Received: 7 February 2023 | Accepted: 14 May 2023

DOI: 10.1002/biof.1976

RESEARCH ARTICLE

Biofactors WILEY

Torpor-responsive microRNAs in the heart of the Monito del monte, *Dromiciops gliroides*

Sarah A. Breedon[1] | **Anchal Varma**[1] | **Julian F. Quintero-Galvis**[2] | **Juan Diego Gaitán-Espitia**[3] | **Carlos Mejías**[2,4] | **Roberto F. Nespolo**[2,4] | **Kenneth B. Storey**[1]

[1]Department of Biology and Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada

[2]Facultad de Ciencias, Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, Valdivia, Chile

[3]The Swire Institute of Marine Science and School of Biological Sciences, The University of Hong Kong, Hong Kong, China

[4]Millenium Nucleus of Limit of Life (LiLi), Valdivia, Chile

Correspondence
Kenneth B. Storey, Department of Biology and Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada.
Email: kenstorey@cunet.carleton.ca

Funding information
Fondo Nacional de Desarrollo Científico y Tecnológico, Grant/Award Number: 1121073; Natural Sciences and Engineering Research Council of Canada, Grant/Award Number: 6793

Abstract

The marsupial Monito del monte (*Dromiciops gliroides*) utilizes both daily and seasonal bouts of torpor to preserve energy and prolong survival during periods of cold and unpredictable food availability. Torpor involves changes in cellular metabolism, including specific changes to gene expression that is coordinated in part, by the posttranscriptional gene silencing activity of microRNAs (miRNA). Previously, differential miRNA expression has been identified in *D. gliroides* liver and skeletal muscle; however, miRNAs in the heart of Monito del monte remained unstudied. In this study, the expression of 82 miRNAs was assessed in the hearts of active and torpid *D. gliroides*, finding that 14 were significantly differentially expressed during torpor. These 14 miRNAs were then used in bioinformatic analyses to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were predicted to be most affected by these differentially expressed miRNAs. Overexpressed miRNAs were predicted to primarily regulate glycosaminoglycan biosynthesis, along with various signaling pathways such as Phosphoinositide-3-kinase/protein kinase B and transforming growth factor-β. Similarly, signaling pathways including phosphatidylinositol and Hippo were predicted to be regulated by the underexpression of miRNAs during torpor. Together, these results suggest potential molecular adaptations that protect against irreversible tissue damage and enable

Abbreviations: AUC, area under the curve; Ct, cycle threshold; ECM, extracellular matrix; GAG, glycosaminoglycan; HS, Hippo signaling; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNAs; MRD, metabolic rate depression; mRNA, messenger RNA; PI3K/Akt, phosphoinositide-3-kinase/protein kinase B; PIS, phosphatidylinositol signaling; ROC, receiver operating characteristic; T_b, body temperatures; TGF-β, transforming growth factor-β.

Sarah A. Breedon and Anchal Varma shares equal coauthorship.

continued cardiac and vascular function despite hypothermia and limited organ perfusion during torpor.

KEYWORDS
epigenetics, gene regulation, hibernation, hypometabolism, microRNA, torpor

1 | INTRODUCTION

When faced with extreme temperature fluctuations and limited food availability, animals must either migrate to areas with more favorable conditions or adapt to endure these harsh environmental conditions. For many mammals, hibernation is a key strategy for surviving these difficult seasonal periods by cycling between periods of torpor characterized by metabolic rate depression (MRD) and brief metabolic arousals.[1–3] Regulation of metabolic rate is a fundamental characteristic of hibernation, enabling animals to reduce energy consumption in ATP-expensive processes such as transcription, translation, and cell cycle control, while reprioritizing critical prosurvival pathways.[2,4] This energy saving strategy helps animals extend the duration of survival supported by fixed fuel reserves during hibernation. However, certain pathways need to be maintained or upregulated during hibernation to provide enhanced cytoprotective mechanisms to promote survival, including antioxidant defenses, anti-apoptosis mechanisms, and chaperone proteins.[2,4]

Monito del monte (*Dromicips gliroides*) is a small marsupial hibernator found in the temperate rainforests of Chile and Argentina.[5] *Dromicips gliroides* is a cold-adapted marsupial capable of undergoing daily torpor bouts as well as multiday hibernation depending on the intensity and duration of low temperatures and food scarcity.[6–9] Previous studies have shown that when animals are food-deprived and temperatures drop to near 1°C–2°C, *D. gliroides* enter into deep torpor with body temperatures (T_b) approaching ambient levels, basal metabolic rate decreasing to ∼1%–5% of the normal euthermic rate, and respiratory rate decreasing from 370 to 3–4 breaths per minute with prolonged periods of apnea.[1,6,7,9] However, when faced with more mild temperatures below their standard range, *D. gliroides* enters a daily shallow torpor to balance energy expenditure by reducing their T_b to between ∼11°C and 28°C and their metabolic rate to ∼10%–60% of euthermic rates.[6,7,9]

The hearts of mammalian hibernators display many interesting adaptations to minimize energy expenditure and tissue damage while experiencing the stresses associated with hibernation including hypothermia and decreased tissue perfusion. In contrast to homeotherms who suffer deleterious arrythmias during hypothermia, heterotherms can maintain cardiac function at low T_b despite increased blood viscosity and peripheral resistance via reversible cardiac hypertrophy and improved contractility.[10–12] Additionally, hibernators utilize tissue preservation strategies including chaperone proteins and antioxidant defenses to prepare for oxidative stress during arousal,[13–19] as well as various transcription factors to regulate gene expression.[20–25] Understanding the molecular mechanisms mammalian hibernators utilize to survive the stresses associated with torpor and arousal could potentially be applied to human medicine, particularly in the development of therapeutics for ischemia/reperfusion injuries and improved organ transplantation.[2,11,26,27]

There are many regulatory mechanisms that contribute to the entrance into and maintenance of hibernation by altering gene expression to suppress nonessential proteins, while allowing for the expression of key prosurvival pathways. MicroRNAs (miRNA) are one such mechanism for fine control of gene expression during these hypometabolic periods.[28–31] miRNAs are short (∼22 nt) noncoding RNAs that inhibit messenger RNA (mRNA) translation through miRNA:mRNA binding leading to either decay or temporary storage of mRNA transcripts in stress granules or processing bodies depending on the degree of complementarity.[32,33] Interestingly, a single miRNA is able to target multiple mRNA transcripts, lending miRNAs the ability to regulate a large proportion of protein-coding genes, subsequently affecting nearly all cellular processes.[32,34] Additionally, miRNA expression is known to change in response to external stimuli, including extreme environmental stress.[35,36] Many previous studies have implicated differential miRNA regulation and expression during MRD in various animals exposed to a variety of environmental stresses.[37–45] These findings underscore the critical role miRNAs play in regulating gene expression to facilitate stress tolerance across vast species.

Furthermore, a previous study on *D. gliroides* characterized tissue-specific changes in miRNA expression during torpor in both liver and skeletal muscle, with the torpor-responsive miRNAs being predicted to affect key cellular pathways associated with metabolic reorganization, thermoregulation, and muscle maintenance.[46] However, the heart of *D. gliroides* remains unstudied in terms of miRNA expression during torpor. As such, the purpose

of this study was to identify torpor-responsive miRNAs, and to predict which downstream processes would be most affected by these differentially expressed miRNAs. The expression of 82 miRNAs was quantified in control (active) and torpid heart tissues, of which, 14 were found to have significantly altered expression during torpor. Subsequent bioinformatic target enrichment analyses predicted that these miRNAs primarily affect various cellular signaling pathways. Overall, these results suggest that altered miRNA expression could be crucial to regulating cardiac gene expression, metabolic reorganization, and various adaptive responses to stress that are characteristic of mammalian hibernation.

2 | MATERIALS AND METHODS

2.1 | Animal treatments

Animal experimentation was performed as previously described,[46] following proper animal capture, handling, and maintenance procedures as outlined by the American Society for Mammalogists.[47] All animal experimentation was authorized by the Chilean Agriculture and Livestock Bureau (SAG: Servicio Agrícola y Ganadero de Chile, permit resolution No. 1054/2014). Adult *D. gliroides* individuals were captured near Valdivia, Chile (39°48′ S, 73°14′ W; 9 m.a.s.l) over January–February in the austral summer of 2014. Tomahawk traps were modified with bananas and yeast as bait and set up 1 m above ground level in trees and shrubs. Once captured, individuals were immediately transported to the laboratory and were transferred to plastic cages ($45 \times 30 \times 20$ cm^3). All cages had 2 cm of bedding and were maintained in a climate-controlled chamber (PiTec Instruments, Chile) at 20°C ± 1°C with a 12:12 h light:dark photoperiod. Individuals were fed a mix of mealworms and fruit with water ad libitum. Following a two-week acclimation period in these conditions, individuals were randomly selected to be in the control or torpor conditions. Control active individuals ($n = 4$) were sampled from the aforementioned conditions with T_b of ~34°C. To induce torpor in the torpid treatment group ($n = 4$), the ambient temperature was gradually decreased (−1°C every 12 h) until a final ambient temperature of 10°C was reached. Verification of torpor incidence using visual observations and T_b measurements was performed several times daily between 09:00 and 17:00 according to previous studies.[46,48] Individuals were kept in torpid conditions for 4 days with food and water being offered daily. Control active and torpid individuals were euthanized according to protocols approved by the Committee on the Ethics of Animal Experiments of the Universidad Austral de Chile. All tissues were rapidly excised and frozen in liquid nitrogen before being packed in a dry shipper and air freighted to Carleton University for subsequent experimentation.

2.2 | Total RNA extraction

Total RNA extraction was performed as previously described.[46] Tissue samples of heart and brain were weighed (~50 mg) and homogenized in 1 mL TRIzol (Invitrogen, Cat no. 15596018; Waltham, Massachusetts) using a Polytron PT1200 homogenizer. Aliquots of chloroform (200 μL) were added to each sample, which were then centrifuged for 15 min at 10,000 rpm (4°C). The resulting upper aqueous phase was transferred to a new centrifuge tube, then 500 μL of isopropanol was added and RNA was precipitated on ice for 10 min. Samples were then centrifuged for 15 min at 12,000 (room temperature). Following centrifugation, the pellets were washed with 1 mL of 70% ethanol and then centrifuged for 5 min at 7500 rpm (room temperature). Leftover ethanol was decanted, and the RNA pellet air dried for 10 min. RNA pellets were resuspended in 50 μL of RNase-free water and RNA quality was assessed via 260/280 nm ratio measurements using a PowerWave XS spectrophotometer with a Take3 microvolume plate (BioTek; Winooski, Vermont). Only samples with a 260/280 nm ratio ≥2 were used for subsequent analysis. The integrity of total RNA isolates was confirmed by the presence of sharp bands corresponding to 18S and 28S ribosomal RNA on 1% agarose gel stained with SYBR Green (Invitrogen, Cat no. S7567). Each RNA sample was standardized to a final concentration of 1 μg/μL with RNase-free water and stored at −20°C until use.

2.3 | Polyadenylation and stem-loop reverse transcriptase-PCR

RNA samples were prepared for qPCR analysis by polyadenylation and RT-PCR as previously described.[46,49] A Poly(A) polymerase tailing kit from Epicenter (Cat no. PAP5104H; Madison, Wisconsin) was used to polyadenylate miRNA. Reactions were prepared with 3 μg total RNA, 1 μL polymerase buffer (0.1 M Tris–HCl pH 8.0, 0.25 M NaCl, and 10 mM $MgCl_2$), 1 mM ATP, and 0.5 μL of *Escherichia coli* poly(A) polymerase (2 U) to a final volume of 10 μL. Reactions were incubated for 30 min at 37°C to allow for polyadenylation and were then terminated by 5 min incubation at 95°C, before being chilled on ice. Reverse transcription of the polyadenylated products was done by combining each sample (10 μL) with 5 μL of 250 pM stem-loop adapter primers (File S1).

These mixtures were incubated for 5 min at 95°C to denature RNA, then 5 min at 60°C to allow for primer annealing, before chilling on ice for 1 min. Next, 4 μL of first-strand buffer (50 mM Tris–HCl pH 8.3, 40 mM KCl, and 6 mM $MgCl_2$), 2 μL 0.1 M dithiothreitol, 1 μL deoxynucleotide triphosphates (25 mM of each base), and 1 μL of Moloney leukemia virus reverse transcriptase (2 U) were added to each sample. RT-PCR reactions proceeded for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. All products were serially diluted and frozen at −20°C until use.

2.4 | MiRNA quantification by qPCR

Quantification of miRNA expression via reverse transcription-quantitative polyermase chain reaction (RT-qPCR) was performed as previously described.[46] The BioRad MyIQ2 Detection System (BioRad, Hercules, California), using MIQE guidelines.[50] Forward primers specific to each miRNA were designed as outlined by Biggar et al.[49] All forward primers and the universal reverse primer (File S1) were synthesized by Integrated DNA Technologies (Coralville, Iowa). Postrun melt-curve analyses were used to ensure amplification of only a single PCR product, and any reactions that amplified multiple products were removed from the analysis.

2.5 | Quantification of relative miRNA expression and statistical analysis

Relative miRNA expression levels were calculated using the comparative ΔΔCt method from qPCR cycle threshold (Ct) data.[51] The mean Ct of 121 miRNAs amplified from the same sample was used as an endogenous control for normalization, as previously described.[52] The stability of the mean Ct value was assessed using NormFinder,[53] which indicated the mean Ct was the most stable reference factor assessed for *D. gliroides* heart under control and torpor conditions. Relative expression data are presented as mean ± SEM ($n = 4$ independent biological replicates from different animals, with two technical replicates for each quantification) relative to the control condition. Statistical analysis was performed using the Student's *t*-test, with $p < 0.05$ considered a significant difference relative to the control group.

2.6 | Bioinformatic analyses

Bioinformatic analyses were performed to assess the functional relevance of torpor-responsive changes in miRNA expression. The DNA Intelligent Analysis (DIANA) miRPath v3.0 program (http://www.microrna.gr/miRPathv3) was used with *Mus musculus* miRNA homologs, microT-CDS (v5.0) threshold of 0.8, and genes union result merging.[54] This platform identifies Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched with target genes using in silico miRNA-mRNA interaction predictions. In this study, we performed pathway enrichment analyses for two groups of torpor-responsive miRNAs: (1) miRNAs with increased expression in torpid hearts (miR-1a-1-5p, miR-144-5p, miR-193b-5p, miR-195a-5p, miR-361-5p, miR-411-5p, miR-7b-3p; miR-545 excluded as it has no *Mus musculus* homolog), and (2) miRNAs with decreased expression in torpid hearts (miR-125a, miR-147-5p, miR-21a-5p, miR-181-5p; miR-3175 and miR-651 excluded as they have no *M. musculus* homolog). Additionally, an unsupervised hierarchical clustering heatmap of all the analyzed miRNAs was performed using the online platform SRPlot (http://www.bioinformatics.com.cn/srplot) with Ward clustering and all other default parameters. Similarly, receiver operating characteristic (ROC) curves were generated for the DE miRNAs using SRPlot with default parameters.

3 | RESULTS

3.1 | Differential expression of miRNA in the heart during torpor

This study utilized qPCR to investigate mRNA expression changes in the heart and brain of the South American marsupial *D. gliroides* during torpor. In the heart, 82 miRNAs were successfully amplified, with 14 being differentially expressed during torpor ($p < 0.05$; Figure 1 and File S2). Specifically, the relative expression of eight of these miRNAs increased significantly as compared to control expression: miR-1a-1 (1.56 ± 0.13-fold), miR-144 (1.48 ± 0.09-fold), miR-193b (1.22 ± 0.04-fold), miR-195 (1.91 ± 0.17-fold), miR-361 (1.62 ± 0.09-fold), miR-411 (1.30 ± 0.08-fold), miR-545 (1.75 ± 0.18-fold), and miR-7a-1 (1.67 ± 0.12-fold). Conversely, six miRNAs showed decreased expression during torpor: miR-125a (0.70 ± 0.09-fold), miR-147 (0.89 ± 0.03-fold), miR-181a-5p (0.64 ± 0.04-fold), miR-21a (0.52 ± 0.06-fold), miR-3175 (0.36 ± 0.03-fold), and miR-651 (0.73 ± 0.08-fold).

3.2 | Bioinformatic analyses

To investigate the cellular processes that may be differentially regulated in *D. gliroides* heart by torpor-responsive

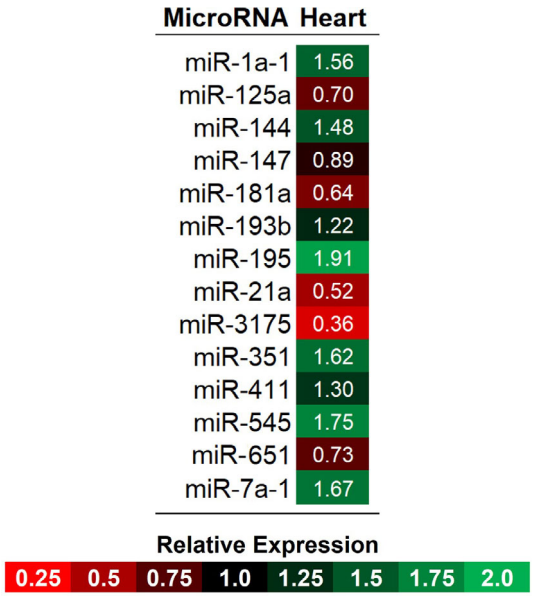

FIGURE 1 Heat map showing torpor-induced changes in the relative expression of 14 miRNAs in heart and four miRNAs in brain of *Dromiciops gliroides*. Relative expression of miRNA was evaluated by RT-qPCR of reverse-transcribed, polyadenylated transcripts. All changes in expression are statistically significant as determined by a two-tailed Student's *t*-test, $p < 0.05$. Data are mean relative expression, $n = 4$ independent samples, with each sample being the mean of two technical replicates. Full relative expression data ± SEM values for all 82 miRNAs can be found in File S2. Shades of red represent downregulation of miRNA during torpor compared with active control, shades of green represent upregulation of miRNA, and black represents no significant change in miRNA expression.

miRNAs, the differentially expressed miRNAs were subjected to pathway enrichment analyses using DIANA miRPath v3.0.[54] The KEGG pathways identified as potential targets of the collective group of query upregulated miRNAs are summarized in Table 1, and downregulated miRNAs are summarized in Table 2. DIANA miRPath indicated that the upregulated miRNAs targeted KEGG pathways associated with signaling such as phosphoinositide-3-kinase/protein kinase B (PI3K/Akt; $p = 5.95E-03$) and transforming growth factor-β (TGF-β; $p = 4.84E-02$; Table 1). Additionally, the glycosaminoglycan (GAG) biosynthesis—chondroitin sulfate/dermatan sulfate pathway ($p = 2.67E-03$) was also predicted to be significantly targeted by these upregulated miRNAs (Table 1). Alternatively, the downregulated miRNAs were found to be primarily associated with KEGG pathways relating to phosphatidylinositol signaling (PIS) system ($p = 3.06E-04$) and Hippo signaling (HS) pathway ($p = 3.06E-04$; Table 2). Hierarchical clustering analysis showed that all control samples clustered together, and all torpor samples clustered together (Figure S1). Furthermore, the ROC curve for the miRNAs found to increase in torpor showed that miR-144, miR-193b, miR-195, miR-1a-1, miR-351, and miR-7a-1 all had area under the curve (AUC) scores of 1.000, with only miR-411 and miR-545 deviating at AUCs of 0.969 and 0.938, respectively (Figure S2). The ROC curve for miRNAs that decrease in torpor found that miR-125a, miR-181a, miR-21a, and miR-3175 all had AUCs of 1.000, whereas miR-147 and miR-651 both had AUCs of 0.938 (Figure S3).

TABLE 1 KEGG pathways predicted by DIANA miRPath v3.0 to be downregulated by torpor-specific upregulated microRNA expression in *Dromiciops gliroides* heart.

KEGG pathway	*p*-Value	MicroRNAs (involved/queried)	No. of genes targeted
Pathways in cancer	1.58E-05	7/7	46
Glycosaminoglycan biosynthesis—chondroitin sulfate/dermatan sulfate	2.67E-03	3/7	3
Axon guidance	2.67E-03	4/7	17
Prostate cancer	5.95E-03	4/7	14
PI3K/Akt signaling pathway	5.95E-03	6/7	37
Signaling pathways regulating pluripotency of stem cells	2.62E-02	5/7	16
TGF-β signaling pathway	4.84E-02	3/7	12
Adherens junction	4.84E-02	4/7	9
FoxO signaling pathway	4.84E-02	6/7	17

Note: Analysis was performed with MicroT-CDS in silico prediction, threshold of 0.8 with results merged by genes union.
Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; PI3K/Akt, phosphoinositide-3-kinase/protein kinase B; TGF-β, transforming growth factor-β.

TABLE 2 KEGG pathways predicted by DIANA miRPath v3.0 to be upregulated by torpor-specific downregulated microRNA expression in *Dromiciops gliroides* heart.

KEGG pathway	*p*-Value	MicroRNAs (involved/queried)	No. of genes targeted
Phosphatidylinositol signaling system	3.06E-04	4/4	14
Hippo signaling pathway	3.06E-04	4/4	20
Endocrine and other factor-regulated calcium reabsorption	2.47E-03	4/4	10
Estrogen signaling pathway	2.61E-03	3/4	15
Fc epsilon RI signaling pathway	1.37E-02	4/4	15
Protein processing in endoplasmic reticulum	1.72E-02	4/4	24
Endocytosis	3.02E-02	4/4	28
Glioma	4.02E-02	4/4	10
Galactose metabolism	4.34E-02	2/4	2
Dorso-ventral axis formation	4.34E-02	3/4	7
Neurotrophin signaling pathway	4.34E-02	4/4	18
T-cell receptor signaling pathway	4.71E-02	4/4	17
GnRH signaling pathway	4.71E-02	4/4	14
Chronic myeloid leukemia	4.71E-02	4/4	13

Note: Analysis was performed with MicroT-CDS in silico prediction, threshold of 0.8 with results merged by genes union.
Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes.

4 | DISCUSSION

Previous studies have characterized the bioenergetics of *D. gliroides* during hibernation, indicating a larger decrease in metabolic rate than was expected for a marsupial.[6,7] While many studies have explored ecological, behavioral, and physiological aspects of hibernation in this "living fossil,"[55,56] only a handful of investigations have focused on changes at the molecular level. To deal with unfavorable environmental conditions, suppression of many energy-expensive cellular processes and reprioritization of ATP use towards support for pro-survival actions is required.[4] This can be achieved by strongly suppressing the expression of various ATP-expensive functions (e.g., gene transcription, protein translation, cell cycle, etc.), which can help the animal prolong the time that a fixed reserve of body fuels can support survival.[4,29,35,57] This is accomplished by a variety of regulatory mechanisms including protein posttranslational modifications, subcellular relocalization of transcription factors, and posttranscriptional gene silencing by miRNA.[4,29,35,57] Recent studies have identified miRNAs as key players of these cellular adaptations by inhibiting mRNA translation or directing mRNA transcripts into degradation.[35,57]

The present study aims to characterize miRNA expression patterns in *D. gliroides* heart to explore their potential involvement in mediating facultative hypometabolism. The relative expression levels of 82 miRNAs in heart were quantified via RT-qPCR with 14 miRNAs found to be significantly differentially expressed during torpor in *D. gliroides* (Figure 1). DIANA miRPath was used to identify potential cellular processes that may be differentially affected by these miRNAs during torpor. As there is very high degree of evolutionary conservation of miRNAs across vertebrates,[34] this analysis relied upon interpretation based on *in silico* predictions for mRNA targets and miRNA-target interactions of mouse miRNA homologs. This was coupled with the relative scarcity of experimentally-validated interactions, providing a rationale for this heterologous approach to pathway-enrichment analysis.[34,58] Additionally, all miRNAs that had significantly altered expression during torpor showed a very high degree of identity with *M. musculus* homologs that were used for bioinformatics analyses, with the exception of miR-545, miR-651, and miR-3175, for which no mouse homolog could be found in miRBase.

Given the criticality of normal functioning of the heart, it follows that maintaining the heart's integrity and activity is crucial during hibernation and afterwards during the arousal period. Mammalian hibernators are able to maintain cardiac contractility over a wide range of core T_b values with associated increases in peripheral resistance as T_b declines, stresses which can cause severe dysfunction for nonhibernating species such as humans.[59] In this study, we identified eight miRNAs with significantly increased expression, and six miRNAs with decreased expression in *D. gliroides* heart tissue during

torpor compared with control (Figure 1). This selective regulation implies that the coordinated action of miRNAs during torpor facilitate the suppression and activation of specific cellular processes, depending on if these miRNAs have increased or decreased expression respectively. It was predicted that the upregulated miRNAs in heart are primarily involved in regulating genes related to GAG biosynthesis (chondroitin sulfate/dermatan sulfate), and PI3K/Akt and TGF-β signaling pathways, suggesting that these pathways become downregulated during marsupial torpor (Table 1). Additionally, it was predicted that the downregulated miRNAs would result in increased expression of various signaling pathways during torpor, including phosphatidylinositol and HS (Table 2). Altogether, the differential expression of these pathways is necessary for regulating metabolic reorganization and proper cardiac function in the hypometabolic torpid state.

4.1 | Pathways predicted to be downregulated by upregulated miRNAs

4.1.1 | GAG biosynthesis

GAGs are a type of polysaccharide that are widely present in the extracellular matrix (ECM) of vertebrate tissues, including the heart.[60,61] The cardiac ECM is a complex fibrous network of proteins, GAGs, and proteoglycans, arranged in a precise 3D framework to provide an elastic and supportive environment for cell membrane electrical impulse propagation and synchronous pump contraction.[62] The ECM plays a critical role in the heart, contributing to numerous essential cellular processes during cardiac development and homeostasis.[63] Though the heart itself is largely incapable of repair, with lost cardiomyocytes being replaced with nonfunctional fibrotic tissue after injury, the ECM undergoes continuous remodeling to control composition and structure.[63–65] This remodeling occurs cyclically as a result of strict metabolic control to regulate cellular processes.[63] ECM remodeling is tightly controlled in homeostasis; however, disordered ECM regulation is associated with many pathological conditions.[63] Indeed, the overaccumulation of chondroitin sulfate and dermatan sulfate—both GAGs identified in the DIANA miRPath KEGG pathway analysis herein—have been previously implicated in myocardial inflammation and fibrosis, valve degeneration, and mitral regurgitation, among others.[66–70]

Given that mammalian hearts display limited regeneration after injury,[71] it is imperative that *D. gliroides* has adapted various cardioprotective processes to mitigate any damage that may occur during torpor to ensure proper functioning of the heart. Since overproduction of cardiac GAGs is deleterious, the strict miRNA-controlled downregulation of GAG biosynthesis likely serves as a key cardioprotective mechanism for torpid *D. gliroides*. Additionally, regulation of ECM composition could also act to control signal transduction pathways over the torpor-arousal cycle. Signal transduction cascades and the control they exert over their downstream pathways contribute to MRD by allowing an organism to respond to the external environment and alter gene expression to minimize unnecessary energy expenditure.[4] As such, altering signal transduction serves to maintain an energy balance while the overall metabolic rate is suppressed. Indeed, GAG levels are known to affect signal transduction in a variety of organisms and pathological states.[72,73] Thus, in addition to protecting cardiac tissue, miRNA-mediated control of GAG biosynthesis would also provide *D. gliroides* with an additional avenue for mediating a reduction in metabolic activity, while preserving the ability to quickly restore basal expression upon arousal from torpor.

4.1.2 | PI3K/Akt and TGF-β signaling pathways

PI3K/Akt and TGF-β signaling pathways regulate various cellular processes, including cell division, proliferation, and turnover .[74,75] Given how energetically costly these processes are, it is unsurprising that these pathways are predicted to be suppressed during torpor. This suppression correlates well with the known reduction of general transcription and translation in various torpid/hibernating mammals.[4,76–78] As such, the signaling pathways that influence these energetically demanding processes, including PI3K/Akt and TGF-β, are critical regulatory points that can be attenuated in response to changing environmental conditions to control gene expression and reduce needless ATP usage. Additionally, apoptosis has also been found to be downregulated in various hibernators during periods of MRD.[79–83] Hence, the inhibition of these signaling pathways among others may also serve to attenuate hibernation-induced apoptosis, further suggesting a role in regulating cardiomyocyte proliferation to maintain cardiac viability during torpor.

While no studies to date have analyzed PI3K/Akt or TGF-β regulation in Monito del Monte hearts during torpor, previous studies on torpid mousebird and hibernating ground squirrel hearts have suggested decreased Akt signaling activity in hearts during MRD.[84,85] Additionally, a previous study found that Akt was downregulated in brain and kidney of torpid *D. gliroides*, whereas liver showed Akt upregulation.[86] Interestingly, these liver findings were supported by an early study which

indicated that the PI3K/Akt signaling pathway would be upregulated by decreased expression of hepatic miRNAs in torpid *D. gliroides*.[46] These studies suggest that Monito del Monte utilizes differential miRNA expression to control gene expression and metabolic processes in a tissue-specific manner. Similarly, decreased TGF-β signaling is likely an energy conserving mechanism to prevent extraneous ATP usage during torpor. Since TGF-β is known to influence energetically costly processes such as cell proliferation, migration, and differentiation, which are not critical for torpor-survival, it makes sense that these pathways would be downregulated via suppression of the TGF-β signaling pathway whilst in a hypometabolic state.[4,87] Moreover, TGF-β functions in the deposition of fibrotic tissue, which often results in the loss of normal organ function.[88] Thus, downregulating TGF-β signaling may act to minimize deleterious cardiac fibrosis and protect the long-term viability of the heart during and after torpor. Altogether, the suppression of various signaling pathways including PI3K/Akt and TGF-β likely helps reduce energy expenditure while maintaining essential cardiac functionality during torpor. However, further research is needed determine the exact expression of these signaling pathways in *D. gliroides* hearts in response to torpor.

4.2 | Pathways predicted to be upregulated by downregulated miRNAs

4.2.1 | Phosphatidylinositol signaling

PIS system plays a major role in a variety of cellular processes. Phosphatidylinositol and its phosphorylated products, phosphoinositides, play important roles in cell survival, membrane trafficking, membrane structure, endocytosis, and cytoskeletal dynamics.[89] Interestingly, phosphoinositides are involved in the PI3K/Akt pathway, which was predicted herein to be downregulated. This curious finding is likely explained by the vast roles of phosphoinositides in a variety of processes outside PI3K/Akt signaling. For instance, phosphoinositides are also critical for the regulation of membrane calcium channels via the calcium signaling pathway.[90,91] Thus, it is likely this Ca^{2+}-related aspect of the PIS pathway that is subject to miRNA-induced upregulation, whereas other aspects of phosphoinositide activity relating to the PI3K/Akt signaling pathway are downregulated.

Heart rate critically depends on the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum and the regulation of cardiac contractility largely depends on changes to intracellular Ca^{2+} concentration via Ca^{2+}-induced Ca^{2+} release.[92] Thus, Ca^{2+} handling is a key mechanism for maintenance of heart rate and contractility.[93] Previous studies on hearts of ground squirrels and hedgehogs showed higher contraction amplitudes at low temperature torpor as compared to normal temperatures.[94,95] Therefore, upregulation of the PIS pathway may be an adaptation to maintain Ca^{2+} homeostasis, such that stable cardiac function is retained during torpor. The PIS-Ca^{2+} signaling system is also crucial for cellular activation in response to various extracellular stimuli, helping to quickly restore plasma membrane and Ca^{2+} levels to maintain the homeostasis and sustain Ca^{2+} signaling.[90,91] Additionally, changes in Ca^{2+} influx may mediate hypertrophic responses.[96–98] Indeed, studies have indicated that reversible cardiac hypertrophy during hibernation/torpor contributes to increased contractility.[25,99–102] Therefore, upregulation of the PIS pathway may also contribute to cardiac remodeling and reversible hypertrophy, which is likely beneficial for forceful contraction during torpor.

4.2.2 | HS pathway

The HS pathway is known to play key roles in regulating organ size, tissue repair, vascular remodeling cell proliferation, and apoptosis.[103–106] Dysregulation of HS can lead to cardiomyopathies, cardiac dysfunction, fibrosis, and heart failure.[107–110] As such, HS is under strict control to maintain a proper balance of cell renewal and cell death to regulate cardiac remodeling and tissue homeostasis. The predicted upregulation of HS suggests that this pathway is critical for establishing and maintaining sufficient cell number and size to facilitate proper heart function during torpor, but further studies are needed to elucidate the specific regulation of HS in Monito del Monte. Though no studies to date have measured the HS pathway on torpid *D. gliroides* or any other hibernating mammals, a previous study on the hearts of *Rana sylvatica* found an overall increase in the HS pathway in response to anoxia exposure.[111] This response was postulated to conserve cellular energy and to function as a cardioprotective/repair mechanism.[111] Thus, HS upregulation may play a similar role in *D. gliroides*, acting to limit downstream ATP consumption and govern heart size.

Given the importance of sustaining proper cardiac function during torpor, which is at least partly regulated via selective hypertrophy, it makes sense that *D. gliroides* would upregulate HS to be able to strictly regulate heart size as needed, depending on the depth/duration of the torpid episode and in preparation for subsequent arousal. While hypertrophy improves heart contractility in hibernators, excessive hypertrophy is known to be

deleterious.[99–101,110] Ergo, the HS pathway is a prime candidate to coordinate a strict level of hypertrophy that would facilitate improved heart function, while mitigating any negative consequences resulting from superfluous growth. Moreover, the HS pathway has also been shown to interact with the TGF-β signaling pathway to activate fibroblasts, potentially resulting in increased myocardial fibrosis.[112,113] This is interesting as TGF-β signaling was predicted to be downregulated in torpid Monito del Monte hearts. However, the simultaneous upregulation of HS and downregulation of TGF-β signaling is likely explained by the need to strictly balance beneficial cardiac hypertrophy while limiting maladaptive fibrotic tissue deposition. Altogether, upregulated HS in this present study suggests that HS is a critical regulator for cardiomyocyte proliferation and apoptosis, thereby determining heart size and maintaining essential cardiac function during torpor.

5 | CONCLUSIONS

This study investigated the expression of 82 miRNAs in the heart of active and torpid *D. gliroides*, with 8 being upregulated in torpor and 6 downregulated in torpor. Subsequent DIANA miRPath bioinformatic analyses identified several pathways predicted to be targeted by this differential miRNA expression during torpor. miRNAs that were overexpressed in the heart were predicted to attenuate GAG biosynthesis, and various signaling pathways including PI3K/Akt and TGF-β signaling. Underexpressed miRNAs were predicted to upregulate the PIS system and the HS pathway. Overall, these results indicate differential miRNA expression in the heart of *D. gliroides* in response to torpor, playing a key role in initiating and maintaining MRD and various cellular adjustments needed to survive a hypometabolic state. These miRNAs regulate the expression of downstream pathways, likely serving as an energy conservation mechanism to limit ATP usage during periods of limited energy production. Additionally, regulation of these pathways may also activate cardioprotective processes to counteract torpor-induced physiological insults to ensure optimal cardiac functioning during and after torpor. However, *D. gliroides* remains understudied to date and further downstream analyses are required to confirm the results presented herein.

AUTHOR CONTRIBUTIONS

Sarah A. Breedon: Conceptualization; investigation; formal analysis; writing—original draft. **Anchal Varma:** Conceptualization; investigation; formal analysis; writing—original draft. Julian **F. Quintero-Galvis:** Investigation. **Juan Diego Gaitán-Espitia:** Investigation. **Carlos Mejías:** Investigation. **Roberto F. Nespolo:** Conceptualization; methodology; supervision; resources; writing—review and editing. **Kenneth B. Storey:** Conceptualization; methodology; supervision; resources; writing—review and editing.

ACKNOWLEDGMENTS

This work was supported by a Discovery grant (Grant no. 6793) from the Natural Sciences and Engineering Research Council (NSERC) of Canada to Kenneth B. Storey and the FONDECYT grant (no. 1121073) and Millennium Science Initiative Program – Center Code NCN2021_050 to Roberto F. Nespolo. Kenneth B. Storey holds the Canada Research Chair in Molecular Physiology, and Sarah A. Breedon holds an Ontario Graduate Scholarship. The authors thank S. Wijenayake and J. A. Moggridge for their experimental contributions and J. M. Storey for editorial review of this article.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Kenneth B. Storey https://orcid.org/0000-0002-7363-1853

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How to cite this article: Breedon SA, Varma A, Quintero-Galvis JF, Gaitán-Espitia JD, Mejías C, Nespolo RF, et al. Torpor-responsive microRNAs in the heart of the Monito del monte, *Dromiciops gliroides*. BioFactors. 2023. https://doi.org/10.1002/biof.1976