

COMMENTARY

Insight into post-transcriptional gene regulation: stress-responsive microRNAs and their role in the environmental stress survival of tolerant animals

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ABSTRACT

Living animals are constantly faced with various environmental stresses that challenge normal life, including: oxygen limitation, very low or high temperature, as well as restriction of water and food. It has been well established that in response to these stresses, tolerant organisms regularly respond with a distinct suite of cellular modifications that involve transcriptional, translational and post-translational modification. In recent years, a new mechanism of rapid and reversible transcriptome regulation, via the action of non-coding RNA molecules, has emerged into post-transcriptional regulation and has since been shown to be part of the survival response. However, these RNA-based mechanisms by which tolerant organisms respond to stressed conditions are not well understood. Recent studies have begun to show that non-coding RNAs control gene expression and translation of mRNA to protein, and can also have regulatory influence over major cellular processes. For example, select microRNAs have been shown to have regulatory influence over the cell cycle, apoptosis, signal transduction, muscle atrophy and fatty acid metabolism during periods of environmental stress. As we are on the verge of dissecting the roles of non-coding RNA in environmental stress adaptation, this Commentary summarizes the hallmark alterations in microRNA expression that facilitate stress survival.

KEY WORDS: Anoxia, Environmental stress, Freeze-tolerance, Hibernation, Hypometabolism, MicroRNA

Introduction

When challenged with severe environmental stresses that impede normal life, a variety of animals are able to survive by suppressing their metabolic rate (i.e. hypometabolism) by 70–99%, which is accomplished by entering a state of dormancy (Guppy and Withers, 1999). Over the past few decades, much of the comparative biochemistry research on stress adaptation has focused on determining the molecular mechanisms that adapt cells and allow organisms to survive a wide array of environmental challenges (Storey and Storey, 2004). As many studies in the comparative biochemistry field are now on the verge of dissecting the role of microRNA (miRNA) and other non-coding RNA (ncRNA) in adaptation to environmental stress, this Commentary summarizes the general function of miRNA and the hallmark alterations in expression that have been implicated in regulation of specific proteins and cellular processes that are known to facilitate survival in the hypometabolic state.

The role of reversible post-translational phosphorylation in the allosteric regulation of protein function is very well established, and has been extensively studied since its initial discovery as a phospho-serine modification (Lipmann and Levene, 1932). Certainly the basic principles governing general cellular signal transduction have been derived primarily through studies of protein phosphorylation. In comparative biochemistry, the reversible phosphorylation of protein has been hallmarked as a central mechanism of metabolic reorganization, affecting the function of a diverse array of cellular proteins (e.g. metabolic enzymes, structural proteins, transcription factors and signaling proteins). To date, reversible post-translational phosphorylation has been shown to be a well-conserved mechanism as a means of modifying cellular function in numerous animal models, including hibernating squirrels (*Ictidomys tridecemlineatus*), frozen frogs (*Rana sylvatica*), anoxic turtles (*Trachemys scripta elegans*) and estivating snails (*Otala lactea*) (Brooks and Storey, 1995; Biggar and Storey, 2012; Dieni et al., 2012; Wu and Storey, 2013).

Control of protein translation

Apart from controlling the function of protein through post-translational modification, the regulation of protein activity can also be influenced through its rate of mRNA transcription and protein translation (i.e. protein abundance). In this regard, the global suppression of protein translation during hypometabolism has been shown to be a vital component of the stress response (Brooks and Storey, 1993; Land et al., 1993; Fraser et al., 2001). As such, the regulation of protein translation appears to be a protective response to deal with environmental stresses that ultimately disrupt cellular ATP production. Several *in vivo* studies have documented the decrease in protein translation rates during hypometabolism in anoxic red eared slider turtles (Brooks and Storey, 1993; Land et al., 1993; Fraser et al., 2001) and marine snails (Larade and Storey, 2002), as well as hibernating thirteen-lined ground squirrels (Frerichs et al., 1998; Hittel and Storey, 2002). One study found that the rate of protein translation in turtles following 1 h anoxia exposure (at 23°C) showed no significant changes from normoxic values. However, these rates soon decreased below measurable values when the duration of anoxia was increased to 3 h (Fraser et al., 2001). In hibernating ground squirrels, the rate of protein translation fell to 34% and 15% of euthermic values in brain and kidney tissues, respectively (Frerichs et al., 1998; Hittel and Storey, 2002). Conceivably, the inhibition of protein translation that is displayed during hypometabolism might be achieved in any combination of three ways: (1) through a reduction of the amount of mRNA substrate available; (2) through differential regulation of the activity of ribosomal translational machinery; or (3) via the recently characterized translational interference from ncRNA, namely miRNA.

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Glossary

Hypometabolism

A decrease in metabolic rate.

Mature microRNA (miRNA)

A functional small RNA (16–24 nt) that regulates the expression of target mRNA transcripts. These small RNA molecules can influence either translational repression (imperfect binding) or degradation (perfect complementarity) of the targeted mRNA. The mature microRNA is cleaved from the precursor microRNA.

Precursor microRNA (pre-miRNA)

A single-stranded hairpin non-coding RNA (60–120 nt) that is exported from the nucleus and is processed into a double-stranded RNA by Dicer endonuclease, where one strand will become the mature microRNA. The precursor microRNA is cleaved from the primary microRNA transcript.

Primary microRNA (pri-miRNA)

A non-coding RNA transcript contains (can be more than 1000 nt in length). The primary microRNA contains an RNA hairpin (precursor microRNA), in which one of the two strands includes the mature microRNA.

Post-transcriptional modification

The regulation of an RNA transcript that influences its function or stability. A classic example is the conversion of RNA transcripts into mature messenger RNA (mRNA), which includes splicing and occurs prior to protein synthesis. Post-transcriptional modification can also include a regulatory influence of non-coding RNA (such as miRNA) over the translation of mRNA.

Post-translational modification

Once translated, proteins can undergo modifications that can influence their function, activity or stability. Post-translational modifications typically include phosphorylation, acetylation, methylation, ubiquitination, among many others.

As a result of the high energetic costs of protein synthesis (approximately 5 ATP equivalents per peptide bond formed, and approximately 24% of basal ATP turnover) and mRNA transcription (2 ATP per nucleotide bond formed), one would expect to see a corresponding downregulation of both protein and mRNA production during periods of cellular stress (Hochachka et al., 1996; Podrabsky and Hand, 2000). However, neither total mRNA content, nor the individual mRNA transcript levels of many constitutively expressed genes are found to be reduced during either hibernation in the thirteen-lined ground squirrels or anoxic submergence in turtles (Epperson and Martin, 2002; Rouble et al., 2014). Furthermore, cDNA array screening of liver tissue of the turtle (control versus 5 h anoxia) revealed that 93–95% of the genes examined showed no change in mRNA transcript levels (Storey and Storey, 2004). Hence, the documented decrease in protein translation rate does not appear to be controlled by mRNA concentration. Instead, reversible control over the rate of protein synthesis during hypometabolism is a process likely to be either mediated by reversible control over translational machinery or mediated specifically through post-transcriptional regulation of mRNA transcripts. Indeed, previous research has documented an increase in the phosphorylation state of the translational elongation and initiation factor eIF2 α and the disaggregation of polyribosomes in the kidneys of hibernating thirteen-lined ground squirrels, indicating a translationally silent state (Hittel and Storey, 2002). Importantly, the phosphorylation of eIF2 α alone has been shown to be sufficient for the suppression of protein synthesis and disaggregation of polyribosomes, and may be a driving force behind

global translational silencing during hypometabolism (Yamasaki and Anderson, 2008). Certainly, tight control of ribosomal machinery is likely to be a critical part of the stress response. However, in light of a global shutdown of translational pathways (e.g. through eIF2 α phosphorylation), how are the few genes that are necessary for survival translated into functional protein? There must be alternative mechanisms of protein translation during periods of cellular stress and mechanisms to select for those genes that should, or should not, be translated. Highlighting the latter point, the regulatory influence of miRNAs provides a rapid and readily reversible mechanism that can elicit selective control over protein translation during periods of cell stress and this area has gained considerable research interest in recent years.

Reversible control of protein translation by microRNA

MicroRNAs are short, ncRNAs that are capable of reversibly regulating the expression of targeted proteins within a cell. These ~22 nt transcripts are able to bind with full or partial complementarity to mRNA targets, resulting in either the inhibition of translation or degradation of the bound mRNA. Although the majority of interactions between miRNA and mRNA result in decreased protein translation, it should be mentioned that several studies have recently documented that miRNAs can also act to increase mRNA-specific translation during specific cellular states, such as quiescence (Vasudevan et al., 2007; Saraiya et al., 2013). In a rather complex regulatory system, a single miRNA sequence may be able to target multiple mRNAs, and a single mRNA may have multiple miRNA binding sites within the transcript (Bartel, 2004). To date, it has been predicted that miRNAs regulate ~60% of the human proteome and are involved in almost every aspect of biological function throughout the animal kingdom (Friedman et al., 2009). In fact, numerous studies have now shown that miRNAs are essential for a number of biological processes, including cell development and differentiation, cell cycle, apoptosis and DNA damage repair, as well as the environmental stress response.

MicroRNA biogenesis

MicroRNA processing involves the progression of the primary (pri-miRNA) through the precursor (pre-miRNA) and finally, the functional mature miRNA molecule (Fig. 1). The pri-miRNA transcripts are typically hundreds to thousands of nucleotides in length and are either transcribed alone as an independent miRNA gene, or present within the intron of a protein-coding gene (Cai et al., 2004; Krol et al., 2010). Interestingly, direct-feedback mechanisms have been shown to exist between the intronic miRNA and the associated protein-coding gene (Zhou et al., 2008; Dill et al., 2012). For example, the zinc finger protein 265 (ZNF265) was found to encode miR-182 within an intron, while also being a regulatory target of miR-182 itself, creating a direct feedback mechanism to control ZNF265 protein translation (Zhou et al., 2008). Additional feedback loops exist between miRNA function in concert with transcription factor regulation. Shedding light on various possible models of regulatory hierarchy, several studies have documented mechanisms where miRNAs transcribed by a specific transcription factor can either act to promote the activity of the transcription factor by selectively targeting its inhibitors (i.e. positive feedback) or select the transcription factor itself for translational repression (i.e. negative feedback) (Kim et al., 2007; Okada et al., 2014). These feedback relationships can also exist directly between miRNAs and transcription factor themselves, serving to up- or down-regulate the miRNA, transcription factor, or both (Krol et al., 2010). This interplay of miRNA and transcription factors illustrates one of the many intricate levels of miRNA-influenced control over various cellular processes.

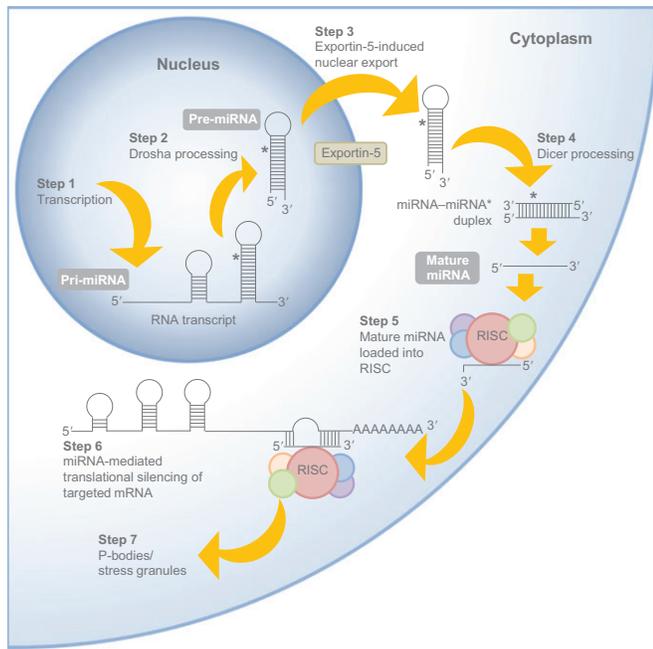


Fig. 1. Pathway of miRNA biogenesis. MicroRNAs are initially transcribed as long pri-miRNA transcripts (step 1) that are processed into pre-miRNA hairpins within the nucleus (step 2). Pre-miRNA hairpins are exported into the cytoplasm (step 3), where they are processed into mature miRNA (step 4) and loaded into the RNA induced silencing complex (RISC; step 5). Mature miRNAs then guide the RISC to target select mRNA transcripts for translational silencing or degradation (steps 6 and 7). Figure modified from Biggar and Storey (2011).

Following pri-miRNA transcription, the ribonuclease protein Drosha recognizes the pri-miRNA terminal stem-loop structure and cleaves the flanking sequences to produce a 50–70 nt double-stranded RNA molecule (i.e. pre-miRNA) (Fig. 1, steps 1 and 2) (Zeng et al., 2005; Morlando et al., 2008; Krol et al., 2010). Interestingly, the pri-miRNA cleavage sites for Drosha bear no sequence homology to each other, and instead, the terminal loop structure of the pri-miRNA is responsible for the recognition of what will become the pre-miRNA molecule (Zeng et al., 2005). Importantly, pri-miRNA processing by Drosha creates the pre-miRNA containing a RNA stem-loop (i.e. hairpin) with a 3' overhang of two nucleotides (Fig. 1, step 2) (Zeng et al., 2005; Krol et al., 2010). Similar to recognition and cleavage by Drosha, sequence specificity is not required for nuclear export, instead the presence of double-stranded RNA with an exposed 3' overhang is enough to allow binding by Exportin-5 and its exit to the cytoplasm (Fig. 1, step 3) (Yi et al., 2003; Zeng et al., 2005).

Upon delivery of the pre-miRNA molecule to the cytoplasm, further processing occurs once recognized by ribonuclease protein, Dicer. Dicer works by cleaving the pre-miRNA molecule 18–25 nt away from the exposed end of the stem-loop, leaving just an RNA duplex (Zeng et al., 2005). It is believed that from this duplex only one strand will function as a mature miRNA, with the passenger strand labeled as miRNA* and subsequently degraded (Krol et al., 2010; Hu et al., 2009). However, miRNA* sequences have also been found to be abundantly expressed and can function as mature miRNAs as well, targeting a different set of mRNA than the corresponding miRNA sequence (Griffiths-Jones et al., 2006; Almeida et al., 2012). To avoid confusion between can function as and degraded miRNA*, the miRNA/miRNA* nomenclature is reserved for duplexes that express mature miRNAs unequally, whereas precursors that equally express two functional mature

miRNAs have been since renamed using miR-5p and miR-3p nomenclature, which is designated according to the 5'- or 3'-arm of the pre-miRNA on which the mature miRNA is processed.

Once processed, the mature miRNA is loaded into the RNA-induced silencing complex (RISC). The RISC is a protein-RNA complex that is guided by mature miRNA to target mRNA, mediating either inhibition of protein translation or degradation of the targeted mRNA (Fig. 1, steps 6 and 7). Importantly, the RISC has been shown to also include a class of GW182 proteins, thought to function in mRNA storage and the repression of mRNA translation (Pauley et al., 2006). Interestingly, GW182 proteins, as well as the complete miRNA-RISC and bound mRNA, have all been found to aggregate into either P-bodies or stress granules during periods of cell stress. Within these aggregates, miRNA-repressed mRNAs accumulate and are eventually either degraded, or re-enter the protein translation process (Pauley et al., 2006).

Recognition of microRNA target sites

MicroRNA function is primarily directed by nucleotide complementarity between the miRNA and target mRNA sequence. In this regard, immediately following the first nucleotide of the mature miRNA, the next seven (i.e. positions 2–8) starting from the 5' end are known to comprise the 'seed region' and are responsible for dictating the binding within the 3' UTR of the target mRNA (Friedman et al., 2009). Five primary types of binding patterns exist, each with varying binding efficacy, and have been defined based on the positions of nucleotide binding between the miRNA and target mRNA (Fig. 2) (Friedman et al., 2009). Canonical sites that show perfect matching between both the mRNA and the seed region of the mature miRNA, in addition to: (1) an adenosine at position 1 (i.e.

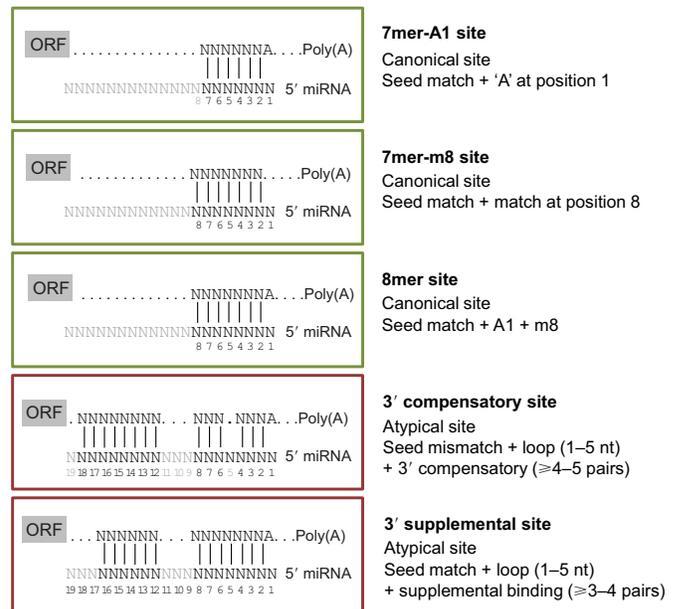


Fig. 2. Types of miRNA–target interaction. Canonical sites show perfect matching between both the mRNA and nucleotides 2–7 (i.e. seed region) of the mature miRNA and either adenosine at position 1 (7mer-A1 site), a match at position 8 with (8mer site) or without adenosine at position 1 (7mer-m8 site). An 8mer site binding can also be supplemented with complementary binding at the 3' end of the miRNA to strengthen miRNA–target interaction and regulate mRNA fate (3' supplemental site). Imperfect binding in the miRNA seed region can also be overcome by a high degree of complementary binding at the 3' end of the miRNA, favorably influencing the overall binding energetics of the miRNA–target interaction (3' compensatory site).

7mer-A1 site); (2) a match at position 8 with (i.e. 8mer site); or (3) without adenosine at position 1 (i.e. 7mer-m8 site). An 8mer site binding can also be supplemented with complementary binding at the 3' end of the miRNA to influence miRNA–target interaction and mRNA fate (i.e. 3' supplemental site). Imperfect binding within the seed region can also be overcome by a high degree of complementary binding at the 3' end of the miRNA, favorably influencing the overall binding energetics of the miRNA–target interaction (i.e. 3' compensatory site).

In general, the mature miRNA and corresponding target binding sequences within the 3' UTR are very well conserved. Within many vertebrate species, both miRNA and mRNA target binding sites are commonly found to be 90–100% conserved, even in the presence of low 3' UTR conservation, suggesting the importance of the miRNA–target interaction (Friedman et al., 2009; Biggar and Storey, 2012). It should be noted that the conservation of miRNA binding within the 3' UTR of target genes can also be confirmed for many species by searching the TargetScan database (<http://www.targetscan.org>). The high degree of conservation between a miRNA and its mRNA binding site suggests the possibility of analyzing the expression patterns of specific miRNA in response to environmental stresses in many

different species that may not have sequence information available. Indeed, our lab has developed the use of stem–loop nucleotide adapter sequences for amplification, expression analysis and sequencing of mature miRNAs in species with or without available genomic information or annotated miRNA sequences (Biggar et al., 2011, 2014). Using these methods, our lab has successfully explored the dynamic regulation of mature miRNA in response to a variety of environmental stress within a range of tolerant animals. For example, the response of mature miRNA has been documented for the tissues of ground squirrels, bats, frogs and turtles (Morin et al., 2008; Biggar et al., 2009, 2012; Biggar and Storey, 2011, 2014a; Kornfeld et al., 2012; Maistrovski et al., 2012; Wu et al., 2014a,b). However, it must be noted that this high degree of mature miRNA conservation is primarily restricted to vertebrate organisms, and invertebrate miRNA studies require further optimization given the limited information and high variability of miRNA sequences (Biggar et al., 2011). Although the detection and quantification of invertebrate miRNAs is typically more difficult than for vertebrates, it has been carried out in both freeze-tolerant intertidal snails (*Littorina littorea*) and in cold-hardy insects (*Epiblema scudderiana* and *Eurosta solidaginis*) (Biggar et al., 2012; Courteau et al., 2012; Lyons et al., 2013).

Table 1. miRNAs that have been shown to be dynamically regulated in response to hibernation, freezing, anoxia or estivation

Stress	Species	Tissue	miRNA	Reference		
Hibernation	<i>Ictidomys tridecemlineatus</i>	Heart	Down: <i>miR-24</i>	Morin et al., 2008		
		Skeletal muscle	Up: <i>miR-1a</i> , <i>miR-21</i> , <i>miR-142-5p</i> , <i>miR-144</i> , <i>miR-451</i> , <i>miR-486</i> Down: <i>miR-24</i> , <i>miR-122a</i> , <i>miR-106b</i>	Morin et al., 2008; Maistrovski et al., 2012		
		White adipose	Up: <i>miR-143</i> , <i>miR-519d</i> Down: <i>let-7a</i> , <i>let-7b</i> , <i>miR-31</i> , <i>miR-107</i> , <i>miR-150</i> , <i>miR-222</i>	Wu et al., 2014b		
		Brown adipose	Up: <i>miR-138</i> Down: <i>miR-21</i> , <i>miR-31</i> , <i>miR-103a</i> , <i>miR-107</i> , <i>miR-125b</i> , <i>miR-221</i>	Wu et al., 2014b		
	<i>Spermophilus parryii</i>	Liver	Up: <i>miR-106b</i>	Maistrovski et al., 2012		
		Liver	Up: <i>miR-1a</i> , <i>miR-142-5p</i> , <i>miR-144</i> , <i>miR-451</i> , <i>miR-486</i> Down: <i>miR-152</i> , <i>miR-184</i> , <i>miR-320</i> , <i>miR-378</i>	Liu et al., 2010		
	<i>Myotis lucifugus</i>	Skeletal muscle	Up: <i>miR-1a</i> , <i>miR-15a</i> , <i>miR-20a</i> , <i>miR-23a</i> , <i>miR-29b</i> , <i>miR-128</i> , <i>miR-181b</i> , <i>miR-206</i> Down: <i>miR-21</i> , <i>miR-106b</i>	Kornfeld et al., 2012		
			Liver	Down: <i>miR-106b</i>	Maistrovski et al., 2012	
		Freezing	<i>Rana sylvatica</i>	Muscle	Up: <i>miR-21</i> Down: <i>miR-16-1</i>	Biggar et al., 2009
			<i>Littorina littorea</i>	Liver	Up: <i>miR-21</i> , <i>miR-16-1</i>	Biggar et al., 2009
Foot muscle	Up: <i>miR-1a</i> , <i>miR-2a</i> , <i>miR-29b</i> , <i>miR-34a</i> , <i>miR-125b</i> , <i>miR-133a</i>	Biggar et al., 2012				
Anoxia	<i>Littorina littorea</i>	Hepatopancreas	Up: <i>miR-1a</i> , <i>miR-29b</i> , <i>miR-34a</i>	Biggar et al., 2012		
		Larvae	Up: <i>miR-1a</i> , <i>miR-92c-3p</i> , <i>miR-284</i> , <i>miR-3791-5p</i> Down: <i>miR-11</i> , <i>miR-34</i> , <i>miR-71</i> , <i>miR-276</i> , <i>miR-277-3p</i> , <i>miR-2543b</i> , <i>miR-3742</i>	Courteau et al., 2012; Lyons et al., 2013		
	<i>Trachemys scripta elegans</i>	Foot muscle	Up: <i>miR-1a</i> , <i>miR-29b</i> , <i>miR-2a</i> , <i>miR-34a</i> , <i>miR-133a</i>	Biggar et al., 2012		
		Hepatopancreas	Up: <i>miR-1a</i> , <i>miR-210</i> Down: <i>miR-2a</i> , <i>miR-29b</i> Up: <i>miR-15a</i> , <i>miR-16-1</i>	Biggar et al., 2012		
Estivation	<i>Xenopus laevis</i>	Kidney	Up: <i>miR-15a</i> , <i>miR-16-1</i>	Biggar and Storey, 2011		
		Liver	Up: <i>miR-21</i> , <i>miR-34a</i> , <i>miR-210</i> Down: <i>miR-1</i> , <i>miR-16-1</i> , <i>miR-125b</i> , <i>miR-133a</i>	Wu et al., 2014a		
		Kidney	Up: <i>miR-21</i> , <i>miR-29b</i> , <i>miR-203</i>	Wu et al., 2014a		
	<i>Apostichopus japonicus</i>	Ventral skin	Up: <i>miR-34a</i> , <i>miR-203</i>	Wu et al., 2014a		
		Respiratory tree	Up: <i>let-7a</i> , <i>miR-124</i> , <i>miR-1b</i> , <i>miR-2010</i> , <i>miR-2013</i> , <i>miR-252a</i> , <i>miR-716b</i> , <i>miR-71a</i> , <i>miR-73b</i> , <i>miR-79</i> , <i>miR-9</i> , <i>miR-92</i> Down: <i>miR-100</i> , <i>miR-133a/b/c/d</i> , <i>miR-222</i> , <i>miR-29b</i> , <i>miR-33a</i>	Chen and Storey, 2014		

MicroRNA regulation during stress adaptation

Highlighting the role of miRNAs in the regulation of the hypometabolic state, studies have documented stress-responsive miRNA expression during mammalian hibernation (Morin et al., 2008; Kornfeld et al., 2012; Maistrovski et al., 2012; Biggar and Storey, 2014a), frog and insect freeze tolerance (Biggar et al., 2009; Courteau et al., 2012) and turtle and marine snail anoxia tolerance (Table 1) (Biggar et al., 2012; Biggar and Storey, 2012). These studies collectively indicate a conserved principle of miRNA stress-response across phylogeny. Since it is vitally important to rapidly and readily reduce the activity of ATP-costly processes in a coordinated and reversible fashion during the stress response, it is possible that miRNAs aid in the reprioritization of ATP use and stress-specific cellular adaptation. In this manner, miRNA can act as a rapid and reversible mechanism to halt the translation of unnecessary proteins (and their associated functions). For example, miRNAs have been shown to be differentially regulated during natural freeze tolerance in the wood frog (Biggar et al., 2009). The expression of two selected miRNA species, *miR-16-1* and *miR-21*, was evaluated in liver and skeletal muscle in response to freezing (24 h at -3°C). Levels of *miR-21* were found to increase significantly during freezing in liver and skeletal muscle, whereas *miR-16-1* transcripts increased significantly in the liver of frozen frogs, but fell by 50% in skeletal muscle. The mRNA transcripts targeted by *miR-21* include caspase-3 (*casp3*), apoptotic protease activating factor-1 (*apaf1*) and programmed cell death protein-4 (*pcna4*) and may have an anti-apoptotic role to facilitate during freeze stress in the frog. The role of *miR-16-1* has also been linked with the repression of cell growth and cell cycle arrest (Biggar and Storey, 2012). Liver and skeletal muscle tissues are two examples of a proliferating and terminally differentiated cell types, respectively, and their associated miRNA expression may be reflective of the tissue-specific function (i.e. repression of cell proliferation by *miR-16-1* in liver tissue). Several miRNAs have also been found to be over-expressed following a 24 h period of either freezing (*miR-1a-1*, *miR-34a*, *miR-133a*, *miR-125b*, *miR-29b* and *miR-2a*) or anoxia (*miR-1a-1*, *miR-34a*, *miR-133a*, *miR-29b* and *miR-2a*) in the foot muscle of a marine intertidal snail (Biggar et al., 2012). The miRNAs assessed in this study focused on those known to be involved in the regulation of cell cycle, cell signaling pathways (such as PI3K/Akt and NF- κ B), carbohydrate metabolism and apoptosis.

Apart from freeze and anoxic stresses, the first study examining the response of miRNA to hypometabolism was explored in response to winter torpor in the thirteen-lined ground squirrel (Morin et al., 2008). This study reported that differential regulation of miRNA occurs in response to mammalian hibernation and that miRNA could provide a mechanism for reversible gene silencing during torpor (Morin et al., 2008). More recently, a study utilized massively parallel Illumina sequencing technology to sequence the small RNAs expressed in the liver of the hibernating Arctic ground squirrel, *Spermophilus parryii* (Liu et al., 2010). This study identified and annotated >200 ground squirrel miRNAs, including 18 novel miRNAs that are currently thought to be specific to the ground squirrel. It is intriguing to hypothesize that the existence of novel miRNAs within the *S. parryii* genome may play a fundamental torpor-specific role in the hibernating animal; however, these functions have not yet been determined.

Reversible gene regulation by microRNA

Many of the initial studies exploring the role of miRNA in response to environmental stresses focused on the dynamic expression of

only a few miRNAs, providing modest insight to their functional role or explored whether miRNA expression was inversely correlated to the protein expression of the proposed target gene (Morin et al., 2008; Biggar et al., 2009). Addressing this concern, more-recent comparative miRNA studies have begun to explore their gene-specific function, yielding greater functional insight into the roles that miRNA may play during stress adaptation (Biggar and Storey, 2012; Maistrovski et al., 2012). For example, one study examining the regulation of *cyclin d1*, a gene that is critical in the initiation and progression of the cell cycle, highlighted the role of two miRNAs, *miR-15a* and *miR-16-1*, in the control of *cyclin d1* translation during anoxia in turtles (Biggar and Storey, 2012). To explore the possibility of post-transcriptional mechanisms of *cyclin d1* regulation, the 3'UTR of *cyclin d1* from the turtle was sequenced and found to contain two conserved miRNA binding sites for *miR-16-1* and *miR-15a*. Both the increased expression of *miR-15a* and *miR-16-1* in liver and kidney tissue under anoxic conditions, and the analysis of turtle-specific RNA binding structure and thermodynamics, implicated miRNA as the likely mechanism mediating a decrease in Cyclin D1 protein expression and subsequent cell cycle control in the anoxic turtle (Biggar and Storey, 2012).

The roles of various types of ncRNAs in the regulation of proteins involved in cell adaptation and metabolic regulation will probably become a crucial area for future study by comparative biochemists. For example, a previous study explored two types of ncRNA [long ncRNA (lncRNA) and miRNA] in regulation of the hypoxia-inducible factor HIF-1 α during hibernation in torpid little brown bats and thirteen-lined ground squirrels (Maistrovski, et al., 2012). This study of ncRNA regulation of HIF-1 α highlighted the role of a natural antisense transcript lncRNA for HIF-1 α (known as *ahif*), as well as a role for miRNA (namely *miR-106b*), both of which repress HIF-1 α protein expression (Thrash-Bingham and Tartof, 1999; Volinia et al., 2006). Furthermore, this study documented for the first time that two different ncRNA types, *ahif* and *miR-106b*, changed significantly when either ground squirrels or bats entered torpor, providing a new mechanisms of natural post-transcriptional regulators of *hif-1 α* gene expression (Maistrovski et al., 2012). Together, these results demonstrate that ncRNA molecules, as a regulatory group, may have widespread importance in multiple comparative models of hypometabolism.

MicroRNA control over cellular pathways and processes

It is now known that a single miRNA molecule has the potential to target hundreds of distinct genes, while a single gene may be the collective target of multiple miRNA. This feature allows for an enormous number of different combinatorial possibilities that could each have a different regulatory outcome. However, this high degree of regulatory potential can be refined when considering that many miRNAs are transcribed in families that collectively target whole cellular processes or pathways. Indeed, many previous comparative biochemistry studies that have examined the stress-induced regulation of miRNA have been justifiably simplified, focusing on the targeting and regulation of a gene(s) by select miRNA(s) (Morin et al., 2008; Biggar et al., 2009; Biggar et al., 2012). For example, *miR-21* has been studied in multiple animals in the context of regulating a number of anti-apoptotic genes in response to environmental stresses and has since been suggested to be a conserved miRNA component of the hypometabolic response to environmental stresses (Table 1) (Biggar et al., 2009; Wu et al., 2014a; Biggar and Storey, 2012, 2011; Morin et al., 2008). However, studies have recently begun

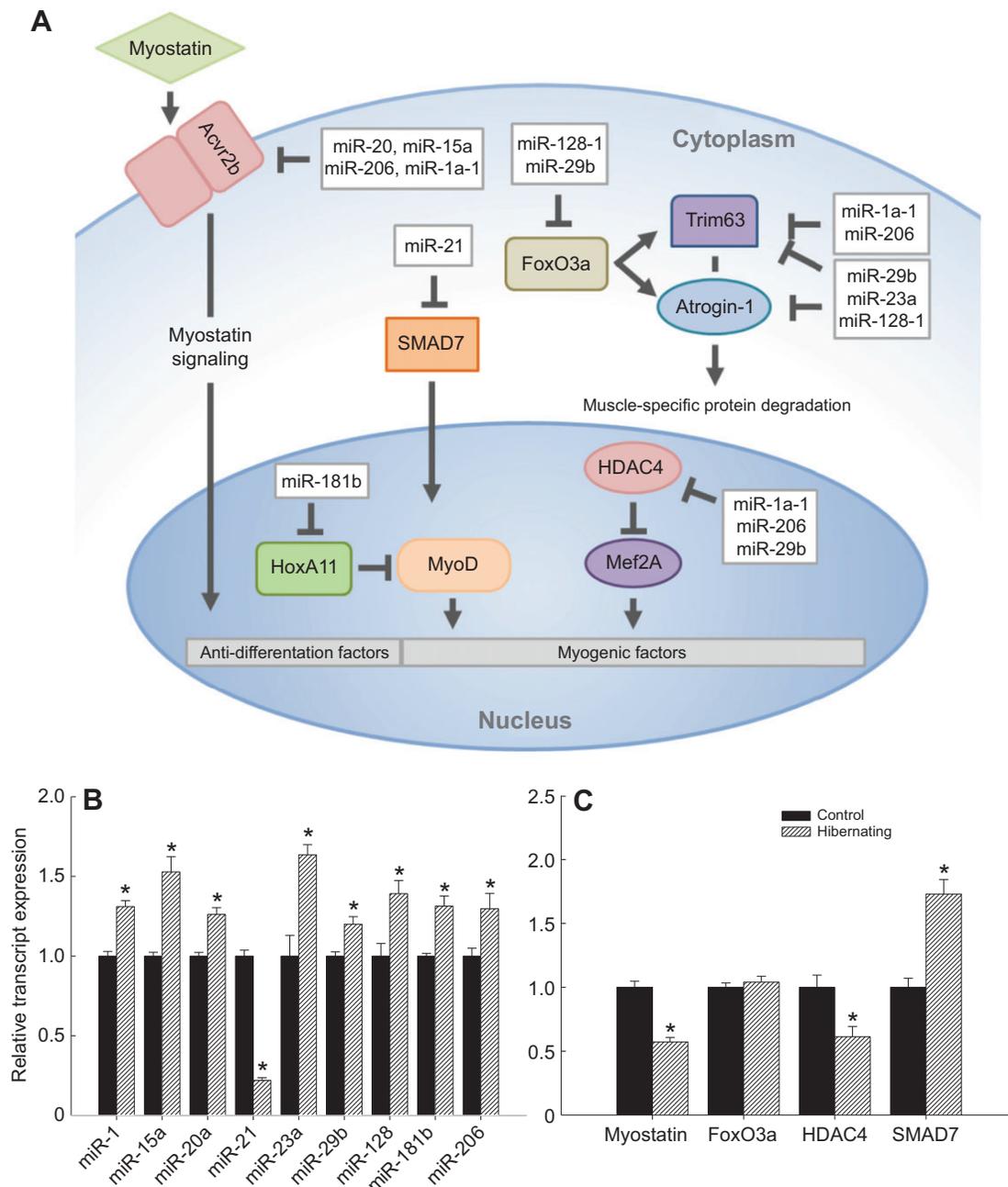


Fig. 3. MicroRNAs proposed to be involved in muscle atrophy resistance in little brown bats. (A) Proposed mechanism of the miRNA-influenced regulation of proteins involved in the expression of muscle regulatory factors. (B) Relative expression of *miR-21*, *miR-1a-1*, *miR-29b*, *miR-107*, *miR-23a*, *miR-181b*, *miR-15a*, *miR-20a*, *miR-128* and *miR-206*. Expression of the indicated miRNAs was normalized to that of 5S rRNA from the same pectoral muscle RNA samples and shown in histogram. (C) Relative expression of Dicer, Myostatin, FoxO3a, HDAC4 and SMAD7 protein between control and hibernating states. Data are presented as means±s.e.m. of independent samples from separate animals (N=3–4). * $P < 0.05$, significantly different from controls. Figure modified from Kornfeld et al. (2012).

to move away from candidate-based miRNA analysis (i.e. whether a particular miRNA is able to regulate a specific target) and are now addressing the ability of miRNA to collectively target and regulate cellular processes (Papadopoulos et al., 2009; Vlachos et al., 2012; Kornfeld et al., 2012; Wu et al., 2014a,b; Biggar and Storey, 2014a). For example, such studies have documented the role of miRNAs in the prevention of muscle atrophy in skeletal muscle (Fig. 3), as well as focal adhesion and axon guidance in the brain of hibernating bats (Biggar and Storey, 2014a; Kornfeld et al., 2012). Another study examining the role of miRNAs in fatty

acid metabolism in the adipose tissues of torpid thirteen-lined ground squirrels found that differentially expressed miRNA collectively regulated mitogen-activated protein kinase (MAPK) signaling in both brown (BAT) and white adipose tissue (WAT), while also possibly regulating transforming growth factor beta (TGF- β) signaling in WAT (Wu et al., 2014b). The pattern of miRNA expression in WAT parallels some of the previous findings of miRNA dysregulation in obese humans; meanwhile, miRNAs regulated in BAT were predicted to target β -oxidative enzymes and uncoupling proteins in the mitochondria, suggesting

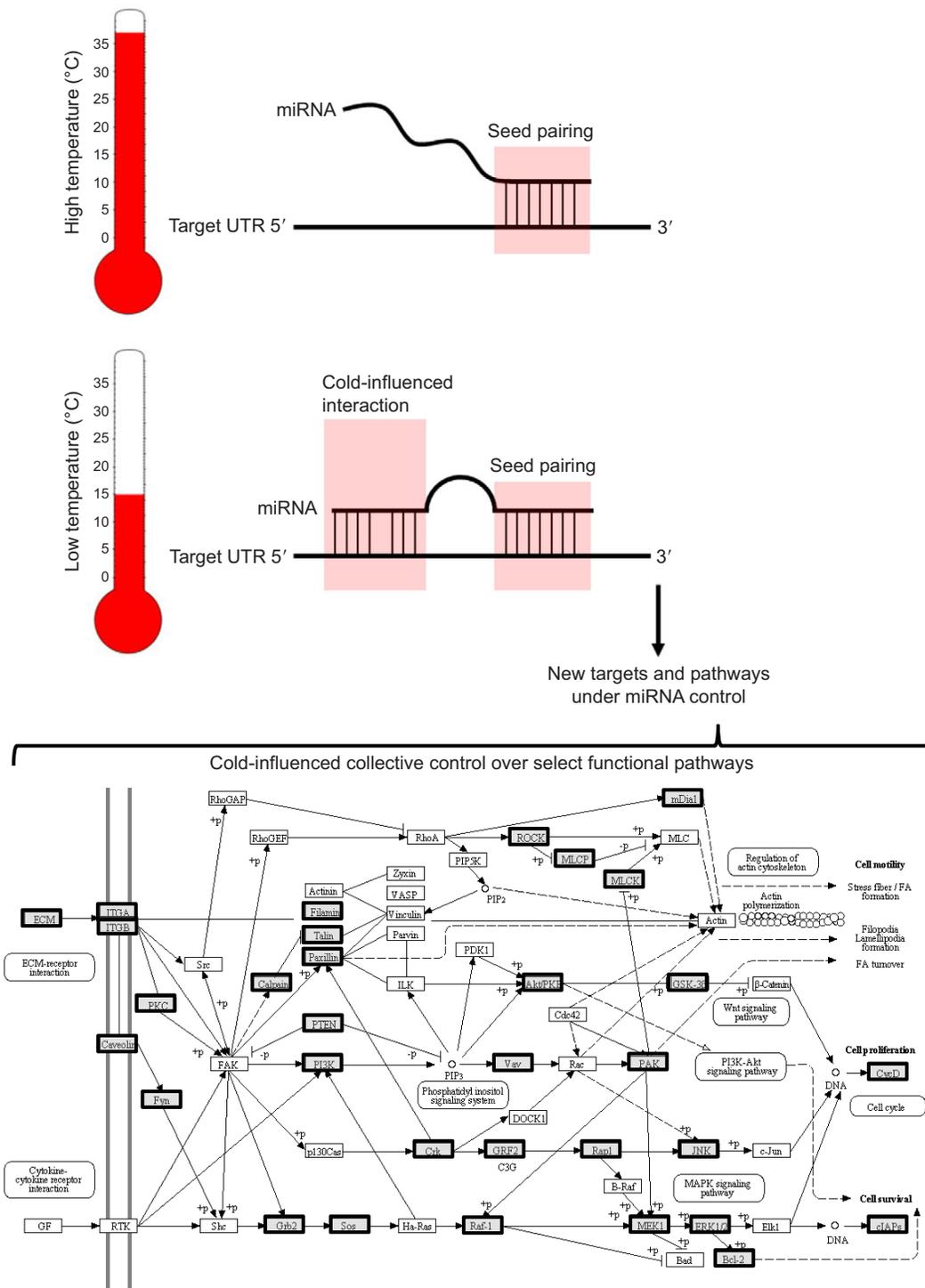


Fig. 4. Mechanism of possible temperature influence on miRNA expression and function. Proposed mechanism of cold-influenced miRNA targeting. Low temperature, such as that experienced during mammalian hibernators, may act to substantially expand upon the possible miRNA–target interactions and collective pathways under miRNA regulation that were once unstable at 37°C. Figure modified from Biggar and Storey (2014b).

a potential role of miRNAs in the regulation of non-shivering thermogenesis.

Perspectives – low-temperature microRNA function

Given the state of miRNA research within the field of comparative biochemistry and the functional insight that it has provided to date, it is becoming clear that these regulatory ncRNAs are an important component to environmental stress survival and the

hypometabolic response. However, several concerns must be noted regarding the prediction of miRNA targets because almost all prediction algorithms are based on a T_b of 37°C – a temperature found only in mammals and not commonly experienced by many other organisms. The temperature dependency behind the thermodynamics of miRNA–target binding has received little attention, but is likely to provide new and critical insight into the stress-responsive, and perhaps novel, miRNA–target interactions

that are part of the environmental stress response of tolerant animals.

As previously discussed, miRNA target recognition relies on nucleotide pairing primarily within the miRNA seed region, with complementary binding from the 3' end of the miRNA acting to stabilize and supplement the interaction (Fig. 2). Critically, the thermodynamic threshold [approximately $-18 \text{ kcal mol}^{-1}$ (mean free energy)] for miRNA–target binding has been determined at a T_b of 37°C for almost all target prediction programs. However, it is likely that a decrease in T_b (such as experienced by hibernating mammals, frozen frogs and many overwintering animals) will favorably stabilize select miRNA–target interactions that were once unfavorable, allowing these interactions to become biologically relevant at low T_b (such as 5°C). Indeed, miRNA target prediction using FindTar3 (a miRNA target prediction program that can account for temperature change) shows a ~ 10 -fold increase in the number of potential mRNA targets when comparing target prediction at 5°C and 37°C (K.K.B., unpublished). The realization that a decrease in T_b can dramatically change the scope of miRNA–target interactions by an order of magnitude, introduces the possibility that distinct cold-influenced miRNA–target interactions could arise and facilitate unique hypometabolic-specific roles to coordinate cellular processes and maintain the stress response (Fig. 4). It is important to note that the seed pairing of the miRNA would still be required and that low temperature would primarily influence the 3'-end complementary binding (i.e. 3' compensatory site) to stabilize and supplement the miRNA–target pair. Further influencing low temperature function through binding of the miRNA, analysis of the average GC content in the seed regions of all mature miRNA (available at miRBase.org) among species with varying T_b showed that GC content decreases with T_b (Carmel et al., 2012). Together, these findings support the hypothesis that there may be adaptations to miRNA binding mechanisms to function at low temperatures, perhaps even altering the mRNAs targeted. Thus, the likelihood that miRNA could play an important role in helping various species to cope with temperature-related stress is likely to attract substantial interest in the future and studies should start to consider the possible influence of temperature on miRNA targeting.

Conclusion

Many human miRNAs (2588 mature and 1881 precursors) have been released in the latest release of miRBase (version 21), yet similar numbers are sparse in non-human species and many miRNA sequences (as well as their function) still remain to be identified. In this regard, the identification of miRNA in many non-human species has recently been receiving significant attention and will play an important role in future comparative miRNA research. As the function of miRNA in the hypometabolic response to environmental stress is further defined, it is likely that the miRNA response will become established as a critical post-transcriptional mechanism to reduce ATP expenditure alongside the post-translational modification of protein. The use of miRNA to influence protein expression would also allow the rapid translation of stored mRNA, reducing the need to re-transcribe mRNA once the stress is removed. Furthermore, the possibly of cold-influenced miRNA targets opens comparative miRNA research to an immense number of regulatory possibilities that will need to be explored in future studies.

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Author contributions

K.K.B. and K.B.S. both conceived and wrote this article.

Competing interests

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