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Original Research

Differential Expression of Mature MicroRNAs Involved in Muscle Maintenance of Hibernating Little Brown Bats, *Myotis lucifugus*: A Model of Muscle Atrophy Resistance

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Abstract

Muscle wasting is common in mammals during extended periods of immobility. However, many small hibernating mammals manage to avoid muscle atrophy despite remaining stationary for long periods during hibernation. Recent research has highlighted roles for short non-coding microRNAs (miRNAs) in the regulation of stress tolerance. We proposed that they could also play an important role in muscle maintenance during hibernation. To explore this possibility, a group of 10 miRNAs known to be normally expressed in skeletal muscle of non-hibernating mammals were analyzed by RT-PCR in hibernating little brown bats, *Myotis lucifugus*. We then compared the expression of these miRNAs in euthermic control bats and bats in torpor. Our results showed that compared to euthermic controls, significant, albeit modest (1.2–1.6 fold), increases in transcript expression were observed for eight mature miRNAs, including miR-1a-1, miR-29b, miR-181b, miR-15a, miR-20a, miR-206 and miR-128-1, in the pectoral muscle of torpid bats. Conversely, expression of miR-21 decreased by 80% during torpor, while expression of miR-107 remained unaffected. Interestingly, these miRNAs have been either validated or predicted to affect multiple muscle-specific factors, including myostatin, FoxO3a, HDAC4 and SMAD7, and are likely involved in the preservation of pectoral muscle mass and functionality during bat hibernation.

Keywords: MicroRNA; Hibernation; Metabolic rate depression; Atrophy; Dicer; Myostatin

Introduction

Skeletal muscle tissue differentiates early in vertebrate embryogenesis and requires the combinatorial actions of multiple signaling pathways. The mechanisms of maintaining and remodeling skeletal muscle are of particular interest, since skeletal muscle tissue is capable of adapting to the changing physiological and environmental conditions [1]. For example, hypertrophy of skeletal muscle is induced through increased muscle use and results in an enlarged size, augmented ability to produce force, increased resistance to fatigue and enhanced oxidative metabolism [1,2]. However, prolonged disuse or immobility of skeletal muscle, such as during prolonged bed rest, various disease states or age-related sarcopenia, typically results in the opposite effect, *i.e.*, atrophy of muscle [2]. Several molecular pathways involved in the development and maintenance of skeletal muscle mass have been well characterized. Major signaling pathways include the phosphoinositol 3-kinase (PI3-K)/Akt/mammalian target of rapamycin (mTOR) pathway, the enzyme histone deacetylase 4 (HDAC4), the secreted protein myostatin and its associated membrane receptor (Acvr2b), as well as muscle-specific E3 ubiquitin ligases tripartite motif-containing protein 63 (TRIM63) and muscle atrophy F-box (Atrogin-1) [3–5] (Figure S1). Transcription factors including the forkhead box O (FoxO3a), myogenin, myogenic differentiation 1 (MyoD), myogenic factor (Myf5), myogenic regulatory factor 4 (Mrf4), and myocyte enhancer factor 2 (MEF2A) are also crucial [2,4,6]. Development of

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skeletal muscle during embryogenesis, maintenance of adult skeletal muscle, and any changes leading to hypertrophy or atrophy typically fall under the control of one or more of these biological factors.

With the discovery of microRNAs (miRNAs), the small non-coding RNAs capable of regulating protein expression within a cell, additional post-transcriptional modes of regulation in skeletal muscle are being elucidated. With the aid of the miRNA-initiated silencing complex (miRISC), these 17–22 nucleotide transcripts are able to bind with full or partial complementarity usually to the 3' UTR of mRNA targets [7–9]. Target binding either inhibits translation or leads to mRNA degradation [7–9]. This form of gene expression control at the post-transcriptional level has been shown to be of great importance in mammalian systems; to date, miRNAs are known to be critically involved in biological development, cell differentiation, apoptosis, cell-cycle control, stress response and disease pathogenesis [7,10–12].

A collection of miRNAs have been shown to be regulated during periods of skeletal muscle atrophy; these include miR-1a-1, miR-29b, miR-181b, miR-15a, miR-20a, miR-206, miR-128-1, miR-21, miR-23a and miR-107 [6,13,14]. Target validation has illustrated some ties, both direct and indirect, between these miRNAs and the host of muscle-specific effects of the aforementioned factors (Figure S1). Thus, miRNAs are critically involved in development, maintenance and adaptation of skeletal muscle [6]. In order to further characterize the roles of miRNAs in muscle metabolism, we turned to an unusual model of mammalian muscle metabolism: hibernation. Many small mammals use hibernation to survive over the winter months when food is scarce and cold environmental temperatures place high demands on thermogenesis if a high constant body temperature (Tb) is to be maintained. For example, body fat reserves of hibernating little brown bats, *Myotis lucifugus*, would last only about 25 days if the animals maintained euthermia but by entering prolonged periods of torpor during which Tb falls to near ambient, the bats readily endure a 7–9 month hibernation season without eating [15]. Indeed, *M. lucifugus* may remain in constant torpor for several weeks at a time during the hibernation season. A recent study reported torpor bout durations of 2–7 weeks for free-ranging little brown bats (interspersed with short arousals back to euthermia) [16]. Much is known about the physiology and biochemistry of hibernation in this species (*e.g.*, [16,17] and references therein). Most mammals that undergo long periods of immobility display significant disuse atrophy of their skeletal muscles [2]. However, hibernators, including *M. lucifugus*, appear to avoid this negative outcome [18] despite spending long weeks in torpor during the winter. Selected molecular mechanisms are clearly at work to preserve both skeletal muscle mass and functionality during hibernation.

The present study was undertaken to evaluate the responses of aforementioned muscle-associated miRNAs in the skeletal muscle metabolism of *M. lucifugus* during hibernation. RT-PCR was employed to investigate expression of these miRNAs in the pectoral muscle that powers flight in bats. The differential expression of several miRNAs was identified during hibernation and suggests an important role for these non-coding RNAs in the maintenance of skeletal muscle. The protein expression of several important miRNA targets (Myostatin, FoxO3a, HDAC4 and SMAD7) were also analyzed by immunoblotting. The data also provide intriguing leads for therapeutic targets that could be investigated to or avoid muscle disuse atrophy in humans. Furthermore, presence of all 10 miRNAs in bats, which until now had only been predicted algorithmically from genome searches, was verified by sequencing Sequences of these miRNAs were identical to those of mouse, indicating that the miRNA sequences in a member of the Chiroptera are highly conserved with those of a rodent, the mouse (*Mus musculus*).

Results

MicroRNA expression during hibernation

To amplify the selected miRNAs, we used a modified stem-loop procedure outlined by Biggar et al. [19]. This protocol allows both amplification and sequencing of mature miRNAs in organisms that have no previous miRNA annotation. Our results show that the uniform FO fiber composition of *M. lucifugus* pectoralis muscle does not change with season, consistent with previous report [20]. We therefore examined the miRNA expression in the pectoral muscle of *M. lucifugus*. Expression levels of miR-1a-1, miR-29b, miR-181b, miR-15a, miR-20a, miR-206, miR-128-1, miR-21, miR-23a and miR-107 in the pectoral muscle of *M. lucifugus* were assessed by RT-PCR and all miRNA PCR products were confirmed by sequencing. The sequences of all mature miRNAs investigated were 100% identical with the known mature miRNA sequences from the house mouse, *M. musculus*.

We further compared the expression of these miRNAs in hibernating bats (Tb $\sim$ 5–6 °C) and aroused euthermic bats (Tb $\sim$ 35–37 °C). Eight miRNAs were significantly upregulated in muscle from torpid bats as compared to euthermic controls ($P < 0.05$) (**Figure 1**). For example, expression of miR-29b increased by 1.2 fold (1.2 ± 0.05) and that of miR-23a increased by 1.63-fold (1.63 ± 0.07). However, expression of miR-21 decreased substantially during torpor, which corresponded to only 22% of the expression in control ($22 \pm 2\%$; $P < 0.05$), whereas expression levels of miR-107 (1.00 ± 0.02) didn't change significantly with hibernation.

Putative miRNA targets of the differentially-expressed miRNAs

Targets of these differentially-expressed miRNAs were analyzed based on the available literature regarding these