treated with 1 μM Dex for 6 h before being harvested.

Transfection of microRNAs into myoblasts was performed when cells were ~70% confluent using 40 nM pre-miR miR-182 or pre-miR negative control mirR precursors (Ambion, Austin, TX) with Lipofectamine 2000 (Invitrogen). After 6 h, the media of the transfected cells were switched to differentiation media for 72 h as described above before harvest for experiments. For some experiments, transfected myotubes were treated with 1 μM Dex for 6 h before being harvested for analysis.

Diabetes model of muscle atrophy. The rat model of muscle atrophy involving acute streptozotocin (STZ)-induced diabetes mellitus (i.e., insulin deficiency) has been described (26, 45). Briefly, male rats (~150 g) received a single intravenous injection of STZ (125 mg/kg) in citrate buffer. STZ-injected and control rats were pair-fed and on the third day after injection, the animals were anesthetized and the gastrocnemius muscle was harvested. Food was withheld during the night before the tissue harvest to avoid the confounding influences of variable food intake on skeletal muscle protein metabolism. All animal studies were approved by the Emory Institutional Animal Care and Use Committee (IACUC).

Quantitative real-time RT-PCR. Total RNA was extracted from cells using the Total RNA Isolation Kit from Norgen Biotek (Norgen Biotek, Thorolod, ON, Canada) which produces RNA that is enriched in small RNAs. Isolated RNA was then reverse-transcribed using the NCode VILO miRNA cDNA synthesis kit (Invitrogen). To evaluate whether miR-182 is packaged into exosomes and released from myotubes, we used a pool of cDNA previously prepared from microRNAs in exosomes isolated from the media of myotubes treated with or without 1 μM Dex for 6 h as described (9). In both cases, quantitative real-time RT-PCR (qPCR) for miR-182 and a control small nuclear RNA U6 were performed. All qPCR reactions were performed using a miR-182-specific LNA PCR primer (Exiqon, Woburn, MA) and a universal PCR primer [NCode VILO miRNA cDNA synthesis kit (Invitrogen)]. Samples were measured in triplicate and normalized using U6 snRNA; U6 has been utilized as an endogenous control RNA for microRNA in muscle, muscle cells, and exosomes (9, 42, 43). Quantification of FoxO3, atrogin-1, microtubule-associated protein light chain 3 (LC3), cathepsin L, and autophagy-related protein 12 (ATG12) mRNAs were performed by qPCR using published primer sets (17, 45); 18S rRNA was used as a normalization control as described previously (26). All qPCR experiments were performed using the Bio-Rad iCycler and iQ SYBR Green (Bio-Rad, Hercules, CA) with the following cycling parameters: 95°C for 2 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were analyzed for fold change (ΔΔCt) using the iCycler software. Melting curve analyses were performed to evaluate and verify the specificity of the reactions.

Western blot analysis. For Western blot analysis of FoxO3, cells were lysed in a buffer consisting of 50 mM HEPES (pH 7.5), 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM Na2VO4, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 μg/ml aprotinin, 10 μg/ml antipain, 5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 1.5 mg/ml benzamidine, and 34 μg/ml PMSF (6). Proteins in cleared lysates were separated by electrophoresis and transferred to a nitrocellulose membrane for detection with polyclonal FoxO3 antibodies from Cell Signaling. Equal amounts (20 μg) of lysate proteins were loaded per lane and equal loading was verified by Ponceau S Red staining and imaging.

TargetScan analysis and 3’-UTR luciferase reporter assay. To identify microRNAs that potentially regulate FoxO3, an in silico analysis was performed using TargetScan (www.targetscan.org). Several candidate microRNAs were predicted to target the 3’-untranslated region (3’-UTR) of FoxO3. We chose to evaluate miR-182 because there are two predicted target sequences in the 3’-UTR of FoxO3. To determine whether miR-182 directly binds to the FoxO3 3’-UTR, we utilized a 3’-UTR luciferase reporter assay. A 50 base pair segment of the mouse FoxO3 3’-UTR sequence containing the proximal predicted miR-182 target site (bases 73–80 of the 3’-UTR) was cloned into the pMIR-REPORT miRNA Expression Report Vector (Invitrogen) by the Emory University Custom Cloning Core Facility. A related reporter plasmid, pMIR-FoxO3-Δ3’-UTR, was also generated that contained the same FoxO3 3’-UTR sequence except that the miR-182 binding site was scrambled. C2C12 myoblasts were plated in a 24-well plate and transfected with 50 ng of either pMIR-FoxO3-3’-UTR, pMIR-FoxO3-Δ3’-UTR, or empty pMIR plasmid (as a control) plus 10 nM of either pre-miR miR-182 precursor or pre-miR negative control miR precursor (both obtained from Ambion) using Lipofectamine 2000 according to the manufacturer’s instructions. After 6 h, the media of the transfected cells were switched to differentiation media. After 48 h, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Fitchburg, WI) and was reported as the percentage of the activity in cells transfected with the negative control pre-miR.

Statistical analysis. Group sample size was determined by performing a power analysis of preliminary data. When results from two groups were compared, a t-test was used to test for significance. When results from more than two groups were compared, a one-way ANOVA was used to determine overall significance. When appropriate, a post hoc Tukey honestly significantly difference (HSD) test was performed. Differences in results were considered significant when P ≤ 0.05. For each outcome, at least 3–5 samples per treatment group, acquired from ≥3 independent experiments, were quantified and analyzed. Specific n values are provided in the figure legends.

RESULTS

MicroRNA-182 regulates FoxO3 in skeletal muscle cells. An in silico analysis was performed with TargetScan to identify candidate microRNAs that potentially regulate FoxO3, and several microRNAs were identified. One candidate, miR-182, was attractive because two predicted miR-182 target sequences are located in the 3’-UTR of FoxO3. Although miR-182 has been reported to be expressed at low levels in a microarray analysis of skeletal muscle, it has been reported to regulate FoxO3 in other cells (18, 34). To confirm that miR-182 targets FoxO3 in C2C12 myotubes, myoblasts were transfected with FoxO3-3’-UTR luciferase reporter plasmids plus miR-182 or a scrambled control microRNA and differentiated into myotubes before measurement of luciferase activity. Compared with the activity in cells transfected with the control pMIR reporter plasmid, miR-182 significantly reduced the activity of pMIR-FoxO3-3’-UTR but not of the mutated reporter pMIR-FoxO3-Δ3’-UTR with a scrambled miR-182 site (Fig. 1A). On the basis of these results, we hypothesized that miR-182 regulates FoxO3 expression in muscle cells and tested this possibility by measuring the levels of FoxO3 mRNA and protein in cells transfected with the miR-182 precursor. miR-182 significantly reduced FoxO3 mRNA by 30% (P < 0.05) (Fig. 1B) and FoxO3 protein by 67% (P < 0.05) (Fig. 1C). Efficient transfection of the miR-182 precursor was verified using a Cy3 labeled mi-
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miR-182 regulates FoxO3 in skeletal muscle cells. A: C2C12 cells were cotransfected with either pMIR-FoxO3-3’-untranslated region (UTR) or pFoxO3-Δ3’-UTR luciferase reporter plasmids and pre-miR miR-182 or control (Con) microRNA precursors as described in METHODS. Luciferase activity is expressed as the percentage of mean luciferase activity for each plasmid with the control microRNA. Shown in the inset is the FoxO3 3’-UTR sequence that contains the more proximal miR-182 interaction site. B, C, and E: C2C12 cells were transfected with either pre-miR miR-182 or control pre-miR microRNA precursors. After 72 h, endogenous FoxO3 mRNA (B) and protein (C) were quantified. D: fluorescent microscopy image of C2C12 cells transfected with a Cy3 dye-labeled precursor miRNA to demonstrate transfection efficiency. E: levels of miR-182 were measured in C2C12 cells that were transfected with either pre-miR miR-182 or control microRNA precursors. In all graphs, results are reported as means ± SE. *P < 0.05 vs. control (n = 3).

croRNA precursor (Fig. 1D), and by confirming an increase in the level of intracellular miR-182 by qPCR (Fig. 1E).

miR-182 is decreased during muscle atrophy. As noted earlier, increased FoxO3 expression and activity is frequently linked to atrophy-inducing conditions, leading us to hypothesize that miR-182 is reduced under these conditions (16, 32, 37, 45). To test this hypothesis, we treated C2C12 myotubes with Dex, a synthetic glucocorticoid, which induces atrophy by increasing FoxO3 activity in C2C12 myotubes (32, 37). After 6 h, FoxO3 mRNA was increased 133% (P < 0.05) (Fig. 2A). Simultaneously, the amount of intracellular miR-182 decreased 44% (P < 0.05) (Fig. 2B).

To further test this hypothesis, we examined if there is a similar relationship between FoxO3 and miR-182 expression in

Fig. 1. MicroRNA-182 (miR-182) regulates FoxO3 in skeletal muscle cells. A: C2C12 cells were cotransfected with either pMIR-FoxO3-3’-untranslated region (UTR) or pFoxO3-Δ3’-UTR luciferase reporter plasmids and pre-miR miR-182 or control (Con) microRNA precursors as described in METHODS. Luciferase activity is expressed as the percentage of mean luciferase activity for each plasmid with the control microRNA. Shown in the inset is the FoxO3 3’-UTR sequence that contains the more proximal miR-182 interaction site. B, C, and E: C2C12 cells were transfected with either pre-miR miR-182 or control pre-miR microRNA precursors. After 72 h, endogenous FoxO3 mRNA (B) and protein (C) were quantified. D: fluorescent microscopy image of C2C12 cells transfected with a Cy3 dye-labeled precursor miRNA to demonstrate transfection efficiency. E: levels of miR-182 were measured in C2C12 cells that were transfected with either pre-miR miR-182 or control microRNA precursors. In all graphs, results are reported as means ± SE. *P < 0.05 vs. control (n = 3).

Fig. 2. Changes in miR-182 and FoxO3 expression during atrophy. FoxO3 mRNA (A and C) and miR-182 (B and D) were measured in either C2C12 myotubes treated with or without dexamethasone (Dex; 1 μM) for 6 h (n = 3; A and B) or gastrocnemius muscles of control or streptozotocin (STZ)-injected, acutely diabetic rats (n = 6 and n = 4, respectively; C and D), 3d, 3 Days. In all graphs, results are reported as means ± SE of the fold change of the respective control values. *P < 0.05 vs. control.
vivo in skeletal muscle during atrophy. Previously, we reported that STZ-induced diabetes mellitus causes muscle atrophy and responses (e.g., increased atrogin-1 expression) that are consistent with FoxO3 activation (11, 22, 45). Therefore, we evaluated FoxO3 expression during STZ-induced diabetes mellitus by measuring FoxO3 mRNA and it was increased 75% \( (P < 0.05) \) (Fig. 2D) whereas the level of miR-182 was decreased 43% \( (P < 0.05) \) (Fig. 2D). These results confirm that a similar inverse relationship exists between FoxO3 and miR-182 in vitro in cultured muscle cells and in vivo in response to atrophy-inducing conditions.

**miR-182 can prevent atrophy-associated gene expression.** Atrogin-1 is a confirmed FoxO3 target gene that is typically increased during skeletal muscle atrophy (35, 36) and FoxO3 alone is able to increase atrogin-1 transcription (31, 32). We therefore reasoned that if miR-182 antagonizes FoxO3 expression, and thus its function, then miR-182 should prevent the Dex-induced increase in atrogin-1 mRNA. As predicted, increasing miR-182 in myotubes prevented an increase in atrogin-1 expression by Dex (Fig. 3A). Furthermore, miR-182 overexpression blocked the induction of LC3, ATG12, and cathepsin-L, three other FoxO3 targets associated with the autophagy/lysosome system (Fig. 3, B–D) (16, 30, 44).

**Dex increases miR-182 in exosomes released from myotubes.** MicroRNAs are present in both tissues and biological fluids (1). They have been shown to be stable even under extreme conditions such as multiple freeze-thaw cycles, long-term room temperature storage, drastic alterations in pH, and RNase activity (19, 38). One mechanism by which microRNAs are protected from degradation is through incorporation into protective exosomes (40). This has led some to propose that the relative abundance patterns of microRNAs in various tissues and fluids as well as in exosomes and other microvesicles are a reflection of underlying pathophysiological processes (1). We recently found that Dex enhanced the exosomal packaging and release of two atrophy-related microRNAs (miR-1 and miR-23a) into the media of C2C12 myotubes (9). This led us to examine whether miR-182 is similarly incorporated into released myotube exosomes. Consistent with our previous findings, the amount of miR-182 in C2C12 myotube media exosomes, normalized to U6 small nuclear RNA, was increased 95% \( (P < 0.05) \) following Dex treatment (Fig. 4).

**DISCUSSION**

Skeletal muscle atrophy is a debilitating response to a variety of conditions and disease states. Activation of FoxO3 is a key aspect of the atrophy process. In models of denervation and cancer, inhibition of FoxO3 activity prevents muscle loss (16, 24, 31, 44, 45). Recent studies demonstrate that microRNAs, by way of their ability to selectively regulate the expression of proteins, play a central role in the pathologies of many human

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**Fig. 3.** miR-182 prevents Dex-induced changes in FoxO3 gene targets. C2C12 cells were transfected with either pre-miR miR-182 or control microRNA precursors. After 72 h, some cells were pretreated with Dex (1 \( \mu \)M) for 6 h before measurement of mRNAs for atrogin-1 (A), microtubule-associated protein light chain 3 (LC3; B), autophagy-related protein 12 (ATG12; C), and cathepsin L (D). Results are reported as means ± SE of the fold change of the respective control values. \( *P < 0.05 \) vs. control; \( #P < 0.05 \) vs. both control and Dex \( (n = 3) \).

**Fig. 4.** Dex increases miR-182 in media exosomes from C2C12 myotubes. Fresh differentiation medium containing 100 nM Dex or vehicle was added to myotubes for 6 h. Afterwards, RNA was prepared from isolated media exosomes as described in METHODS; miR-182 and small nuclear U6 RNA (control) were measured by quantitative real-time PCR. Results are reported as means ± SE of the fold change relative to the mean untreated control value. \( *P < 0.05 \) vs. control \( (n = 3) \).
diseases including muscle disorders (5, 27). For example, we recently found that miR-23a, which targets atrogin-1 and MuRF1, is decreased by diabetes or glucocorticoids (9) while Wada et al. (42) reported that ectopic expression of miR-23a prevents glucocorticoid-induced muscle atrophy. miR-1 also impacts the atrophy process by indirectly inhibiting FoxO3 via regulation of HSP70 and consequently, Akt activity (12).

On the basis of our in silico target analysis, a number of microRNAs may target FoxO3. miR-182 was chosen for detailed investigation because there are two predicted binding sites in the 3′-UTR of FoxO3. Evidence is presented that miR-182 directly targets FoxO3 via binding to at least one of these sites (bases 73–80 of the 3′-UTR) and that increasing the level of miR-182 decreases both FoxO3 mRNA and protein in C2C12 myotubes. Next, we found that miR-182 is decreased in muscle during diabetes-induced atrophy and in cultured myotubes by glucocorticoid administration. Transfection of miR-182 prevents the glucocorticoid-induced increase in FoxO3-responsive genes that are necessary for increased activity of the ubiquitin-proteasome and autophagy/lysosomal systems in C2C12 myotubes. These findings are in agreement with microRNA array data indicating that miR-182 is decreased in skeletal muscle from type 2 diabetic rats and humans (10). They are also consistent with a report by Segura et al. (34) that miR-182 represses the FoxO proteins, including FoxO3, in melanoma cells. Although miR-182 is less abundant than other microRNAs highly enriched in skeletal muscle (e.g., miR-1), our data indicate that miR-182 expression is altered during muscle atrophy and contributes to the regulation of FoxO3 during this process. We suggest that changes in miR-182, likely along with other microRNAs, contribute to the regulation of FoxO3 in skeletal muscle during chronic diseases (e.g., diabetes, chronic kidney disease) that are associated with elevated glucocorticoid production.

Recently, we reported that two microRNAs involved in the atrophy process, miR-1 and miR-23a, were highly enriched in exosomes isolated from the culture media of myotubes undergoing Dex-induced atrophy (9). This finding is notable because there were opposing changes in their intracellular levels; Dex increased intracellular miR-1 while miR-23a was decreased. Moreover, the process that is stimulated by Dex involves exosome packaging since the steroid did not cause an increase in media exosome number. The present study adds to our data indicating that miR-182 is decreased in skeletal muscle (e.g., miR-1), our data

ACKNOWLEDGMENTS

The authors thank Sara Zoromsky for technical assistance and Dr. Charles Searles for thoughtful scientific advice.

GRANTS

This work was supported by National Institutes of Health (NIH) National Institute of Diabetes and Digestive and Kidney Diseases Grants T32 DK-007656 and RO1 DK-095610 (to S. R. Price), American Heart Association Grant GRNT7660020 (to S. R. Price), and Veterans Affairs Grant X01BX001456 (to S. R. Price).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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