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Low-temperature microRNA expression in the painted turtle, *Chrysemys picta* during freezing stress

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ABSTRACT

Natural freeze tolerance depends on cellular adaptations that address the multiple stresses imposed on cells during freezing. These adaptations preserve viability by suppressing energy-expensive cell processes in the frozen state. In this study, we explore the freeze-responsive expression of microRNA in hatchling painted turtles exposed to 20 h freezing. Furthermore, we also explore the possibility of unique temperature-sensitive microRNA targeting programs that aid in adapting turtles for survival in the frozen state. Interestingly, two freeze-responsive 'cryo-miRs' (*cpm-miR-16* and *cpm-miR-21*) were found to have unique low-temperature mRNA targets enriched in biological processes that are known to be part of the stress response.
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1. Introduction

For a number of ectothermic animals, freeze tolerance is a strategy of winter survival in subzero temperatures. In these animals, a number of biochemical and cellular changes take place to permit the freezing of extracellular body fluids. Since the first report in 1988, a number of studies have documented the freezing of some species of hatchling turtles while overwintering in the natal nests [32]. Painted turtles (*Chrysemys picta*) hatch from their eggs in late summer, but do not emerge from their nest until the following spring. This delayed emergence allows the hatchling protection from predation until juvenile growth conditions are more favorable. However, this also exposes the hatchling turtles to subzero terrestrial temperatures throughout the winter months. During the cold winter months, the hatchlings are able to endure the freezing of 53% of their total body water converted to ice [32]. As a result of such extreme environmental stress, these turtles utilize strong metabolic and cellular controls that reprioritize cellular processes and ATP demand until conditions are more favorable [33,34].

Despite global reprioritization and reductions in the majority of cellular functions, several survival pathways that act to preserve and stabilize cellular macromolecules are enhanced (such as antioxidant defense and chaperone proteins) [35,18]. However, the entrance into the frozen state does not appear to involve extensive changes to genes expression [34], undoubtedly because an energy-limited frozen state is not the time for a major reorganization of the cellular environment. Instead, the types of molecular mechanisms that adapt the cell for survival are most likely those that are broadly applicable to all types of cellular processes, readily coordinated by extracellular stimuli, and also easily induced and reversed to allow for rapid transitions to and from the stressed state [33]. It has been widely acknowledged that reversible post translational modification to protein plays an important role to rapidly adapt protein function, however less studied is the function of microRNA (miRNA), whose regulatory mechanisms also match the requirements needed for metabolic depression.

In response to freezing and other external stress stimuli, it has been well established that stress-responsive cellular modifications include transcriptional, translational, post-translational, and allosteric mechanisms of regulation [33,19,29]. Recently, studies have begun to document the role of miRNA as a new rapid and reversible mechanism of stress adaptation [4,10]. These 18–25 nucleotide transcripts are able to bind with full or partial complementarity to

Abbreviations: miRNA, microRNA; mRNA, message RNA; GO, gene ontology; RT-PCR, real-time polymerase chain reaction; UTR, untranslated region

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mRNA targets, resulting in either translational inhibition or degradation of that target [22,16,30]. Indicative of their large regulatory potential, a single miRNA may target many mRNAs, and a single mRNA may contain multiple miRNA binding sites [1]. The degree and extent of miRNA binding to its target creates a complex regulatory system that has been predicted to be involved in nearly every aspect of biological function. Importantly, there is a remarkably high conservation between miRNA and its corresponding target binding sequences (typically within the 3′ UTR) in vertebrate species (90–100%), even in the presence of a low overall 3′ UTR conservation between distantly related vertebrate species [15,7,20]. This high degree of conservation demonstrates the importance of miRNA influenced regulation of protein expression and of the miRNA–mRNA interaction. Recently, it has been proposed that ectothermic species that regularly experience a wide range of body temperatures (T_b) might also experience temperature influence over the thermodynamics of the miRNA-mRNA interaction, with a low T_b reducing the free energy of RNA duplex formation and favoring mRNA binding [36,8,10]. As such, miRNA regulated biological processes may change dynamically once an organism experiences a dramatic change in T_b, such as that experienced during freezing in the turtle.

Since the initial discovery of miRNAs in 2001, many studies have documented the stress-responsive role of miRNAs in the regulation of the cell survival in a diverse number of animal and environmental stresses, including the regulation of mammalian hibernation [26,17,25,9,38] aestivation in frogs and sea cucumbers [24,12,11,37], frog and insect freeze tolerance [3,14], and turtle and marine snail anoxia tolerance [6,7]. This collection of studies indicates a conserved miRNA response to periods of environmental stress across phylogeny. As animal survival during periods of such extreme stress depends on the ability to rapidly and readily reorganize cellular processes to conserve vital ATP supplies [33], it is possible that miRNA function as a mechanism to aid in the reprioritization of ATP use and stress-specific cellular adaptation. We therefore reasoned that miRNAs are likely to play a role in freeze tolerance in the hatchling painted turtle, Chrysemys picta, and that changes in miRNA expression, and temperature-influenced miRNA-mRNA targeting, may be crucial to transducing stress signals into appropriate changes in mRNA translation to aid cryoprotection. In this study, we explored the freeze-response of select miRNA that have previously been shown to be highly stress-responsive in animal models of metabolic rate depression, namely *cpm-miR-21*, *-16*, *-29b*, *-34a*, *-133*, and *-15a*, to evaluate their role in hatchling turtle freeze tolerance [3,17,6,9].

2. Materials and methods

2.1. Animals

Animal care and treatment procedures were similar to that previously described by Churchill and Storey [13]. Briefly, eggs of midland painted turtles, *C. picta marginata*, were obtained from Algonquin Provincial Park, Ontario in June and were reburied in nearby Ottawa, Ontario in artificial nests to control natural predation, ensuring near 100% hatching success. Hatchling turtles were excavated in early November and transferred to a laboratory incubator set at 5 °C. All turtles were held at a constant 5 °C for at least 2 weeks before use. All animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and all experimental procedures had the prior approval of the Carleton University Animal Care Committee. Control turtles were sampled from the 5 °C acclimated group. Animals were killed by decapitation and tissues were quickly excised, individually frozen in liquid nitrogen, and stored at 80 °C. For freezing exposure, hatchling turtles were placed in plastic containers lined with damp paper towels and then transferred to an incubator set at −3 °C. Turtle body temperature was cooled and animals began to freeze within 25 min. Freezing exposure was timed from this point and turtles were sampled after 20 h of freezing. All animals survived the freezing experiments.

2.2. RNA isolation

Total RNA was isolated from liver and heart muscle of turtles using Trizol™ (Cat# 15596-018, Invitrogen). Briefly, 50 or 100 mg of heart and liver tissue, respectively, from independent animals were homogenized as separate samples in 1 mL Trizol using a Polytron homogenizer followed by the addition of 200 μL of chloroform and centrifugation at 10,000×*g* for 15 min at 4 °C. The upper aqueous layer (containing RNA) was removed and placed in a fresh microcentrifuge tube containing 500 μL of isopropanol and incubated at room temperature (RT) for 10 min to allow for RNA precipitation. Samples were then centrifuged at 10,000×*g* for 15 min at 4 °C. The RNA pellet was washed with 70% ethanol and centrifuged again. The RNA pellet was then allowed to dry for 10–15 min and then resuspended in 50 μL RNase-free H_2O. RNA quality was assessed by the 260/280 nm ratio, as well as gel electrophoresis on a 1% agarose gel stained with 2× Sybr Green I (Cat# S7563; Invitrogen) to check for integrity of the *18S* and *28S* rRNA bands. All RNA samples were then diluted to a concentration of 1 μg/μL using RNase-free ddH_2O.

2.3. Reverse transcription and amplification

Reverse transcription and PCR was performed as previously described [5]. A 10 μL sample of RNA (1 μg total) was incubated with 5 μL of 250 nM miRNA-specific stem-loop reverse transcription primer (Table 1). The reaction was heated at 95 °C for 5 min to denature the RNA, and then incubated for 5 min at 60 °C to anneal the stem loop primer. After cooling on ice for 1 min, the remaining reagents (4 μL of 5× first strand buffer, 2 μL of 0.1 M DTT, 1 μL of dNTP mixture containing 25 mM of each nucleotide, and 1 μL of M-MLV reverse transcriptase) were added. The reaction proceeded for 30 min at 16 °C, followed by 30 min at 42 °C, and 85 °C for 5 min. Following reverse transcription, the reverse transcribed product was serial diluted and stored at −20 °C. Real-time PCR (RT-PCR) was performed on a BioRad MyiQ2 Detection System (BioRad; Cat#170-9790). The 10 μL RT-PCR reaction included 2.5 μL of diluted reverse transcribed product, 5 μL ssoFast EvaGreen Supermix (BioRad; Cat#172-5201), 1.25 μL of 12.5 μM forward primer (Table 1), and 1.25 μL of 12.5 μM reverse primer (5′-CTCACAGTACGTTGGTATCCTTGTG-3′). Reactions were incubated in a 96-well thin-walled PCR plate at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was performed for each miRNA analyzed. Select PCR products were run on a 2% agarose gel and sent to Bio-Basic (Markham, ON, Canada) for sequencing using a shortened universal reverse primer (5′-CTCACAGTACGTTGG-3′) to increase sequence coverage.

2.4. MicroRNA target identification

Mature miRNAs were searched against the 3′ UTR sequences from the *Chrysemys picta* reference genome available in the UCSC table browser (v.3.0.1/chrPic1) using FindTar3 (v.3.11.12). To search the predicted miRNA target genes the FindTar3 software was used within the following parameters and conditions: AT and GC weight of 5, GT weight of 2, a gap opening penalty of −8, a gap extension penalty of −2, target duplex with maximum threshold free energy −20 kcal/mol, and demand strict 5′ seed pairing. Target predictions were carried out with a temperature

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