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Dehydration mediated microRNA response in the African clawed frog *Xenopus laevis*

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ABSTRACT

Exposure to various environmental stresses induces metabolic rate depression in many animal species, an adaptation that conserves energy until the environment is again conducive to normal life. The African clawed frog, *Xenopus laevis*, is periodically subjected to arid summers in South Africa, and utilizes entry into the hypometabolic state of estivation as a mechanism of long term survival. During estivation, frogs must typically deal with substantial dehydration as their ponds dry out and *X. laevis* can endure >30% loss of its body water. We hypothesize that microRNAs play a vital role in establishing a reversible hypometabolic state and responding to dehydration stress that is associated with amphibian estivation. The present study analyzes the effects of whole body dehydration on microRNA expression in three tissues of *X. laevis*. Compared to controls, levels of *miR-1*, *miR-125b*, and *miR-16-1* decreased to 37 ± 6, 64 ± 8, and 80 ± 4% of control levels during dehydration in liver. By contrast, *miR-210*, *miR-34a* and *miR-21* were significantly elevated by 3.05 ± 0.45, 2.11 ± 0.08, and 1.36 ± 0.05-fold, respectively, in the liver. In kidney tissue, *miR-29b*, *miR-21*, and *miR-203* were elevated by 1.40 ± 0.09, 1.31 ± 0.05, and 2.17 ± 0.31-fold, respectively, in response to dehydration whereas *miR-203* and *miR-34a* were elevated in ventral skin by 1.35 ± 0.05 and 1.74 ± 0.12-fold, respectively. Bioinformatic analysis of the differentially expressed microRNAs suggests that these are mainly involved in two processes: (1) expression of solute carrier proteins, and (2) regulation of mitogen-activated protein kinase signaling. This study is the first report that shows a tissue specific mode of microRNA expression during amphibian dehydration, providing evidence for microRNAs as crucial regulators of metabolic depression.

1. Introduction

During periods of environmental stress, many animal species employ adaptive biochemical strategies to promote survival when their habitats are unfavorable. A widely used strategy is metabolic rate depression that provides energy savings and life extension under severe environmental conditions (e.g. too hot, too dry, too cold, etc.). Entry into a hypometabolic state is a common feature of phenomena including estivation, hibernation, diapause, torpor and freeze tolerance, among others (Storey, 2010; Storey and Storey, 1992, 2007, 2012). The African clawed frog, *Xenopus laevis*, is an example of an animal that utilizes hypometabolism to endure the dry months of Southern African summers. Primarily an aquatic animal, during the dry season *X. laevis* can be stranded as ponds dry out and relocates into the cool and damp subsoil. There frogs undergo estivation that is frequently characterized by a substantial loss of body water (as much as 35%) as the soil dries out (Romspert, 1975). During estivation, plasma urea rises by nearly 30-fold and this increases osmolality to help resist the loss of body fluids and, when possible, promote water uptake across the skin (Malik and Storey, 2009). Glucose levels and hematocrit also rise, the latter suggesting that *X. laevis* can experience hypoxia stress due to circulatory impairment caused by a decrease in blood volume and an increase in blood viscosity in dehydrating animals (Storey and Storey, 2012).

During a state of estivation, species such as *Otala lactea* have been shown to undergo strong reductions in protein synthesis and proteolysis along with activation of stress responsive proteins such as AMP-activated protein kinase (AMPK) that functions as an energy sensor of the cell (Ramnanan et al., 2009, 2010). However, the molecular regulation of estivation in *X. laevis* remains largely unexplored, with little known about the mechanisms that regulate dehydration survival in a reversible manner. Recent studies in our lab and others have implicated a role for microRNA regulation in hypometabolism, with a number of microRNAs showing differential expression in response to cellular stresses including hibernation, anoxia, and freezing (Biggar and Storey, 2011, 2012; Biggar et al., 2012; Kornfeld et al., 2012; Liu et al., 2010). These studies have demonstrated a crucial role of microRNAs in reversible metabolic rate depression, and that this stress response is largely conserved in a number of different species.

MicroRNAs are short single stranded non-coding RNA molecules that are 17–22 nucleotides in length. When incorporated into a microRNA-induced silencing complex (miRISC) they have been

Abbreviations: AMPK, Adenosine monophosphate-activated protein kinase; ERK, Extracellular signal-regulated kinases; HIF-1α, Hypoxia inducible factor-1α; JNK, C-Jun N-terminal kinases; KEGG, Kyoto encyclopedia of Genes and Genomes; MAPK, Mitogen activated protein kinase; MFI, Median fluorescent intensity; miRISC, MicroRNA induced silencing complex; P53, Tumor protein 53; PI3K, Phosphoinositide 3-kinase; PTEN, Phosphatase and tensin homolog; SAPE, Streptavidin–phycoerythrin; SLC, Solute carrier.

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shown to generally inhibit gene expression at a post-transcriptional level via complementary binding of specific microRNAs to the 3′ UTR of their target mRNA transcripts (He and Hannon, 2004; Leung and Sharp, 2010). Interacting primarily with the 5′ seeding regions of microRNA, microRNA-mediated repression of target mRNAs is dictated based on the degree of complementarity that exists between the microRNA and its target mRNA (Grimson et al., 2007). Depending on the level of complementarity, mRNA translation is arrested by targeting transcripts either for degradation or for storage in cytoplasmic loci, such as P-bodies or stress granules (Kedersha and Anderson, 2007; Liu et al., 2005). It is well documented that individual microRNAs can regulate a number of different targets and a single target can be regulated by a number of microRNAs (Bartel, 2004). This creates a highly complex regulatory network that involves microRNA in regulation of virtually all biological processes, ranging from metabolic functions, cellular signaling, stress responses, to disease pathogenesis (Ivey and Srivastava, 2010; Rottiers and Näär, 2012; Shi and Jin, 2009; Wu et al., 2013).

In this study, we assessed the expression of 10 well-characterized microRNAs that have previously been shown to play a role in hypometabolism (Biggar and Storey, 2012; Biggar et al., 2012; Kornfeld et al., 2012; Liu et al., 2010) to determine if they are also differentially regulated when *X. laevis* undergo dehydration. MicroRNAs, such as *miR-29b*, *miR-21*, and *miR-34a* have been previously documented to be a direct regulator of both insulin signaling and apoptosis, by targeting genes such as p85α (regulatory subunit of phosphoinositide 3-kinase), phosphatase and tensin homolog (PTEN), and vesicle-associated membrane protein 2 (VAMP2) (Lovis et al., 2008; Park et al., 2009; Zhang et al., 2010). Meanwhile, *miR-210* and *miR-203* have both been shown to be up-regulated in response to various environmental stresses, including hypoxia and dehydration. *MiR-210* has been shown to be hypoxia responsive, functioning to stabilize the expression of the hypoxia inducible factor-1α (HIF-1α) through a reduction in glycerol-3-phosphate dehydrogenase 1-like expression, resulting in a reduction of HIF-1α hyper-hydroxylation (Kelly et al., 2011; Shen et al., 2013). Additionally, *miR-203* has been shown to be involved in the typical dehydration response and skin morphogenesis by targeting the transcription factor, p63 (Lena et al., 2008; Viticchie et al., 2012). The epidermis, the outer layer of the skin, contains keratinocytes and is a stratified epithelium that functions as a barrier to protect the organism from dehydration and develops depending on the transcription factor p63, a member of the p53 family of transcription factors. Using a relatively new approach, we utilized Luminex-based multiplex technology to measure the expression of 10 microRNAs (Luminex applications reviewed in Storey and Wu, 2013). In this study, we provide molecular data that show a tissue-specific mode of microRNA expression between liver, ventral skin, and kidney of frogs during dehydration stress.

2. Materials and methods

2.1. Animal preparations

Procedures used for animal experiments were comparable to previously described (Malik and Storey, 2009). Male *X. laevis* were received as donations from the University of Toronto, each with a body mass between 30 and 55 g. All were acclimated for about 10 days in tanks of dechlorinated water maintained at 22 °C. Control animals were sampled from this condition. For dehydration, other frogs were transferred to dry plastic buckets and water loss was monitored over several days until a target value of approximately 30% of total body water lost was reached. To determine water loss, frogs were removed from the buckets at intervals and quickly weighed. The percentage water loss of frogs was calculated as:

$$\% \text{ Changed} = [(W_i - W_d)/(W_i \times BWC_i)] \times 100$$

where W_i represents initial body weight, W_d represents dehydrated body weight, and BWC_i represents mean body water content of frogs before experimentation in gram H_2O per gram body mass. For tissue collection, control and dehydrated frogs were sacrificed by pithing and then tissues (liver, kidney, and ventral skin) were quickly dissected out and frozen in liquid nitrogen before transfer to long term storage in a −80 °C freezer.

2.2. Extraction of total RNA

Briefly, ~50 mg samples of frozen tissue were homogenized in 1 mL of TRIzol reagent using a Polytron PT1000 homogenizer; samples were then centrifuged at 10,000 ×*g* for 15 min at 4 °C. The aqueous layer containing total RNA was removed and transferred to an RNAse-free microcentrifuge tube and precipitated by the addition of 500 μL isopropanol. Samples were allowed to precipitate over a period of 10 min at room temperature, followed by centrifugation at 10,000 ×*g* for 15 min at 4 °C. RNA pellets were then washed twice with 70% ethanol, allowed to dry, then re-suspended in 100 μL of diethylpyrocarbonate-treated ddH_2O. RNA quality was assessed by absorbance measurements at 260 and 280 nm, meanwhile RNA integrity was determined by separation on a 1% agarose gel to visualize both 18S and 25S ribosomal RNA bands.

2.3. MicroRNA expression

Procedures used for Luminex Vantage™ microRNA analysis were performed as described by the supplied protocol (Origene; Cat# AM500007). Samples containing 2 μg of total RNA were first incubated with xMAP® beads at 60 °C in a heating block for 120 min under continuous shaking at 100 rpm. The xMAP® bead mixture contained 12 different beads with unique regions used for identification; bead regions for each microRNA probe are described in Table 1; bead 49 contained probe detecting 5.8S ribosomal RNA used for microRNA expression normalization, whereas bead 99 contained a positive control. After incubation, samples containing both RNA and bead mixtures were transferred to a membrane-bottom microplate, and were washed three times in streptavidin–phycoerythrin (SAPE) diluent with gentle vacuum filtration between each wash. Samples were then incubated with 100 μL of SAPE detection reagent in the dark for 30 min while shaking at 400 rpm, followed by three more wash steps. Samples are then re-suspended in 100 μL of SAPE diluent, and analyzed by the Luminex 100 instrument at high gain setting with events/bead set to 50 with minimum events to 20.

2.4. TargetScan and DIANA-miRPath microRNA analysis

Putative microRNA targets were predicted based on two web-based bioinformatic tools TargetScan (http://www.targetscan.org) and DIANA lab (http://diana.imis.athena-innovation.gr). TargetScan is

Table 1
Mature *Xenopus* microRNA sequences. MicroRNA sequences from *X. tropicalis* (very closely related to *X. laevis*) are shown along with the corresponding xMAP® regions used in the Luminex assay and the percent homology with the comparable human microRNA species.

	XMAP® bead region	Mature sequence	Homology to human
Xtr-mir-1	1	UGGAAUGUUAAGAAGUAUGUA	90.91%
Xtr-mir-16	2	UAGCAGCACGUAAAUAUUGGUG	94.45%
Xtr-mir-21	3	UAGCUUAUCAGACUGAUGUUGA	100%
Xtr-mir-29b	4	UAGCACCAUUUGAAAUCAGUGUU	100%
Xtr-mir-34a	13	UGGCAGUGUCUUAGCUGGUUGUU	95.65%
Xtr-mir-125b	16	UCCCUGAGACCCUAACUUGUGA	100%
Xtr-mir-133a	7	UUGGUCCCCUUCAACCAGCUGU	91.30%
Xtr-mir-181a	18	AACAUUCAACGCUGUCGGUGAGU	100%
Xtr-mir-203	26	GUGAAAUGUUUAGGACCACUUG	95.45%
Xtr-mir-210	17	CUGUGCGUGUGACAGCGGCUAA	95.45%