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

Matthew B. Hudson,1 Jill A. Rahnert,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

Hudson MB, Rahnert JA, Zheng B, Woodworth-Hobbs ME, Franch HA, Price SR. miR-182 attenuates atrophy-related gene expression by targeting FoxO3 in skeletal muscle. Am J Physiol Cell Physiol 307: C314–C319, 2014. First published May 28, 2014; doi:10.1152/ajpcell.00395.2013.—Skeletal muscle atrophy occurs in a variety of 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.

microRNA; atrophy; FoxO3; muscle; glucocorticoids

SKELETAL MUSCLE ATROPHY occurs in many conditions including mechanical ventilation, chronic kidney disease, cancer cachexia, disuse, cast immobilization, and glucocorticoid-induced insulin resistance (7, 8, 15, 20, 23, 24, 39). It decreases the quality of life for patients and increases their risk of mortality. Atrophy typically results from activation of the ubiquitin-proteasome (13, 14, 29) and autophagy-lysosome systems, leading to the degradation of muscle proteins (16). A common feature of muscle atrophy includes activation of the forkhead box O (FoxO) family of transcription factors of which FoxO1, FoxO3, and FoxO4 are the three major FoxO proteins in skeletal muscle (33). Evidence indicates that both FoxO1 and FoxO3 have roles in various models of muscle atrophy. Overexpression of either FoxO1 or FoxO3 produces skeletal muscle atrophy in vivo, and reducing FoxO activity attenuates glucocorticoid, cachexia, and disuse-induced muscle atrophy (25, 32, 35). In particular, work over the past decade has identified a key role for FoxO3 during atrophy including the coordination of the activities of the proteasomal and autophagic/lysosomal systems through increased expression of key component genes (21, 31–33, 35–37, 44, 45). Prevailing evidence indicates that the level and activity of FoxO3 is tightly controlled in a variety of ways that involve phosphorylation, acetylation, ubiquitination, and protein interactions (4, 41). Given its role in the pathogenesis of skeletal muscle atrophy, it is important to gain a thorough understanding of the mechanisms that control FoxO3 function.

Recent studies have demonstrated that microRNAs can have key regulatory roles in a variety of pathological conditions (12, 27, 28, 42, 43). MicroRNAs are a class of short, noncoding RNAs that alter the levels of specific proteins by inhibiting the translation of specific mRNA or promoting mRNA degradation (2). Over 500 unique microRNAs have been reported to date, and many of them are evolutionarily conserved (3). Recent work indicates that microRNAs participate in the regulation of muscle atrophy, thereby adding another layer of control to a process that already involves several mechanisms. For example, microRNA-1 or miR-1, a muscle-specific microRNA, is increased during dexamethasone (Dex)-induced muscle atrophy (12). miR-1 indirectly increases FoxO3 activity by targeting heat shock protein 70 (HSP70), leading to a reduction in the phosphorylation of Akt and subsequent upregulation of FoxO3 activity. Wada et al. (42) reported that miR-23a suppresses the translation of muscle RING-finger protein-1 (MuRF1) and atrogin-1 mRNAs, and we recently found that miR-23a is suppressed during diabetes and Dex-induced muscle atrophy (9). The mechanisms by which Dex reduces miR-23a involves suppression of calcineurin activity and increased packaging and release in exosomes (9).

Considering the central role of FoxO3 in the pathogenesis of skeletal muscle atrophy, identification of microRNA(s) that target FoxO3 could be a significant advancement in the atrophy field. Therefore, the objectives of this study were to identify a microRNA that targets FoxO3 in skeletal muscle and to determine whether it impacts the functional role of FoxO3 in the atrophy process. On the basis of a preliminary in silico analysis, we hypothesized that miR-182 targets FoxO3 in skeletal muscle and that its level is reduced by conditions that induce muscle atrophy.1

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 miR 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 for fold change (Ct) using the iCycler software. Melting curve analysis was performed 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 expression in skeletal muscle.** 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 miRNA.
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. 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
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 understanding because there are two predicted binding sites in the 3′-UTR of FoxO3, which 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 earlier findings by demonstrating that, similar to miR-23a, Dex decreases intracellular miR-182 and increases the incorporation of miR-182 into exosomes released from muscle cells. The data further support the hypothesis that intracellular microRNA levels are regulated through selective packaging of microRNAs into exosomes followed by their release from muscle cells.

The importance of our current findings is underscored by the central role that FoxO3 has in controlling the expression of key components of the ubiquitin-proteasome and autophagy-lysosomal systems. Consequently, several regulatory mechanisms have been identified that exert control over FoxO3 activity. Our results contribute to the growing evidence that a number of microRNAs, including miR-182, represent a posttranscriptional mechanism of regulation by which the muscle atrophy process is controlled during a variety of diseases and catabolic conditions.

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-076565 and R01 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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