

ORIGINAL RESEARCH

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



Cheng-Wei Wu, Kyle K. Biggar [§], Kenneth B. Storey ^{*}

Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada

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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 (TGF β) 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

^{*} Corresponding author.

E-mail: kenneth_storey@carleton.ca (Storey KB).

[§] Current address: Biochemistry Department, Schulich School of Medicine and Dentistry, Western University, London, ON N6A 5C1, Canada.

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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 eliminated/ O_2 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 Droscha 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 re-prioritization 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 $89.70\% \pm 2.91\%$ and $97.58\% \pm 0.36\%$ 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

Table 1 Thermodynamic and structural characteristics of pre-miRNAs in *I. tridecemlineatus*

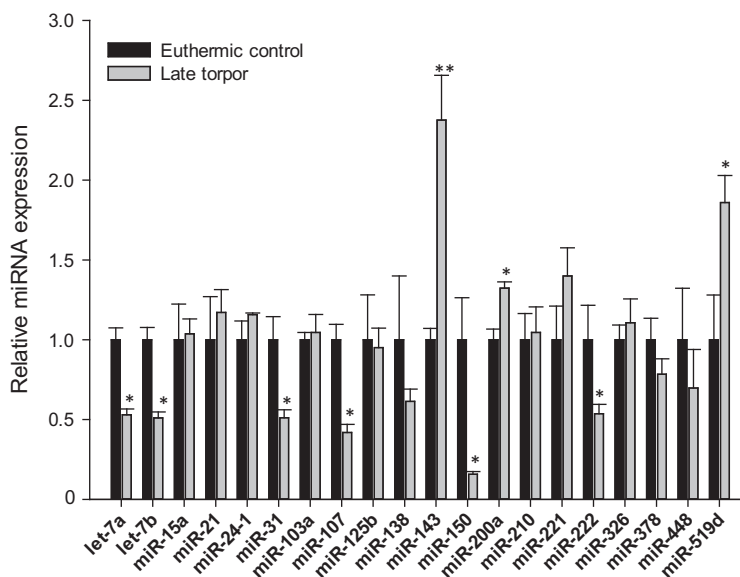
miRNA	Length	MFE (kcal/mol)	GC (%)	AU (%)	U/A ratio	G/C ratio
itr-let-7a-1	94	-43.2	42.6	57.4	1.35	0.74
itr-let-7b	82	-44.7	50.0	50.0	1.00	1.00
itr-mir-15a	84	-29.4	41.7	58.3	1.40	0.72
itr-mir-21a	92	-42.7	45.7	54.3	1.19	0.84
itr-mir-24-1	68	-26.3	50.0	50.0	1.00	1.00
itr-mir-31	105	-44.5	51.4	48.6	0.95	1.06
itr-mir-103-1	86	-35.6	51.2	48.8	0.95	1.05
itr-mir-107	87	-33.3	46.0	54.0	1.17	0.85
itr-mir-125b-1	77	-29.3	51.9	48.1	0.93	1.08
itr-mir-138-1	84	-37.7	57.1	42.9	0.75	1.33
itr-mir-143	63	-36.6	55.6	44.4	0.80	1.25
itr-mir-150	59	-26.0	61.0	39.0	0.64	1.56
itr-mir-200a	90	-41.4	51.1	48.9	0.96	1.04
itr-mir-210	100	-43.8	69.0	31.0	0.45	2.23
itr-mir-221	95	-42.9	45.3	54.7	1.21	0.83
itr-mir-222	79	-38.7	50.6	49.4	0.98	1.02
itr-mir-326	88	-48.8	65.9	34.1	0.52	1.93
itr-mir-378a	97	-27.9	50.5	49.5	0.98	1.02
itr-mir-448	107	-42.4	53.3	46.7	0.88	1.14
itr-mir-519d	58	-25.2	48.3	51.7	1.07	0.93

ratio) to the known pre-miRNAs [20], displaying MFE values of -25.2 to -48.8 kcal/mol (Table 1; Figure S1).

miRNA expression during torpor

To identify potential regulatory roles of the 20 selected miRNAs in adipose metabolism of hibernating ground squirrels, we used a modified miRNA stem-loop amplification technique to evaluate the expression of the selected miRNAs. In WAT, we identified three miRNAs that showed significantly upregulated expression during torpor, including miR-143

(2.34 ± 0.28 -fold, $P < 0.005$), miR-200a (1.32 ± 0.04 -fold, $P < 0.05$) and miR-519d (1.86 ± 0.17 -fold, $P < 0.05$) (Figure 1). Meanwhile, expression of six miRNAs was significantly downregulated during torpor. These include let-7a, let-7b, miR-31, miR-107, miR-150 and miR-222 ($P < 0.05$; Figure 1). Among them, expression of miR-150 was reduced the most, which is only $16\% \pm 2\%$ of the euthermic state, whereas about 50% reduction was observed for the other 5 miRNAs. We did not observe significant alteration in the expression of the remaining 11 miRNAs such as miR-103a during torpor, albeit modest variations exist.

**Figure 1** miRNA expression in white adipose tissue during torpor

Expression of miRNAs in white adipose tissue samples from the euthermic control (black bar) and torpid ground squirrels (gray bar) was evaluated by RT-PCR. Relative expression of indicated miRNAs was normalized to the expression of 5S rRNA from the same sample. The relative miRNA expression in the torpid animals was further normalized to that in the euthermic control, which was arbitrarily set as 1.0. Data are mean \pm SEM ($n = 4-5$ independent trials from different animals). Significant difference in miRNA expression in torpid compared to that of the euthermic control according to Student's t -test was indicated with * ($P < 0.05$) or ** ($P < 0.005$).

Similarly, we examined the expression of these 20 miRNAs in BAT during torpor. As shown in **Figure 2**, miR-138 was the only miRNA with significantly-upregulated expression in BAT during torpor (2.91 ± 0.8 -fold, $P < 0.05$). On the other hand, we observed significantly-reduced expression for six miRNAs including miR-21, miR-31, miR-103a, miR-107, miR-125b and miR-221. However, expression of the affected miRNAs is reduced to a less extent in general compared to that of the miRNAs affected in WAT during torpor. For example, expression of miR-107 during torpor is $61\% \pm 6\%$ of the control in BAT ($P < 0.005$), compared to $42\% \pm 5\%$ observed in WAT ($P < 0.05$). No significant alteration in expression was observed in BAT for all three miRNAs that were significantly upregulated in WAT (miR-143, miR200a, or miR-519d; **Figures 1 and 2**). Among the significantly-downregulated miRNAs in BAT (miR-103a, miR-107, miR-125b, miR-21, miR-221 and miR-31), only miR-107 was downregulated in both tissues.

Dicer expression during torpor

Immunoblotting was performed to determine if the miRNA processing enzyme Dicer is regulated during torpor. The Dicer antibody detected a single band of approximately 200 kDa, which is the expected monomer size of the human Dicer protein. As seen in **Figure 3**, the relative expression of Dicer in both WAT and BAT did not change significantly between euthermic control and torpid squirrels.

Pathway enrichment of torpor-responsive miRNAs

To help determine the collective impact of the torpor-responsive miRNA, we used DIANA-miRPath to predict the cellular pathways targeted during torpor in WAT and BAT. miRPath

generates a list of predicted miRNA targets and defines for target enrichment using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. In both BAT and WAT, the top KEGG pathway found to be targeted by significantly-downregulated miRNAs during torpor was the mitogen-activated protein kinase (MAPK) signaling cascade, with putative targets of 36 and 45 genes, respectively (**Figures S2 and S3**, **Table S4**). Interestingly, the top signal transduction pathway targeted by the three miRNAs upregulated in WAT was the transforming growth factor β (TGF β) signaling pathway with 19 putative targets (**Figure S4 and Table S3**).

Discussion

Adipose tissues function as a storage depot for lipid molecules and act as a major endocrine signaling organ that is crucial for regulating whole-body energy homeostasis [21]. Several studies have shown a dependence on lipid as the primary source of metabolic fuel during torpor in several species of hibernators including bats, squirrels and lemurs [6,22,23]. Both WAT and BAT are specialized organs that are activated during metabolic depression, with transcriptional upregulation of transcription factors in peroxisome proliferator-activated receptor (PPAR) family [24]. Meanwhile, expression of both heart and adipose-specific fatty acid binding proteins has been shown to be upregulated in the BAT during torpor to promote fatty acid transport [24,25]. Although some similarities in gene expression patterns have been observed for the two adipose tissues during torpor, these two adipose tissues are distinct in terms of their biological roles. WAT primarily function as energy storage in the form of triglycerides, while BAT is involved in heat dissipation via non-shivering thermogenesis. In humans, the development of pre-adipocytes to mature

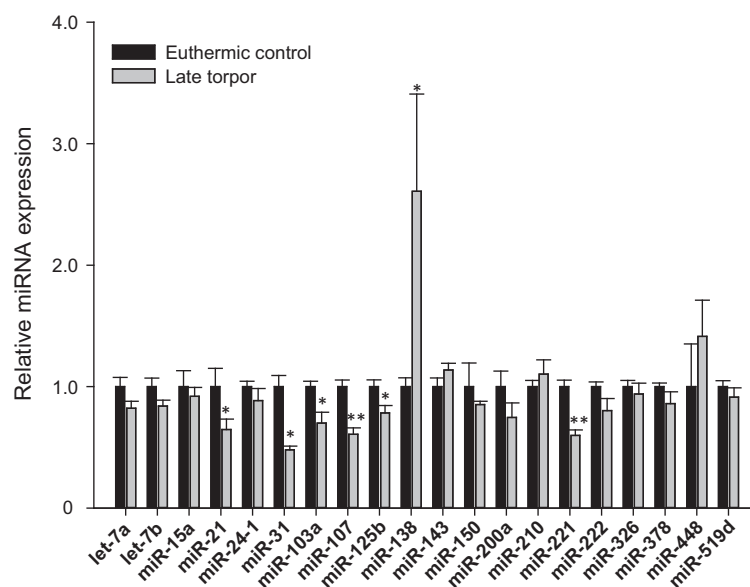


Figure 2 miRNA expression in brown adipose tissue during torpor

Expression of miRNAs in the brown adipose tissue samples from euthermic control (black bar) and torpid ground squirrels (gray bar) was evaluated by RT-PCR. Relative expression of indicated miRNAs was normalized to the expression of 5S rRNA from the same sample. The relative miRNA expression in the torpid animals was further normalized to that in the euthermic control, which was arbitrarily set as 1.0. Data are mean \pm SEM ($n = 4-5$ independent trials from different animals). Significant difference in miRNA expression in torpid compared to that of the euthermic control according to Student's t -test was indicated with * ($P < 0.05$) or ** ($P < 0.005$).

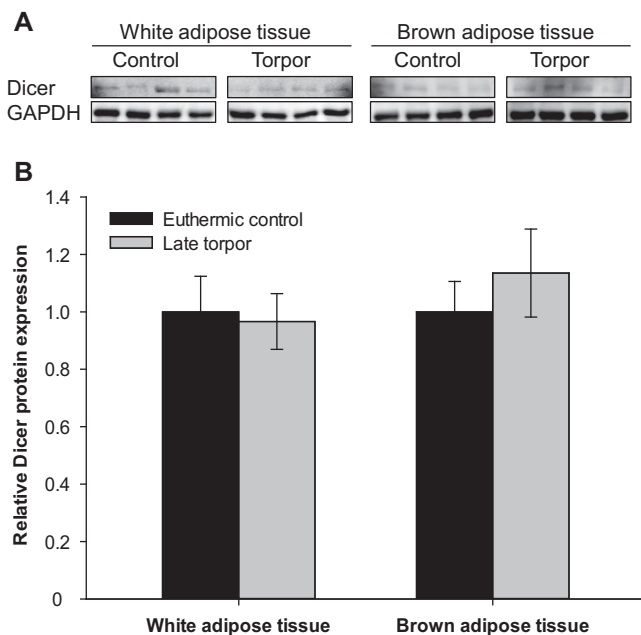


Figure 3 Dicer protein expression during torpor

A. Protein expression of Dicer in the white and brown adipose tissue samples from euthermic control (black bar) and torpid ground squirrels (gray bar) was examined using immunoblotting. **B.** Relative expression of Dicer was normalized to the expression of GAPDH from the same sample. The relative miRNA expression in the torpid animals was further normalized to that in the euthermic control, which was arbitrarily set as 1.0. Data are presented as mean \pm SEM ($n = 4$ independent trials from different animals). Statistical analysis was performed using Student's *t*-test.

adipocytes is a tightly regulated multi-step process [26]. When adipogenesis becomes dysregulated, aberrant accumulation of fat can lead to the onset of obesity, increasing the health-related risks of metabolic diseases such as diabetes, atherosclerosis, and cancer [27,28]. In recent years, with the revealed importance of miRNAs in many life processes, multiple study groups began to characterize miRNAs whose expression is dysregulated in obesity phenotypes (such as body composition and body fat amount) [18].

Our data show that during torpor, WAT and BAT both exhibit tissue-specific patterns of miRNA regulation and this could potentially be attributed to their distinct biological roles. Of the 20 miRNAs studied in WAT (Figure 1), we observed a significant decrease in the torpor-expression of six miRNAs, while significant torpor-responsive increase was observed for three miRNAs. Interestingly, several of the downregulated miRNAs during torpor have also been shown with decreased expression during adipogenesis in humans [29,30]. For example, let-7 has been shown to inhibit clonal expansion of pre-adipocytes through targeting the gene encoding high-mobility group AT-hook 2 (HMGA2). HMGA2 is a transcription factor that can promote adipogenesis and mice lacking HMGA2 exhibit significant reduction in adipose tissue [29,30]. In addition, the decreased expression of both miR-31 and miR-150 in WAT has also been linked to adipogenesis [31]. Overexpression of miR-31 has been shown to suppress adipogenic

markers including PPAR- γ , C/EBP and adipocyte protein-2 (AP2) [32], whereas miR-150 has recently been shown to directly target and repress PR domain-containing 16 (prdm16) and PPAR- γ -coactivator 1 α [31]. PPAR- γ function as a master regulator for adipogenic differentiation by promoting expression of downstream genes that are important for triglyceride uptake, including AP2, glucose transporter 4, lipoprotein lipase, phosphoenolpyruvate carboxykinase and more [33]. In addition, the upregulation of miR-143 in WAT was previously linked to promotion of adipose differentiation, while upregulation of miR-200a and miR-138 has been shown to be negative regulators of adipogenic differentiation [34–36]. The combination of elevated expression of miRNAs that accelerate adipogenesis along with a decrease in expression of miRNAs that inhibit such process would suggest that miRNAs could play a prominent role in WAT regulation during torpor.

When examining the miRNA regulation in BAT, results suggest a limited overlap in regulation patterns as compared to WAT. Interestingly, several miRNAs that were downregulated during torpor in BAT have been shown to negatively target genes in the mitochondria (miR-103 and miR-107). Silencing of miR-103 using antagomir-103 in mice led to an increased expression of genes involved in β -oxidation, including carnitine palmitoyltransferase 1A (cpt1a), peroxisomal acyl-coenzyme A oxidase (acox) and very long chain acyl-coenzyme A dehydrogenase. Meanwhile, miR-107 expression has been negatively correlated with the expression of mitochondria uncoupling protein 2 [37,38]. Recent studies have shown that expression of both cpt1a and acox is upregulated in squirrel during torpor, suggesting a potential role of miR-103 in regulation of β -oxidation [39,40]. The link between miR-103 and miR-107 to mitochondria oxidation is of particular interest to hibernation research, as the decrease in both of these miRNAs could be linked to the increased β -oxidation that is known to occur during torpor [41]. Although several miRNAs characterized in this study have each been previously experimentally validated to regulate adipogenesis in mice and humans, we used miRPath to predict possible cellular processes and signaling pathways that could be affected in a combinatorial manner by these torpor-responsive miRNA. Interestingly, although WAT and BAT showed distinct patterns in miRNA expression during torpor, all the miRNAs that were downregulated were predicted to target a large number of proteins in the MAPK signaling pathway, with 45 genes collectively targeted in WAT and 36 genes target in BAT. The decrease in expression of these miRNAs could suggest a reduced inhibitory effect on the expression of these genes in the MAPK signaling pathway during torpor. Recent studies have also found that MAPK signaling may be a predicted target for miRNAs altered in estivation-induced metabolic depression, suggesting MAPK signaling cascade as a prominent pathway targeted by miRNAs that are regulated during metabolic depression [42]. In addition, prediction of putative targets of miRNAs that were upregulated in WAT during torpor (miR-143, miR-200a and miR-519d) identified TGF β as the top candidate signaling pathway with 19 putative targets. In the adipose tissues, TGF β has been found to inhibit adipogenesis by repressing the transcriptional activity of C/EBP [43]. The upregulation of these three miRNAs along, with the down-regulation of miR-31 (negative regulator of C/EBP),

would suggest that the differential expression of miRNAs in WAT during torpor is likely to be aimed at promoting adipose tissue development.

Changes in RNA secondary structure have been shown to allow efficient precursor processing at the low temperatures, such as that experienced during torpor [19,44]. To identify potential structural mechanisms that regulate differential miRNA expression in torpor, we investigated the sequence and structural characteristic of the terminal stem-loop region of the squirrel miRNAs examined in this study. Previous studies have also found that single nucleotide substitutions of miR-29b at the stem-loop terminal region in freeze-tolerant Western painted turtle (*Chrysemys picta bellii*) lead to a more flexible stem-loop, which could be linked to its increased expression during freezing, suggesting miRNA nucleotide substitution as a potential mechanism to allow efficient processing at low temperature [19,44]. Of the 20 miRNAs we examined in this study, we were unable to identify unique nucleotide substitution of the miRNA at the terminal stem-loop region using BLAST analysis. In addition, no qualitative correlation was found between increased mature miRNA expression and any unique structural characteristics of its pre-miRNA when compared to homologous structures in *H. sapiens* or *M. musculus*. Moreover, we did not observe any changes in the protein expression of Dicer between euthermia and torpor. The mechanisms controlling differential expression of miRNAs during torpor remains to be determined, and future studies examining the transcription factors controlling the expression of these miRNAs would shed more light in their regulatory mechanism during torpor.

In conclusion, the present study measured the expression of 20 miRNAs in BAT and WAT during ground squirrel torpor and observed differential expressions of miRNAs between the two tissues. The pattern of miRNA expression in WAT is consistent with some of the previous findings of miRNA dysregulation in obese humans. Our findings suggest that although the difference in miRNA expression patterns are likely linked to the distinct biological roles between WAT and BAT, signaling cascades such as the MAPK pathway could be regulated in both tissues during torpor. In summary, our data suggest the involvement of miRNAs in the regulation of adipose metabolism during torpor.

Materials and methods

Identification of pre-miRNA sequences

We screened the *I. tridecemlineatus* genome (SpeTri2.0 Ref. assembly top-level) to select the pre-miRNA candidates using the BLASTn tool (available at ftp://ftp.ncbi.nlm.nih.gov/genomes/Ictidomys_tridecemlineatus). The query sequences used for the genome screening were conserved pre-miRNAs from *H. sapiens* and *M. musculus* (miRbase v.20). Once putative miRNA candidates were found, the respective sequences that matched were retrieved from the SpeTri2.0 assembly. For further analysis, a set of structural characteristics and thermodynamic parameters were selected and analyzed in the identified *I. tridecemlineatus* pre-miRNAs. These include length, minimal free energy (MFE), GC content, AU content, G/C ratio and A/U ratio. The MFE of the secondary structures was measured using mfold (available at <http://mfold.rna.albany.edu/?q=mfold>).

Animal treatment

The 13-lined ground squirrels used for this study were captured by USDA licensed personnel (TLS Research, Bartlett, IL, USA) and transported to the Hibernation Facility at the National Institute of Neurological Disorder and Stroke (NINDS), National Institute of Health (NIH), Bethesda, MD. All protocols used for animal procedures were approved by the NINDS Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke (Animal protocol No. ASP 1223-02), with procedures described in detail previously [45]. Briefly, a temperature transponder (IPTT-300, Bio Medic Data System) was injected into the intrascapular region of the ground squirrel in order to monitor the body temperature (T_b) of individual animals. Torpor was induced by keeping the animals at 4–5 °C in constant darkness, whereas the euthermic animals used as the control group were kept in a cold room (4–5 °C) for 3 days while maintaining a constant T_b of 36–38 °C. Tissues for late torpid ground squirrels were collected when T_b of the animal was reduced to 5–8 °C and maintained for >3 days. During tissue collection, the animals were anesthetized with 5% isoflurane and sacrificed by decapitation for excision of BAT and WAT. Tissues collected were rinsed in ice-cold phosphate-buffered saline (PBS) and immediately frozen in 2-methylbutane chilled in dry ice and stored at –80 °C until use.

miRNA polyadenylation and cDNA synthesis and real-time PCR

Total RNA was isolated from WAT and BAT of euthermic control and torpid 13-lined ground squirrels as previously described [46]. Polyadenylation of miRNAs was carried out using the Poly(A) Polymerase Tailing Kit (Epibio, Madison, WI, USA) as instructed by the manufacturer. Reverse transcription (RT) of polyadenylated miRNAs was performed as previously described [47] using universal stem-loop RT adapter (Table S4) and the resulting product was serially diluted and stored at –20 °C until use.

Real-time PCR was performed using a BioRad MyiQ2 Detection System (BioRad, Hercules, CA, USA) using the sense primer specific to individual miRNA and the universal reverse primer (Table S1). A melting curve analysis was performed for each miRNA as a quality check.

Protein extraction and immunoblotting

Samples of frozen BAT and WAT (0.5 g) were homogenized using a Polytron PT 1000 homogenizer at a 1:2 (weight/volume) in buffer containing 20 mM HEPES pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4 , and 10 mM β -glycerol-phosphate, supplemented with 1 μL of protease inhibitor cocktail (BioShop, Burlington, ON, Canada). Tissues were centrifuged at $10,000 \times g$ for 15 min at 4 °C to collect supernatant containing soluble proteins. Samples containing 30 μg of protein were separated on 6% SDS-PAGE gel and electroblotted by wet transfer onto PVDF membrane. The blots were incubated overnight at 4 °C with rabbit anti-Dicer polyclonal primary antibody (1:1000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or rabbit

anti-glyceraldehyde 3-phosphate dehydrogenase (1:1000; GAPDH; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) polyclonal antibody. After extensive wash, the blots were subsequently incubated with HRP-linked anti-rabbit IgG secondary antibody (1:4000; BioShop, Burlington, ON, Canada) and developed using enhanced chemiluminescence reagent (BioRad, Hercules, CA, USA) after wash.

Pathway analysis

The DIANA miRPath web-based bioinformatics program (<http://diana.cslab.ece.ntua.gr/pathways/>) was used to determine pathway enrichment for miRNAs found to be altered during torpor [48].

Data and statistical analysis

For real-time PCR analysis, cycle threshold (Ct) of each miRNA was normalized to the Ct of endogenous control 5S rRNA from the same sample. The comparative $\Delta\Delta C_t$ method was used to quantify relative expression of miRNA expression. For immunoblots, band densities were analyzed using ChemiGenius Bio-Imaging System of GeneTools software (SynGene, Fredrick, MD, USA). Band intensities of Dicer in each lane were normalized against the corresponding band intensities of endogenous control protein GAPDH from the same sample. For both qRT-PCR and immunoblot results, data were expressed as mean expression (mean \pm SEM, $n = 4$ –5 independent samples from different animals at each sampling point) and were plotted relative to euthermic control that was arbitrarily set as 1.0. A statistical analysis of the data was carried out using student's t -test with $P < 0.05$ for significant change (SigmaPlot 12.0 statistical package).

Authors' contributions

CWW, KKB and KBS conceived and designed the project, while CWW and KKB designed and performed the experiments, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that there are no competing interests.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.gpb.2014.08.003>.

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