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ORIGINAL RESEARCH

Expression Profiling and Structural Characterization of MicroRNAs in Adipose Tissues of Hibernating Ground Squirrels

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Abstract MicroRNAs (miRNAs) are small non-coding RNAs that are important in regulating metabolic stress. In this study, we determined the expression and structural characteristics of 20 miRNAs in brown (BAT) and white adipose tissue (WAT) during torpor in thirteen-lined ground squirrels. Using a modified stem-loop technique, we found that during torpor, expression of six miRNAs including let-7a, let-7b, miR-107, miR-150, miR-222 and miR-31 was significantly downregulated in WAT ($P < 0.05$), which was 16%–54% of euthermic non-torpid control squirrels, whereas expression of three miRNAs including miR-143, miR-200a and miR-519d was found to be upregulated by 1.32–2.34-fold. Similarly, expression of more miRNAs was downregulated in BAT during torpor. We detected reduced expression of 6 miRNAs including miR-103a, miR-107, miR-125b, miR-21, miR-221 and miR-31 (48%–70% of control), while only expression of miR-138 was significantly upregulated (2.91 ± 0.8-fold of the control, $P < 0.05$). Interestingly, miRNAs found to be downregulated in WAT during torpor were similar to those dysregulated in obese humans for increased adipogenesis, whereas miRNAs with altered expression in BAT during torpor were linked to mitochondrial β-oxidation. miRPath target prediction analysis showed that miRNAs downregulated in both WAT and BAT were associated with the regulation of mitogen-activated protein kinase (MAPK) signaling, while the miRNAs upregulated in WAT were linked to transforming growth factor β (TGFβ) signaling. Compared to mouse sequences, no unique nucleotide substitutions within the stem-loop region were discovered for the associated pre-miRNAs for the miRNAs used in this study, suggesting no structure-influenced changes in pre-miRNA processing efficiency in the squirrel. As well, the expression of miRNA processing

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enzyme Dicer remained unchanged in both tissues during torpor. Overall, our findings suggest that changes of miRNA expression in adipose tissues may be linked to distinct biological roles in WAT and BAT during hibernation and may involve the regulation of signaling cascades.

Introduction

Mammalian hibernation is a natural phenotype that involves the re-programing of metabolic function in response to changes in animals' surrounding environment. Hibernators are able to undergo extreme depression of their metabolic rate (< 5% euthermic values), which is characterized by reduced body temperature (from ~37 °C to ~5 °C), heart rate (from 200–300 to < 5 beats/min), respiration (from 100–300 to 4–6 breaths/min) and brain activity. However, all physiological and metabolic adaptations can be reversed upon arousal [1–4]. Programed increase in body weight perhaps is one of the most fascinating changes for hibernators [5]. During the fall, hibernators undergo periods of hyperphagia that can increase their total body mass by ~40% in males and ~60% in females, primarily via accumulation of triglycerides in the white adipose tissue (WAT) [5]. This increase in fat storage functions as the primary source for metabolic fuel during hibernation, as evident by a respiratory quotient value (*i.e.*, measurement of basal metabolic rate determined by $CO_{2\ \text{eliminated}}/O_{2\ \text{consumed}}$) of 0.70 during torpor, which indicated the exclusive fat catabolism of the metabolically depressed state (where a value of 1.0 would represent pure carbohydrate oxidation) [6]. In addition to white adipose storage, hibernators also experience a substantial increase in brown adipose tissue (BAT) mass during torpor. However, BAT is functionally distinguished from WAT, which acts to dissipate energy through production of heat and gets involved in regulating adaptive thermogenesis during hibernation cycle [7,8]. The major role of BAT in hibernators is to provide non-shivering thermogenesis (NST) during periods of torpor. The mitochondria in BAT are able to uncouple the electron transport chain and disrupt oxidative phosphorylation via uncoupling proteins (UCPs), resulting in the generation of heat that is used to provide NST [9].

A major driving force in understanding the mechanisms underlying 'mammalian life in the cold' is the potential for medical applications of cryopreservation, which would allow prolonged storage of human organs for transplantation. Less well known are the implications of hibernation to many metabolic diseases, by comparing the known molecular changes in hibernators to those observed in obese and diabetic phenotypes in humans [10]. For example, the observed increase in lipid accumulation in hibernators is coupled with periods of insulin resistance of the adipose tissues, which contributes to the development of an obesity-like phenotype that is characterized by hyperinsulinemia and increased adipocyte diameter [11,12]. Interestingly, this obese state and periods of insulin resistance are reversed at the end of the hibernation season [11,12].

MicroRNAs (miRNAs) are short (18–25 nt) non-coding single-stranded RNA molecules that function to post-transcriptionally regulate gene expression [13]. miRNAs are initially transcribed as primary transcripts that contain secondary hairpin structures, which are subsequently processed by a class 2 RNase III enzyme Drosha into precursor miRNAs (pre-miRNAs). The pre-miRNA is then exported out of the nucleus, and is further processed into mature miRNAs in the cytoplasm by another RNase III enzyme called Dicer [14]. Mature miRNAs have been estimated to regulate at least 60% of human protein-coding genes [15]. Not surprisingly, recent findings have linked miRNA dysregulation to many metabolic diseases such as obesity and diabetes [16,17]. In adipose tissues, miRNAs have been reported to both accelerate and suppress the rate of adipocyte differentiation, implicating multiple roles for miRNAs in fat cell development [18].

As it is vitally important to rapidly and readily reduce the activity of ATP-costly processes in a coordinated and reversible fashion during hibernation, miRNAs may aid in the reprioritization of ATP use and stress-specific cellular adaptation [13]. In this regard, it is possible that miRNAs act with a rapid and reversible mechanism to dynamically regulate critical cellular processes. In this study, we used the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) as a well-studied model with a known genome and known roles for miRNA during hibernation [13]. To better understand the regulatory mechanisms of adipose metabolism in hibernators, we chose to characterize 20 miRNAs in ground squirrels during torpor that have been shown to be altered in human obesity.

This present study provides a comparative analysis of the expression and characterization of adipose-associated miRNAs in hibernation. A stem-loop miRNA amplification protocol was employed to measure the expression of these miRNAs in both WAT and BAT of euthermic and torpid ground squirrels. As it has been previously reported that pre-miRNA can contain unique nucleotide substitutions that influence secondary structure to promote low-temperature mature miRNA processing [19], we also analyzed the pre-miRNA sequence and secondary structure to identify any potential nucleotide substitutions that are unique to hibernators.

Results

Structural characterization of squirrel miRNAs

We searched the *I. tridecemlineatus* genome (SpeTri2.0 squirrel assembly) against *Homo sapiens* and *Mus musculus* precursor sequences in miRbase. A total of 20 miRNAs were selected based on their roles in human adipogenesis and obesity. These pre-miRNAs showed high sequence homology with their counterparts in *H. sapiens* and *M. musculus*, which are 89.70% ± 2.91% and 97.58% ± 0.36% on average, respectively (Table S1). The genomic miRNA-coding regions from *I. tridecemlineatus* were also identified using BLAST (Table S2). The sequence length, base composition (GC%, AU%, U/A ratio and G/C ratio) and minimal free energy (MFE) are important features in identifying pre-miRNAs, so the structural and thermodynamic characteristics of the *I. tridecemlineatus* pre-miRNA were analyzed (**Table 1**). The 20 *I. tridecemlineatus* pre-miRNAs identified in our study folded into stem-loop structures that fit expected structural characteristics (average of 51.9% GC, 0.96 U/A ratio and 1.13 G/C