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REVIEW

The OxymiR response to oxygen limitation: a comparative microRNA perspective

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ABSTRACT

From squid at the bottom of the ocean to humans at the top of mountains, animals have adapted to diverse oxygen-limited environments. Surviving these challenging conditions requires global metabolic reorganization that is orchestrated, in part, by microRNAs that can rapidly and reversibly target all biological functions. Herein, we review the involvement of microRNAs in natural models of anoxia and hypoxia tolerance, with a focus on the involvement of oxygen-responsive microRNAs (OxymiRs) in coordinating the metabolic rate depression that allows animals to tolerate reduced oxygen levels. We begin by discussing animals that experience acute or chronic periods of oxygen deprivation at the ocean's oxygen minimum zone and go on to consider more elevated environments, up to mountain plateaus over 3500 m above sea level. We highlight the commonalities and differences between OxymiR responses of over 20 diverse animal species, including invertebrates and vertebrates. This is followed by a discussion of the OxymiR adaptations, and maladaptations, present in hypoxic high-altitude environments where animals, including humans, do not enter hypometabolic states in response to hypoxia. Comparing the OxymiR responses of evolutionarily disparate animals from diverse environments allows us to identify species-specific and convergent microRNA responses, such as miR-210 regulation. However, it also sheds light on the lack of a single unified response to oxygen limitation. Characterizing OxymiRs will help us to understand their protective roles and raises the question of whether they can be exploited to alleviate the pathogenesis of ischemic insults and boost recovery. This Review takes a comparative approach to addressing such possibilities.

KEY WORDS: Anoxia, Hypoxia, High altitude, Metabolic rate depression, miR-210, miRNA

Introduction

Oxygen is the basis for life on Earth. However, animals have evolved to survive varying degrees of acute and chronic oxygen deprivation, on both short and long timescales (Larson et al., 2014). From anaerobes that thrive in complete anoxia by relying on creative fermentative mechanisms to satisfy their energetic requirements, to those that have made hypoxic high-altitude mountains their home: where there is an environment, there are organisms that have evolved a suite of adaptations to thrive and survive. In the case of oxygen-limited systems, animal adaptations have been shown to be either reactive, in immediate response to an acute oxygen-limited bout, or preparatory, where animals have evolved complex mechanisms to endure prolonged hypoxic/anoxic periods (Larson et al., 2014; Giraud-Billoud et al., 2019). In both cases, the metabolic consequences and anaerobic end-products resulting from partial or total restriction of oxygen must immediately be dealt with. This typically occurs through the activation of pro-survival mechanisms, coupled with the suppression of metabolic rate in an effort to re-prioritize ATP expenditure for vital cellular functions (Krivoruchko and Storey, 2010). Suppression of metabolic rate reduces ATP demand to a level that can be met by the less-efficient anaerobic ATP-generating pathways, giving vertebrates and invertebrates a method to circumvent oxygen limitation (Krivoruchko and Storey, 2015).

Large-scale genomic and transcriptomic studies of animals exposed to oxygen deprivation have identified networks of protein-coding genes and non-coding RNA sequences implicated in organismal stress responses, highlighting both the unified and species-specific nature of these responses (Valero et al., 2019preprint). These stress responses require the coordination of biochemical and molecular mechanisms from all cellular regulatory levels, including epigenetic modification of DNA and histones, transcription factor activity, post-transcriptional regulation, translational capacity and post-translational modifications (Storey, 2015). One regulatory factor that is capable of targeting all biological systems and modulating the complex networks required for successful and reversible stress responses is the microRNA (miRNA) system.

MicroRNAs are short (~22 nt) non-coding RNA molecules that rely on sequence complementarity to temporarily or permanently suppress the translation of specific mRNA transcripts (Bartel, 2004) (Fig. 1). In humans, this large group of RNAs is predicted to target more than 60% of protein-coding genes, thereby allowing them to impact virtually all biological functions (Ebert and Sharp, 2012). Indeed, miRNAs have been implicated in development, cell growth, metabolism, apoptosis, cancer and numerous other central processes (Gebert and MacRae, 2019). Furthermore, miRNAs are highly conserved across metazoans, which is also demonstrated by the 90–100% conservation of miRNA-binding regions in mRNA 3′ untranslated regions (3′-UTRs) despite overall low 3′-UTR sequence conservation (Friedman et al., 2009). Refer to Box 1 for more information on miRNA biogenesis and their mode of action.

MicroRNAs can post-transcriptionally regulate gene expression by either temporarily suppressing mRNA translation by transcript storage, or through the permanent degradation of mRNA transcripts (Bartel, 2004) (Fig. 1); but what is it about miRNAs that makes them such excellent stress regulators? From the research available, there appear to be five key inherent properties that make miRNAs good regulators of metabolic reprogramming: (1) they show reversible regulation, (2) they are capable of rapid targeting, (3) they have broad control over virtually all biological processes, (4) they are energetically inexpensive to synthesize and (5) there is a high degree of miRNA sequence conservation among different species (Storey,

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Glossary

Brackish water
When saline water (35–50 p.p.t. dissolved salt) from the sea or ocean mixes with fresh water (<0.5 p.p.t.), this results in brackish water (0.5–35 p.p.t.) of intermediate saltiness. This water-mixing phenomenon typically occurs in estuaries and mangroves.

Estivation (or aestivation)
This is an animal survival strategy characterized by inactivity, which involves entry into a hypometabolic state to survive hot, arid conditions. This dormancy occurs in aquatic and terrestrial animals, both in vertebrates and invertebrates, to prevent desiccation through the use of numerous physiological and biochemical adaptations that conserve energy and water.

Exosomes
Exosomes are small lipid vesicles (30–200 nm) produced from endosomal compartments that are excreted by a variety of cells. They are enriched with selected proteins, lipids and nucleic acids, including microRNAs. They have recently emerged as intracellular communicators that can be exploited for the development of non-invasive diagnostics and therapeutic drug delivery.

Hypoxic preconditioning
This is a phenomenon whereby animals exposed to short periods of moderate hypoxia are protected against cerebral and cardiac ischemia.

Ischemic injury
Ischemia is the restriction of blood flow to tissues, typically caused by damage to blood vessels. This leads to an oxygen shortage that affects the tissue's ability to meet its metabolic needs, thereby resulting in the dysfunction of the affected area.

OxymiRs
These are a subset of oxygen-responsive miRNAs that are differentially expressed under hypoxia and/or anoxia. Chronic or acute oxygen limitation can result in pro-survival or maladaptive miRNA and gene network regulation.

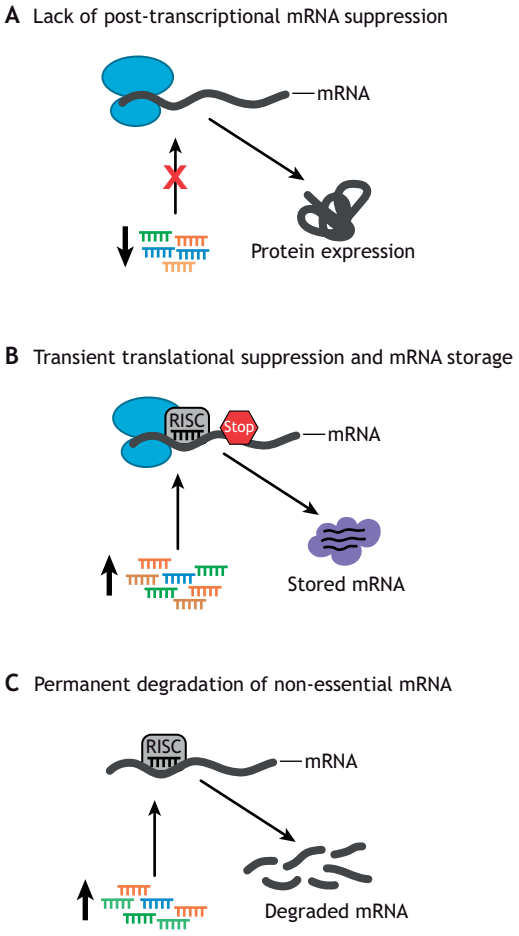

Fig. 1. Consequences of microRNA (miRNA) targeting for gene and protein expression. (A) The reduced expression of miRNAs during stress conditions can act to promote the translation of mRNAs that encode protective, pro-survival proteins, by removing miRNA-induced inhibition. (B) Imperfect miRNA complementarity with mRNA transcripts temporarily suppresses translation and directs mRNA transcripts into temporary storage in stress granules and processing bodies until normoxic conditions return. (C) Perfect miRNA complementarity to target mRNA transcripts induces permanent mRNA degradation of non-essential genes. This allows miRNAs to limit energy-expensive protein translation to vital genes. RISC, RNA-induced silencing complex.

2015). Herein, we discuss the involvement of miRNA in supporting anoxia and hypoxia tolerance in diverse environments, ranging from underwater to terrestrial all the way up to high-altitude habitats (Table 1). By broadening the scope of this Review to include both uniquely adapted anoxia-tolerant animals and more sensitive hypoxia-tolerant species, we can draw conclusions regarding the nature and evolutionary conservation of oxygen-responsive miRNAs (OxymiRs, see Glossary). We also focus on the involvement of OxymiRs in metabolic rate depression and the various functions they facilitate in hypometabolic states. Finally, we consider the potential involvement of OxymiRs in medical applications.

OxymiRs and oxygen restriction in the marine environment

Jumbo squids

We begin our exploration of anoxia- and hypoxia-responsive microRNAs at the bottom of the ocean: specifically, 300 m deep, at the oxygen minimum zone, where organisms are confronted with frigid temperatures, high pressure and low dissolved oxygen levels of ∼10 µM O_2, compared with ∼280 µM dissolved O_2 at the ocean's surface (Rosa and Seibel, 2010). Investigations of miRNAs from inhabitants of the ocean's depths are scarce, as are most studies on these elusive creatures, but a relevant study was performed on hypoxic jumbo squid (*Dosidicus gigas*, also known as red devil squid). At night, jumbo squid feed at the ocean's surface but, come daylight, these squid descend hundreds of meters, where they slow down, digest and avoid predators (Seibel et al., 2014). This vertical migration exposes them to daily episodes of severe hypoxia that they survive by entering a hypometabolic state characterized by the suppression of metabolic rate by 52%, relative to normoxic levels, measured as the sum of ATP derived from pathways that result in anaerobic energy equivalents and ATP derived from oxidative phosphorylation (Rosa and Seibel, 2010). By examining a group of 39 highly conserved miRNAs in the brain, branchial hearts and mantle muscle of jumbo squid, researchers identified a miRNA subset that appears to be partially responsible for the metabolic flexibility observed in response to hypoxic conditions (Hadj-Moussa et al., 2018).

All hypoxia-responsive miRNAs in the squid brain are up-regulated during exposure to hypoxia. Similarly, in heart tissue, all differentially expressed miRNAs display hypoxia-induced increases, and a subset of these up-regulated miRNAs (miR-133, miR-2a and miR-2) are involved in modulating protective anti-apoptotic functions (Xu et al., 2007; Zhang and Cohen, 2013). In both hypoxic brain and heart, miR-133 is significantly up-regulated, and it is suggested to serve protective functions in each tissue. In the brain, this OxymiR plays a neuroprotective role, by facilitating

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Box 1. MicroRNA biogenesis and mode of action

MicroRNA biogenesis

Canonical microRNA biogenesis is a multi-step process that begins in the nucleus with the transcription of long imperfect dsRNA hairpins known as primary-miRNAs (pri-miRNAs). These are then processed by the microprocessor complex (consisting of Drosha and DGCR8) that cleaves the 5′- and 3′-ends, leaving behind the ∼70 nt precursor-miRNA (pre-miRNA) transcript (O'Brien et al., 2018). Pre-miRNA transcripts are exported out of the nucleus to the cytoplasm, where Dicer cleaves the loop structure to yield a ∼21 nt miRNA duplex that contains both the inactive passenger strand and the guide strand that is used for mRNA targeting. Most of the literature focuses on the expression of miRNAs themselves; however, recent work is showing that biogenesis is also under tight spatial and temporal control during stress. For a more detailed description of miRNA biogenesis, refer to the excellent review by Ha and Kim (2014).

Mode of action

Mature miRNAs function by directing the RNA-induced silencing complex (RISC), consisting of Argonaute endonucleases and various other proteins, to the appropriate mRNA transcript(s). Targets are selected based on sequence complementarity: the 5′ seed region – nucleotides 2–8 of mature miRNA guide strands – target complementary motifs in mRNA 3′-UTRs. Imperfect complementary binding results in the translational suppression of the mRNA transcript: it is directed into cytoplasmic stress granules and processing bodies (P-bodies), where it is sequestered until cellular conditions shift to promote translation (O'Brien et al., 2018). When miRNAs bind to mRNAs with perfect complementarity, this ultimately leads to the degradation of the bound mRNA. Single miRNAs are capable of targeting multiple mRNA transcripts and single mRNA transcripts can be under the regulation of more than one miRNA species (Bartel, 2004).

recovery from ischemic injury (see Glossary), whereas in the heart it is probably also linked to the inhibition of maladaptive cardiac hypertrophy (Carè et al., 2007; Xin et al., 2013). It should be noted that whole-brain miRNA studies potentially mask miRNA responses that are specific to brain regions, neuron types and cell compartments (O'Carroll and Schaefer, 2013). This applies to the other brain findings discussed in this Review and warrants future, deeper investigations of these neuronal miRNA fingerprints. In comparison, mantle muscle, which is responsible for the jet propulsion required for the daily vertical migrations and escape from predators, displays several hypoxia-responsive miRNAs, including miR-100, expression of which is increased in response to hypoxia (Hadj-Moussa et al., 2018). This miRNA has been implicated in energy preservation and the suppression of cell growth via the inhibition of mTOR and selected growth factors during hypoxia (Blick et al., 2013; Wang et al., 2015).

Epaulette sharks

Research has also addressed the miRNA responses of what is arguably the most hypoxia-tolerant cartilaginous fish, the epaulette shark (*Hemiscyllium ocellatum*) (Nilsson and Renshaw, 2004). Native to the Great Barrier Reef of Australia, epaulette sharks have evolved to survive in a naturally cycling hypoxic environment and have even been reported to survive acute bouts of complete anoxia (approximately 2 h at 19°C) (Renshaw et al., 2002; Dowd et al., 2010). Although precise values for the reduction in metabolic rate when sharks enter a hypometabolic state are not available, studies have shown that oxygen-deprived shark brains switch to anaerobic ATP production, as indicated by the accumulation of lactate and other fermentation by-products. Under these conditions, sharks also display overall reduced consumption of ATP and have reduced cytochrome oxidase activity (Mulvey and Renshaw, 2000). Globally, anoxia-exposed sharks demonstrate a dynamic miRNA response (Riggs et al., 2018). This is to be expected based on their extensive hypoxic preconditioning (see Glossary) to anoxic survival, as demonstrated by various physiological and biochemical findings, including their ability to maintain brain ATP throughout the first hour of anoxia (Renshaw et al., 2002; Dowd et al., 2010).

In epaulette sharks, many of the miRNAs differentially expressed in response to anoxia are up-regulated (miR-92, -181a, -146b, -140, -20a, -17, -138 and -143). All these miRNAs have previously been implicated in responses to hypoxia – although in some cases their up-regulation would not be expected to elicit an adaptive response to oxygen limitation. For example, miR-143 inhibition has previously been shown to protect against ischemic cerebral injury and has been strongly implicated in remodelling cerebral vasculature; however, this miRNA is up-regulated in anoxic sharks (Müller et al., 2015; Zeng et al., 2017). Similarly, the up-regulation of miR-140 has previously been shown to aggravate hypoxia-induced cell injury and enhance apoptosis (Xing et al., 2018). However, other miRNAs up-regulated in response to anoxia in the epaulette shark are likely to provide protective effects against oxygen deprivation; some up-regulated miRNAs, including miR-92, are known to be direct and indirect regulators of the hypoxia-inducible factor (HIF) pathway (Taguchi et al., 2008; Valera et al., 2011). As will become evident throughout this Review, the targeting of HIF by OxymiRs is a conserved response in various models of oxygen deprivation. Furthermore, miR-146b has been shown to attenuate hypoxia-induced apoptosis and protect against ischemia/reperfusion injury (Li et al., 2015b; Di et al., 2017).

Oxygen limitation and miRNA responses in the intertidal zone

Common periwinkles

The squid and shark discussed above experience oxygen deprivation in the water; however, intertidal organisms inhabit a unique transitional environment that renders them vulnerable to oxygen limitation when exposed to air. Throughout the year, intertidal species such as the common periwinkle (*Littorina littorea*) experience low tides that leave them exposed to the atmosphere in their native rocky shores across the northern Atlantic Ocean. In periwinkles, the metabolic suppression and reorganization required to survive these dynamic conditions are under tight regulation in order to manage the accumulation of molluscan anaerobic end-products (McMahon et al., 1995; Storey et al., 2013). MiRNA responses to anoxia have been preliminarily explored in two tissues in periwinkles: the foot muscle (used for locomotion and anchorage) and the hepatopancreas (responsible for the functions performed by the liver and pancreas in mammals) (Biggar et al., 2012). After exposure to anoxia for 24 h, miR-210 is up-regulated in the hepatopancreas. This well-characterized OxymiR has been implicated in the adaptive response to oxygen limitation in other studies, as a result of its ability to negatively regulate mitochondrial metabolism and its relationship with HIF-1α signaling: HIF-1α acts to induce miR-210 expression and additionally promote anti-apoptotic and anti-proliferative roles (Biggar et al., 2012; Chan et al., 2012; Ivan and Huang, 2014) (Fig. 2). The miRNA-mediated suppression of mitochondrial energetics during anoxia probably acts to shunt fermentable fuels to anaerobic pathways in order to minimize oxidative stress.

Foot muscle also displays signs of miRNA-mediated protection against the challenges of anoxia: miR-133 is up-regulated in response to anoxia in this tissue (Biggar et al., 2012). Previous work

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Table 1. Species and tissues in which the OxymiR response to hypoxia/anoxia has been assessed

Environment	Animal	Tissue	Method	Reference
Marine	Jumbo squid (*Dosidicus gigas*)	Brain Brachial heart Mantle muscle	RT-qPCR	Hadj-Moussa et al. (2018)
	Epaulette shark (*Hemiscyllium ocellatum*)	Cerebellum	miRNA-Seq	Riggs et al. (2018)
Intertidal	Common periwinkle (*Littorina littorea*)	Hepatopancreas Foot muscle	RT-PCR	Biggar et al. (2012)
Estuary	Sea cucumber (*Apostichopus japonicus*)	Respiratory tree	miRNA-Seq	Huo et al. (2017)
Freshwater	Northern crayfish (*Orconectes virilis*)	Hepatopancreas Tail muscle	RT-qPCR	English et al. (2018)
	Red-eared slider turtle (*Trachemys scripta elegans*)	Liver Kidney	RT-PCR	Biggar and Storey (2012, 2017)
		Skeletal muscle Spleen		Zhang et al. (2013)
	Painted turtle (*Chrysemys picta belli*)	Telencephalon	miRNA-Seq	Riggs et al. (2018)
	Annual killifish (*Austrofundulus limnaeus*)	Embryo	miRNA-Seq	Riggs et al. (2018)
	Crucian carp (*Carassius carassius*)	Whole brain	miRNA-Seq	Riggs et al. (2018)
Subterranean	Naked mole rat (*Heterocephalus glaber*)	Whole brain	RT-qPCR	Logan et al. (2020)
	Cape dune mole rat (*Bathyergus suillus*)	Whole brain	RT-qPCR	Logan et al. (2020)
	Common mole rat (*Cryptomys hottentotus hottentotus*)	Whole brain	RT-qPCR	Logan et al. (2020)
	Common mole rat (*C. h. mahali*)	Whole brain	RT-qPCR	Logan et al. (2020)
	Common mole rat (*C. h. pretoriae*)	Whole brain	RT-qPCR	Logan et al. (2020)
	Cape mole rat (*Georychus capensis*)	Whole brain	RT-qPCR	Logan et al. (2020)
Terrestrial	Leopard frog (*Rana pipiens*)	Whole brain	miRNA-Seq	Riggs et al. (2018)
	Goldenrod gall fly (*Eurosta soldigans*)	Whole larva	PCR	Lyons et al. (2015)
High altitude	Human (*Homo sapiens*)	Plasma	Microarray	Yan et al. (2015)
		RBC	miRNA-Seq	Sun et al. (2018)
		Plasma	Microarray	Liu et al. (2016)
	Tibetan pig (*Sus scrofa*)	Heart	miRNA-Seq	Zhang et al. (2015)
	Yak (*Bos primigenius*)	Heart Lung	miRNA-Seq	Guan et al. (2017)
	Jersey cattle (*Bos taurus*)	Plasma	Microarray	Kong et al. (2019)
	Sprague–Dawley rat (*Rattus norvegicus*)	Plasma	Microarray	Chen et al. (2018c)
	Great tit (*Parus major*)	Heart	miRNA-Seq	Chen et al. (2018a)

has implicated miR-133 in the attenuation of hypoxia-induced apoptosis through the suppression of pro-apoptotic caspases 3, 8 and 9, suggesting an anti-apoptotic 'tissue preservation' role for miR-133 in oxygen-deprived tissues (Li et al., 2015a). It should be noted that miR-133 is also up-regulated in hypoxic squid tissues (Hadj-Moussa et al., 2018).

The role of OxymiRs in hypoxic estuarine habitats

Sea cucumbers

Before we consider the freshwater models of OxymiR function, we will explore the miRNAs that regulate the response to anoxia in a second transition zone. Estuaries are partially enclosed bodies of brackish water (see Glossary) where rivers connect to open seas. These environments pose unique challenges, including transient hypoxic exposures, that estuarine organisms, such as the Japanese sea cucumber (*Apostichopus japonicus*), must overcome. Sea cucumbers are widely cultured and economically valuable in the shallow temperate coasts of Southeast Asia. Changing global climates are currently threatening their populations in northern China, forcing them to rapidly adjust to non-ideal higher summer temperatures, lower salinity and, of relevance to our review, limited oxygen availability (Plagányi et al., 2013; Huo et al., 2017). Previous work has examined the miRNA responses of sea cucumbers during estivation (see Glossary; Chen et al., 2013; Chen and Storey, 2014; Wang et al., 2019), and Huo and colleagues built on these results by using high-throughput sequencing to examine miRNA responses to hypoxia in the sea cucumber respiratory tree, an organ used for oxygen extraction (Huo et al., 2017). Of the 26 miRNAs differentially expressed (12 up-regulated and 14 down-regulated) during severe hypoxia, a subset are associated with hypoxia-adaptive mechanisms important for redox, transcription, small molecule and ion transport, and hydrolysis (Huo et al., 2017).

Key miRNAs that are differentially regulated in response to hypoxia in sea cucumbers are hypoxia-up-regulated miR-1, miR-31, miR-153, miR-184 and miR-375 (Huo et al., 2017). MiR-31 and miR-153 antagonistically regulate the HIF pathway: miR-31 promotes its activation via the ablation of the HIF repressor, whereas miR-153 directly binds to the HIF-1α 3′-UTR and inhibits HIF-1α translation (Liu et al., 2010). This interaction demonstrates

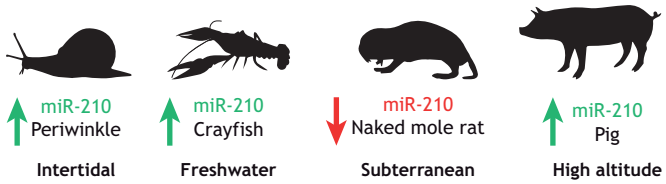

Fig. 2. Regulation of the miR-210 OxymiR in natural models of oxygen deprivation. One of the most well-characterized OxymiRs is miR-210, which has been implicated in numerous hypoxia-associated functions (reviewed in Ivan and Huang, 2014). MiR-210 specifically targets HIF-1α and downstream genes, thus regulating cell survival, mitochondrial oxidative metabolism, DNA repair, angiogenesis and growth. It is implicated in cardiac cerebrovascular diseases, cancer, stroke and ischemia. miRNA-210 is differentially expressed in response to hypoxia in various environments – it is up-regulated in anoxic intertidal periwinkles (Biggar et al., 2012), anoxic freshwater crayfish (English et al., 2018) and hypoxic high-altitude Tibetan pigs (Zhang et al., 2015), and is down-regulated in hypoxic subterranean naked mole rats (Logan et al., 2020).

the complex nature of hypoxic networks. Increased levels of miR-184 and miR-375 reduce cellular autophagy, but are also associated with higher levels of oxidative products, whereas increased levels of miR-375 are linked to increased apoptosis (Chang et al., 2012; Liu et al., 2015). The fact that this pair of hypoxia-up-regulated OxymiRs act both to promote and prevent hypoxia-related injury in the sea cucumber illustrates the complex crosstalk involved in the miRNA response to hypoxia: the overall response depends on the cellular microenvironment. Another miRNA worth mentioning is miR-1: the increased expression of this miRNA in hypoxic sea cucumbers could be associated with facilitating cell cycle arrest (Nohata et al., 2011).

OxymiRs aid survival of hypoxia and anoxia in fresh water

Similar to those living in saline environments, freshwater species are also vulnerable to both acute and chronic oxygen deprivation. We begin this section by taking a comparative multi-species approach to understand the miRNA responses of phylogenetically diverse anoxia-tolerant fish. We will discuss the highly anoxia-tolerant crucian carp (*Carassius carassius*) and annual killifish (*Austrofundulus limnaeus*) (Riggs and Podrabsky, 2017; Riggs et al., 2018). We also discuss the anoxic responses of northern crayfish and red-eared slider turtles, both of which have evolved to overcome the challenges of overwintering in oxygen-deprived ice-locked ponds.

Crucian carp and annual killifish

Crucian carp are extremely anoxia-tolerant bony fish that are routinely exposed to months of anoxia in the ice-locked lakes of northern Europe (Nilsson and Renshaw, 2004). To survive, carp globally reduce metabolic rates by 70% relative to normoxic rates, and couple this with glycolytic anaerobic adaptations that include ethanol production as a means to avoid lactic acidosis (Johansson et al., 1995). As for killifish, their extremely anoxia-tolerant embryos can survive months of complete oxygen deprivation (Johansson et al., 1995). The miRNA responses of crucian carp and annual killifish brains to anoxia were compared by Riggs et al. (2018). Rather than identifying a conserved OxymiR response, Riggs and colleagues identified species-specific differential expression changes, suggesting that each species may have evolved distinct miRNA expression patterns to cope with oxygen limitation (Riggs et al., 2018). For example, crucian carp had fewer global small non-coding RNA changes in response to anoxia, compared with killifish, a concept that is discussed in detail in the multi-species comparison section below.

Another observation made by Riggs et al. (2018) is that a majority of the most abundant miRNA sequences under normoxia and anoxia in carp and killifish are known stress-responsive miRNAs. This emphasizes the importance of stabilizing the expression of these miRNAs that appear to not only be crucial for anoxia survival but also for normal brain homeostatic functions (Riggs et al., 2018). Two specific miRNAs that are up-regulated in response to anoxia in highly anoxia-tolerant carp brain are: miR-182, the expression of which is known to increase in other brain models of ischemia preconditioning (Lee et al., 2010), and miR-6497, which is less characterized. These miRNAs are also up-regulated in the brain of anoxia-exposed red-eared slider turtles, a highly anoxia-tolerant species that is discussed below (Riggs et al., 2018). Another change reported in carp was the up-regulation of miR-10 in anoxia; this miRNA is possibly associated with mitigating hypoxia-induced apoptosis (Wu et al., 2019). One of the main takeaway messages from this comparative study is that rather than conserve a specific subset of OxymiRs, these diverse species promote global stabilization of small non-coding RNAs between normoxic and anoxic profiles, seeing that less than 1% of miRNA sequences are differentially expressed (Riggs et al., 2018). This suggests that the maintenance of normal brain homeostasis may be just as important for anoxia tolerance as the dynamic regulation of anoxia-protective OxymiRs. The expression of these miRNAs under normoxic conditions begs the question of whether these miRNAs are involved in normal brain homeostasis or instead play a role in anoxia tolerance or preconditioning, a question that cannot be presently answered without deeper analyses. The unique miRNA responses of each of these species, and of others discussed in this Review, may reflect the fact that many of these species evolved anoxia tolerance independently and that miRNAs did not evolve to be at the vanguard of this response.

Northern crayfish

Another organism that has evolved to endure prolonged periods of hypoxia and anoxia is the northern crayfish (*Orconectes virilis*) (English et al., 2018). These animals experience oxygen deprivation both in their warm stagnant summer waters and in their cold ice-locked winter waters. Previous work on crayfish has revealed that they survive periods of reduced oxygen by using anaerobic fermentation of glycogen to lactate (Gäde, 1984), modulating anti-oxidant defences (Lant and Storey, 2011) and regulating enzymatic controls of metabolism (Dawson and Storey, 2012). Recently, researchers have shown that oxygen-deprived crayfish also modulate miRNA expression during anoxia-induced hypometabolic retreats. Researchers exposed crayfish to acute (2 h) and chronic (20 h) periods of anoxia, and examined the response of 76 miRNAs in tail muscle and hepatopancreas. This revealed a strong tissue-specific response: 22 miRNAs were significantly regulated in hepatopancreas but only four changed in anoxic muscle tissue (English et al., 2018). Despite the fact that crayfish undergo global metabolic rate depression during exposure to hypoxia/anoxia, changes in miRNA expression indicate the activation of various processes in anoxic hepatopancreas. These anoxia-responsive hepatopancreas miRNAs target cell proliferation and apoptotic signaling pathways in a HIF-1α-dependent manner: down-regulation of the HIF-1α-targeting miRNAs (miR-133-3p, miR-33-5p, miR-125-5p and miR-190-5p) and up-regulation of HIF-1α-promoting miRNAs (miR-210) contributes to enhanced levels of HIF-1α mRNA during anoxic episodes.

Red-eared slider turtles

During winter, red-eared slider turtles (*Trachemys scripta elegans*) can survive up to 4 months at the bottom of ice-locked ponds, where oxygen levels are quickly depleted, by drastically reducing metabolic rates to 10% of normoxic rates. Many of the adaptations used by this champion anoxia-tolerant vertebrate to survive oxygen deprivation have been extensively reviewed (Storey, 2007). Briefly, similar to other oxygen-deprived animals, turtles suppress non-essential ATP-expensive processes. This is coupled with a switch to anaerobic glycogen fermentation that leads to the accumulation of lactate. To accommodate the increased levels of lactate fermentative products, turtles make use of carbonates in their shell to store lactate and to enhance their buffering capacity, thereby minimizing metabolic acidosis (Jackson et al., 2000; Reese et al., 2004). These processes are supplemented with various anti-oxidant defences to minimize oxygen reperfusion injury. Here we focus solely on the miRNA response of anoxic red-eared slider turtles, but Riggs et al. (2018) have examined the miRNA responses of the

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western painted turtle (*Chrysemys pita belli*), another highly anoxia-tolerant tetrapod with a similar overwintering strategy.

Biggar and Storey (2012) investigated anoxia-responsive miRNAs in the context of cell cycle regulation, which is known to be inhibited in anoxic turtles (Fig. 3). Protein levels of cyclin D1 (responsible for cell cycle initiation) are down-regulated in anoxia, whereas cyclin D1 transcript levels are unaffected. Deeper investigations revealed that miR-15a and miR-16, which directly target cyclin D1, are up-regulated and could be behind the mismatch between mRNA and protein expression (Biggar and Storey, 2012) (Fig. 3). In addition to being regulated by miRNAs, the cell cycle is also under tight control by various signaling cascades, including the p53 network, which itself is a regulator of miRNA expression (Chen, 2016). Induction of p53 modulates translation, apoptosis, autophagy and various other cellular pathways, and the protein itself has been shown to be heavily phosphorylated during anoxia and during exposure to other stressors that cause metabolic rate depression, suggesting that it is stabilized and activated under these conditions (Zhang et al., 2013; Hefler et al., 2015; Luu et al., 2018). In a study by Zhang and colleagues, levels of miR-34a, a miRNA that promotes cell cycle arrest, were significantly up-regulated in both liver and muscle of anoxic turtles (Zhang et al., 2013; Gang et al., 2017). Both these studies underline the contribution of miRNA-mediated suppression of cell growth in anoxic turtles, demonstrating the multiple levels of regulation required at the post-transcriptional and post-translational levels.

In addition to overcoming anoxic conditions in their ice-locked ponds, red-eared sliders must also endure low temperatures, and an aspect of miRNA functionality that is understudied is their temperature sensitivity (Wu et al., 2002; Hadj-Moussa and Storey, 2018). Large fluctuations and drops in body temperature experienced by ectotherms affect the thermodynamic nature of miRNA–mRNA interactions, resulting in decreases in the free energy required for RNA duplex formation, thereby favoring more

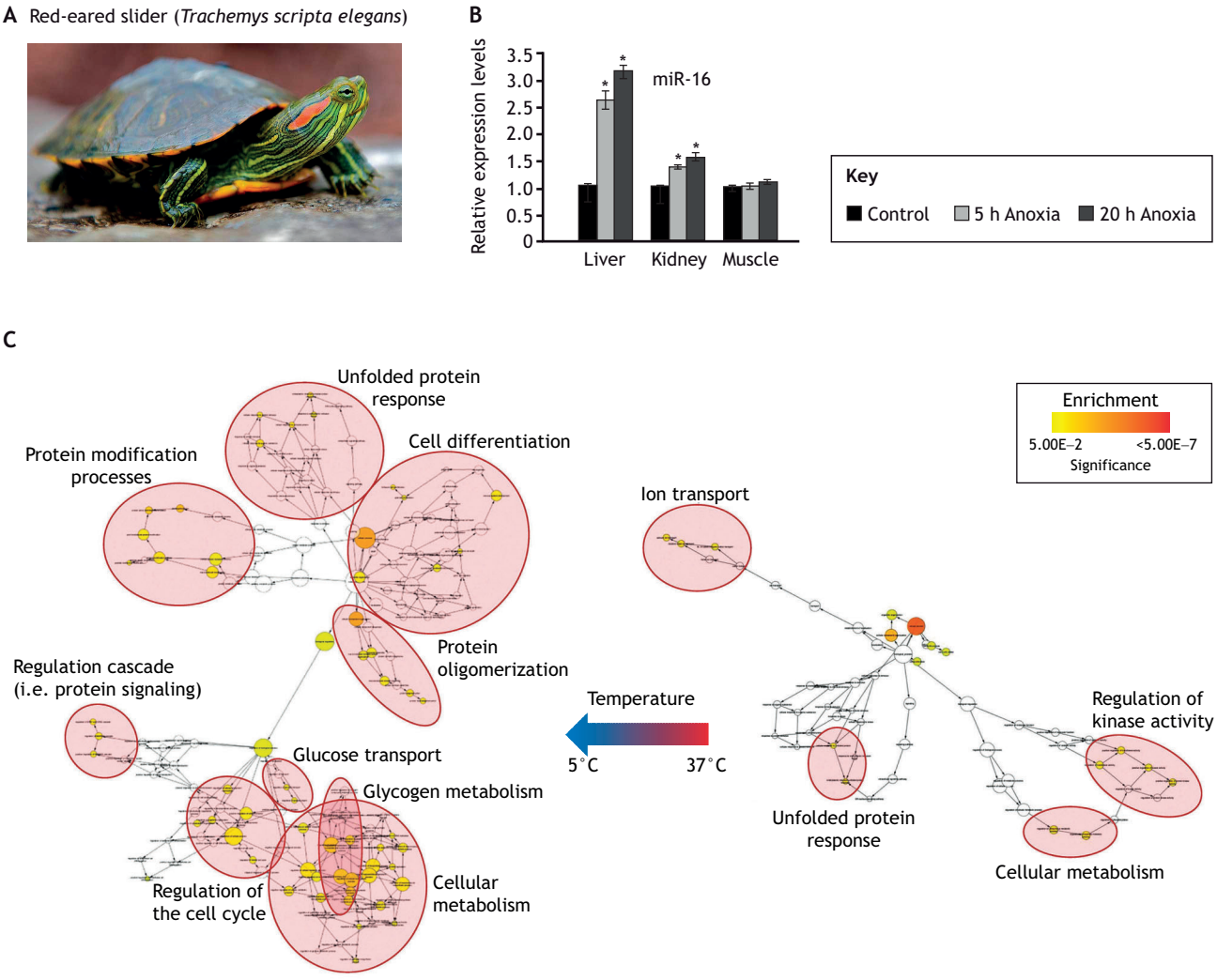

Fig. 3. Anoxia-responsive miRNAs and the effect of temperature in red-eared slider turtles. (A) The red-eared slider turtle (*Trachemys scripta elegans*) is among the most anoxia-tolerant vertebrates. These turtles experience anoxic conditions while overwintering at the bottom of their ice-locked ponds. (B) Histogram showing relative miR-16 expression levels in turtles exposed to anoxia for 5 h and 20 h, relative to control levels, and as determined by RT-PCR. Data are means±s.e.m. (n=3–4). The asterisks indicate a significant difference from the corresponding control (P<0.05) (Biggar and Storey, 2012). The activation of miR-16 inhibits cyclin D, thus inhibiting cell cycle progression. (C) Temperature influence of miRNA–mRNA targeting at 5°C compared with predictions at 37°C. The biological processes that are predicted to be targeted are drastically changed by lowering the prediction temperature. Anoxia-responsive liver miRNA targets were predicted using FindTar3. Predicted targets were analysed for enrichment in biological processes (GO terms) and visualized using the BiNGO function with Cytoscape software (Biggar and Storey, 2017).

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miRNA–mRNA binding (Wu et al., 2002; Biggar and Storey, 2015; Hadj-Moussa and Storey, 2018). Traditional miRNA-targeting bioinformatics enrichment programs rely on default RNA binding temperatures of 37°C; this is not representative of winter temperatures. Therefore, to investigate the effects of low temperatures on miRNA–mRNA targeting in anoxic turtles, Biggar and Storey simulated RNA-binding interactions at the more biologically relevant temperature of 5°C to mimic winter interactions (Biggar and Storey, 2017). When high temperatures were used to map the interactions of anoxia-responsive miRNAs, relatively few mRNA targets with non-anoxia-related functions were predicted compared with the hundreds of targets predicted when 5°C was substituted into the prediction model (Biggar and Storey, 2017). Functional clustering of low-temperature-predicted targets revealed miRNA-enriched targets that were biologically relevant for successful anoxia tolerance, including glycolytic pathways, cell cycle and protein-modifying processes; these results corroborate findings from previous studies (Greenway and Storey, 2000; Biggar and Storey, 2012; Krivoruchko and Storey, 2013; Biggar and Storey, 2017) (Fig. 3).

Involvement of OxymiRs in hypoxic subterranean environments

Naked mole rats

Naked mole rats (*Heterocephalus glaber*) are one of the most hypoxia-tolerant mammals, capable of enduring high levels of endogenous oxidative damage, hypoxia and, incredibly, up to 18 min of complete anoxia (Edrey et al., 2014; Park et al., 2017). They live in crowded conditions in extensive underground burrows in Africa, ultimately resulting in hypoxic and hypercapnic tunnel systems (Chung et al., 2016); however, it should be mentioned that there is debate regarding the ‘hypoxic’ nature of their burrows (Roper et al., 2001; Holtze et al., 2018). Nonetheless, researchers have explored the unique mechanisms that allow this extraordinary long-lived mammal to survive these conditions; much focus has been on their inherent resistance to neurodegeneration and ability to withstand acute brain ischemia (Triplett et al., 2015; Hawkins et al., 2019).

To explore the role of OxymiRs in naked mole rat neuroprotection, an international team of researchers examined a group of 27 hypoxia-associated miRNAs (Logan et al., 2020). Their results present miRNAs as modulators of neuroprotection in hypoxic mole rats; induction of hypoxia revealed that a subset of miRNAs are involved in inhibiting apoptosis and neuroinflammatory regulation (Logan et al., 2020) (Fig. 4). Oxygen limitation is associated with the up-regulation of five miRNAs (miR-24-3p, -24-5p, -207, -27 and -592), whereas miR-210, -126 and -26 are down-regulated in response to hypoxia (Logan et al., 2020). As with the other hypoxic environments and animal models discussed above, Logan et al. also reported that stress-responsive miRNAs such as miR-24 and miR-210 are differentially regulated in response to hypoxia, but the study also identified species-specific hypoxia-inducible miRNAs. For example, the induction of miR-24, an inhibitor of apoptosis (Kulshreshtha et al., 2007), combined with the suppression of miR-210 (Bavelloni et al., 2017), an indirect stabilizer of HIF-1, could potentially act to both promote neuronal preservation during bouts of hypoxia as well as effectively mount an adaptive hypoxic response (Figs 2 and 4).

Oxygen-deprived terrestrial animals use OxymiRs

Frogs

Land dwellers adapted to air-breathing can also experience episodes of anoxia. The freeze-tolerant wood frog (*Rana sylvatica*), native to

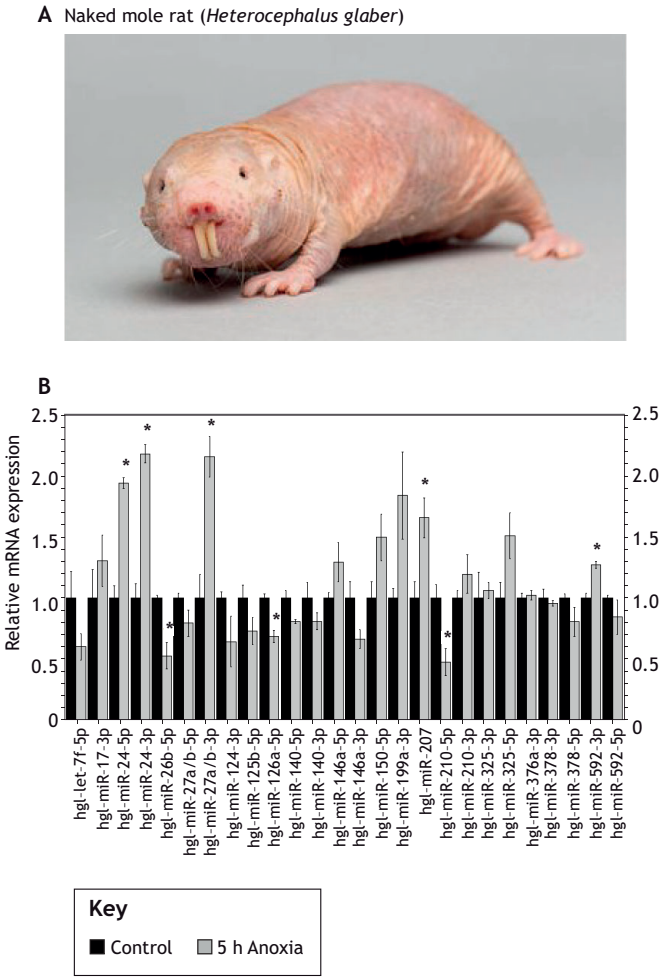

Fig. 4. Regulation of hypoxia-responsive miRNAs in naked mole rat brains. (A) Naked mole rats (*Heterocephalus glaber*) are native to East Africa; they live in underground burrows where they must overcome hypoxic and hypercapnic tunnel conditions. (B) Relative miRNA expression levels in hypoxic brains. The histogram represents data as means±s.e.m., n=4 isolations of RNA from different brains. The asterisks indicate data that are significantly different from the control condition (P<0.05) (Logan et al., 2020). Naked mole rats rely on select miRNAs to protect against hypoxia-associated challenges. Up-regulation of miR-24 inhibits apoptosis, and miR-592 regulates glycolytic metabolism.

North American forests, can survive freezing of up to 65% of total body water as extracellular ice for months at a time; simultaneously, anoxia is induced by the restriction of oxygen uptake and diffusion caused by ice formation (Schmid, 1982; Storey and Storey, 2017). miRNAs have been implicated in managing freeze tolerance, and we have shown that differential expression of miRNAs, such as up-regulation of miR-451, protects the animals against anoxia/reoxygenation injury (Hadj-Moussa and Storey, 2018); despite this, the involvement of miRNAs in the adaptive response to wood frog anoxia has yet to be explored. However, miRNA responses have been investigated in response to oxygen limitation in another frog species: the anoxia-tolerant leopard frog (*Rana pipiens*). At 25°C, leopard frogs can withstand approximately 5 h of anoxia – compare this with their enhanced anoxic survival capacity at 5°C, which has been recorded as 48 h (Holden and Storey, 1997; Milton et al., 2003). In nature, these frogs are capable of overwintering at 3°C in complete anoxia for several days (Bickler and Buck, 2007). When miRNA responses were examined in leopard frogs, the majority of

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differentially expressed miRNAs were down-regulated during anoxia, which led authors to suggest that leopard frogs may be unable to appropriately respond to anoxia (Riggs et al., 2018). We elaborate on this further in our conclusions below.

Gall flies

An insect model that has been used extensively in research on both oxygen limitation and cold hardiness is the goldenrod gall fly (*Eurosta solidaginis*). Gall fly larvae overwinter within galls of goldenrod stems and can survive sub-zero temperatures by freezing up to 65% of total body water. Similar to wood frogs, gall fly freezing also results in anoxia (Storey and Storey, 1983). Researchers examined the miRNA responses of this system using a targeted approach to measure a subset of known stress-responsive miRNAs, and found that only levels of miR-92b are down-regulated during anoxia (Lyons et al., 2015). Anoxia-responsive miR-92b is a potent regulator of mTOR signaling that directly binds to its negative regulator (TSC1), therefore the lack of translational suppression of TSC1 during anoxia in the goldenrod gall fly could promote the inhibition of mTOR pro-proliferative functions (Lee et al., 2019). Follow-up high-throughput investigations focused on the ability of *E. solidaginis* to tolerate freezing, but seeing that freezing is a multi-factorial stressor that also requires adequate anoxia tolerance, it is interesting to note that the freeze-induced up-regulation of miR-137 and miR-31a is associated with the mitigation of reactive oxygen species and modulation of HIF-1α, respectively (Lyons et al., 2016). Future studies are required to determine if the same response would be elicited in an insect exposed to anoxia in the absence of freezing.

Hypoxia and OxymiRs at high altitude

The final oxygen-limited environments we will explore are high-altitude mountains and plateaus. At elevations above 3500 m, organisms must endure low atmospheric oxygen pressure, low temperatures and limited resources. High altitudes are the only hypoxic environment where we humans have ‘successfully’ settled; we will begin our discussion by considering what we know about human OxymiRs, and then go on to consider the miRNA responses of two other mammalian species and a bird.

Humans

Although humans are not resilient to anoxic exposures – denial of oxygen for just a few minutes results in irreversible brain damage, likely to end in death – we are surprisingly adept at coping with lower oxygen levels for short time periods. However, prolonged oxygen deprivation leads to chronic mountain sickness, which is characterized by a proliferation of red blood cells (RBCs) resulting in headaches, sleep problems, breathlessness and fatigue. If left untreated, this can lead to heart failure and death. Despite the negative effects of hypobaric and hypoxic high altitudes, for hundreds of generations, populations such as the Andeans, the Ethiopians and the Tibetans have adapted to life at the top of the world.

Recent reports have stated that approximately 17 million individuals live 3500 m or more above sea level, where they are exposed to hypoxic and hypobaric conditions (Chen et al., 2018c). Most studies to date have focused on the pulmonary adaptations, oxygen delivery and pathological effects relating to life in the mountains (Azad et al., 2017; Moore, 2017), which has led to the identification of both short-term (acclimatization and developmental) and long-term (genetic) responses to high-altitude environments (Valero et al., 2019preprint). In the last decade, scientists have begun to characterize the signature of genes at the core of high-altitude adaptations responsible for the complex physiological changes observed in these populations (Julian and Moore, 2019; Valero et al., 2019preprint). Fortunately for us, recent work has also begun to explore the potential role and influence that miRNAs play in the adaptation to hypoxic high-altitude environments. In humans, this has focused on the study of circulating miRNAs (see Box 2), a population of miRNAs that can be examined in a non-invasive manner in order to provide us with biomarkers implicated in adaptive or maladaptive responses.

When researchers examined 754 miRNAs in over 500 plasma samples from native Tibetans (residing ∼3560 m above sea level), new migrant Tibet Han (at ∼3560 m or higher) and Nanjing Han people (at ∼8.9 m), they found that differentially expressed miRNAs were positively correlated with RBC counts and hemoglobin levels (Yan et al., 2015). Compared with the lowland Nanjing Han, 172 miRNAs were differentially expressed (105 up-regulated and 67 down-regulated) in the Tibet Han (new highland migrants). An examination of the functional targets of high-altitude responsive miRNAs revealed several hypoxia-related genes including *HIF-3*, *VEGFA* and *DDX6*. Yan and colleagues focused on four particular miRNAs up-regulated at high altitude, two of which – miR-130a and miR-572 – have known pathogenic functions in hypoxic cells, where they promote vascular remodelling and apoptosis, thereby exacerbating hypoxia and reoxygenation injuries (Brock et al., 2013; Fang and Yeh, 2017). This demonstrates a maladaptive response in the new highland

Box 2. Circulating blood microRNA origins and functions

Origins and targeting of blood microRNA

Extracellular/circulating miRNAs are synthesized intracellularly in tissues and are then expelled into body fluids. Whether their exit from the cell is via specific RNA export mechanisms, or as a result of non-specific exocytosis as a by-product of cellular activity and death, remains unknown. Accumulating evidence suggests that both possibilities are true (Sohel, 2016). Circulating miRNAs have been reported in all body fluids, but in this Review we focus on miRNAs found in blood plasma, serum and red blood cells (Turchinovich et al., 2012). These miRNAs are stabilized in the blood by being either bound to proteins or enclosed in apoptotic bodies, high-density lipoproteins, exosomes or in microvesicles (Sohel, 2016). A question currently plaguing the field is how these circulating miRNAs are taken up by recipient cells. The leading theory is that, once enclosed in membrane vesicles, either actively or passively, the miRNAs enter cells by direct membrane fusions or endocytosis (Turchinovich et al., 2012).

Functions of blood miRNA

The targeting capacity of blood miRNAs is still being investigated, and although studies have shown that they indeed impart specific functions, new work is showing that functions of the majority of these miRNAs remain unknown (Sohel, 2016). Other confirmed functions include: (1) cell–cell communication, (2) regulation of translational activity of distant cells, (3) triggering of inflammatory responses and (4) promotion of neurodegeneration (Sohel, 2016).

Applicability of blood miRNA

The ease and non-invasive nature of studying blood miRNAs and their differential expression during disease states has rendered them popular biomarkers for a plethora of diseases, for both diagnosis and prognosis. Various cancers, neurodegenerative disorders and autoimmune diseases, among others, are reported to be associated with subsets of miRNAs from early detection to treatment monitoring (Wang et al., 2018). Future work should look at the presence and feasibility of using circulating miRNAs as biomarkers of environmental stress and adaptions, a feat that could prove useful for conservation work, as well as in other areas.

migrants, who have not had generations to adapt to increased altitude, unlike their native Tibetan counterparts. Another main finding was that several blood cell-related genes – *SMAD5*, *ETV6*, *VEGFA* and *erythropoietin* – are predicted to be targets of hypoxia-up-regulated miR-130a and miR-302b, and plasma levels of VEGFA and erythropoietin are elevated in individuals at high altitude (Yan et al., 2015). This indicates that miR-130a and miR-302b are likely to play a role in erythroid acclimatization and adaptation to high altitude.

At high altitudes, RBC counts drastically increase. Although modest rises in RBC and hemoglobin levels are beneficial in hypoxic high-altitude environments, profound pathological increases elevate blood viscosity and aggravate cellular anoxia, leading to multiple organ dysfunction (Villafuerte and Corante, 2016). Given that erythropoiesis is known to be regulated by miRNAs, studying RBC-derived miRNAs at high altitudes could identify potential miRNA-mediated mechanisms involved in hypoxic adaptations (Felli et al., 2005; Sun et al., 2015, 2018). Below are findings from a study that examined RBC-derived miRNAs from three populations in the Tibet Plateau: (1) native low-altitude Sichuan Han Chinese (residing at ∼500 m), (2) high-altitude native Tibetans (residing at ∼3658 m), and (3) high-altitude migrant Tibet Han (residing at ∼3658 m). Of the 516 RBC-derived miRNAs analysed, 49 were found to be differentially expressed (17 up-regulated and 32 down-regulated) between low-altitude Sichuan Hans and high-altitude migrant Tibetan Hans. Slightly fewer differential miRNA changes were identified between native high-altitude Tibetans and the low-altitude Sichuan Hans population (12 up-regulated and 21 down-regulated) (Sun et al., 2018).

Two of the hypoxia-up-regulated RBC-derived miRNAs, miR-30b and miR-144, stand out for their roles in erythroid, hypoxia and nitric oxide signaling pathways, and their association with the formation and stability of RBCs (Sun et al., 2018). One of the targets of miR-30b is ERG (E-twenty-six related gene), a transcription factor that regulates erythrocyte differentiation and hematopoietic stem cell function (Carmichael et al., 2012). Blood-specific targets are also observed for miR-144 – this miRNA positively regulates erythropoiesis and increases RBC abundance through its interactions with the RAB14 (RAS-associated protein 14) transcription factor (Papapetrou et al., 2009). Fittingly, it has been recently reported that miR-144 is commonly loaded into blood exosomes (see Glossary), and this process can be altered by hypoxia in non-adapted individuals. Exosomes are small cell-derived extracellular vesicles that transport miRNAs (and other cargo) around the body, implicating them in cell–cell communication and miRNA delivery (Johansson et al., 1995). Native Tibet highlanders have lower levels of miR-144 in their blood, compared with the new migrant Tibetan Han highlanders, who have not physiologically or genetically adapted to high altitudes (Guduric-Fuchs et al., 2012; Sun et al., 2018). This is possibly an adaptation to prevent pathological erythropoiesis in native Tibet highlanders.

The above two studies focused on the miRNAs of native high-altitude acclimatized populations, and both plasma and RBC miRNA profiles revealed RBC-specific functionality, emphasizing the importance of tightly regulating miRNAs during hypoxic exposures. However, it is interesting to note that miRNAs have also been implicated in facilitating the acclimatization process and the restoration of oxygen delivery to tissues for individuals at high altitudes (Liu et al., 2016). The physiological adjustments observed during acclimatization – including those affecting hematology, stress hormones and lipid molecules – were found to be strongly correlated with hypoxia-mediated regulation of circulating miRNAs (Liu et al., 2016), where 86 miRNAs (79 up-regulated and seven down-regulated) were significantly differentially expressed upon exposure to hypoxia. Key functional targets of these circulating miRNAs include low-density lipoprotein-cholesterol, cholesterol metabolism and cortisol synthesis. It is no surprise to find that HIF-1α signaling is significantly suppressed by miRNAs in unacclimated individuals (Liu et al., 2016). This inhibition of HIF-1α is likely to contribute to the pathogenesis and high-altitude sickness that unacclimated individuals experience, as HIF-1α signaling acts to promote adaptive responses to high altitude: one of its roles is to facilitate an increase in erythropoietin and aid oxygen delivery (Bigham and Lee, 2014).

Highland pigs

The Tibetan highland pig (*Sus scrofa*) has evolved adaptations to thrive in chronic hypobaric hypoxic environments ∼2500–4300 m above sea level. When compared with lowland Yorkshire pigs raised at high altitude, miRNA-Seq analysis identified 20 significantly differentially expressed miRNAs (10 up-regulated and 10 down-regulated) in cardiac muscle (Zhang et al., 2015). In particular, miR-210, -142, -194b and -421 are up-regulated, and miR-101, -214, -206 and -320 are down-regulated in Tibetan pigs, compared with lowland pigs (Zhang et al., 2015). For more information on the up-regulation of miR-210, known to be induced by HIF-1α and regulated by vascular endothelial growth factor (VEGF) under hypoxic conditions (Huang et al., 2009), refer to the below section on HIF-1α and miR-210 targeting during oxygen deprivation. Bioinformatic target enrichment of global miRNA levels using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that differentially expressed miRNAs in Tibetan pigs are involved in central signaling cascades, including MAPK, Akt/mTOR and VEGF-related pathways (Zhang et al., 2015). Collectively, these miRNA-mediated processes appear to be contributing to the enhanced hypoxic adaptations of Tibetan pigs.

Yaks

The yak (*Bos grunniens*) is another species that is native to the highland Tibetan plateaus (∼2500–6000 m). Previous work has characterized the contribution of yak anatomical and physiological traits (such as an enlarged heart and lungs) to their survival at high altitude (Wiener et al., 2003). Recently, researchers have performed comparative miRNA transcriptomic studies between the yak and lowland cattle to better characterize these adaptations (Guan et al., 2017). Of the 808 miRNAs characterized, 85 were found to be differentially expressed between yaks and cattle. Lung tissue has greater numbers of differentially expressed miRNAs than cardiac tissue (70 versus 29 miRNAs) (Guan et al., 2017). The majority of differentially expressed miRNAs in yaks are down-regulated, similar to other high-altitude systems, suggesting that this pervasive miRNA down-regulation corresponds to the potential activation, or lack of inhibition, of target pathways. As has been the case for other species covered in this Review, functional analysis identified the targets of these miRNAs to be enriched in hypoxia-related pathways including HIF-1α and Akt, as well as DNA repair, cell cycle control and apoptotic regulation (Guan et al., 2017). miRNAs differentially expressed in yaks also target metabolic pathways including fatty acid metabolism, indicating a role for these miRNAs in metabolism at high altitude.

Great tits

The last animal discussed in this Review, which has physiologically adapted to life at high altitudes, is a species of bird. A small RNA

transcriptome study was conducted on a species of wild songbird, the great tit (*Parus major*), which is distributed between sea level and ∼4500 m across Europe and Asia. This study identified 14 hypoxia-regulated miRNAs in cardiac tissue: six miRNAs were up-regulated and eight were down-regulated in high-altitude great tits, relative to low-altitude birds (Chen et al., 2018a). Levels of miR-19b-3p were down-regulated in response to hypoxia and, as a result of its direct targeting to the MAPK1 3′-UTR, mRNA and protein levels of MAPK1 were up-regulated during hypoxic exposures, thus regulating cell cycle in response to hypoxia (Chen et al., 2018a). It is also of note that miR-133a and -b are up-regulated at high altitude: these miRNAs were previously reported to attenuate hypoxia-induced apoptosis (Li et al., 2015a).

Conclusions, cross-species comparisons and future directions

Major conclusions

One of the major conclusions we can glean from this review of over 20 diverse miRNA animal responses to oxygen limitation is that there does not appear to be a clear universal 'OxymiR signature' observed in all, or even a majority, of the animals discussed – a conclusion we are not the first to come to (Riggs et al., 2018). The lack of a conserved OxymiR fingerprint highlights the animal-, stress- and tissue-specific nature of oxygen-sensitive miRNAs. This is at odds with the many physiological, biochemical and molecular studies that have demonstrated conserved survival mechanisms that facilitate anoxia and hypoxia tolerance in the animal kingdom (Bickler and Buck, 2007; Storey, 2007). Knowing that oxygen limitation poses the same core cellular and physiological challenges to all organisms, the lack of a conserved OxymiR response is unexpected, but not impossible. Indeed, the diverse nature of the animals considered here may be responsible for the differences observed. As suggested by Riggs et al. (2018), the identities of miRNAs may not be the only deciding factor for miRNA targeting; the downstream targets and physiological responses being regulated by these changing miRNAs may differ between species, and this could be equally, if not more, important (Riggs et al., 2018). However, Riggs and colleagues were quick to state a caveat to this scenario, resulting from the profound sequence conservation of miRNAs, a conclusion that we agree with (Bartel, 2004; Riggs et al., 2018). In addition, as has been shown above, the stabilization of the miRNA population between normoxic and hypoxic/anoxic conditions may be more critical than the identity and expression of individual miRNA species. Therefore, perhaps we should focus on the global changes, or lack thereof, when looking for miRNA commonalities and differences across species in response to hypoxia or anoxia. Collectively, these studies suggest that although miRNAs appear to play important roles in facilitating responses to oxygen deprivation, individual species are likely to have evolved their responses independently and, as such, a conserved suite of miRNAs did not develop at the vanguard of this response.

Less hypoxia-tolerant organisms mount more dynamic miRNA responses

It is interesting to note that, in general, less hypoxia-tolerant organisms mount more dynamic miRNA responses to hypoxia and anoxia. This was the case in two very different multi-species studies looking at the brains of anoxia/hypoxia-tolerant and -sensitive animals. The first multi-species study looked at the miRNA responses to anoxia in the epaulette shark, crucian carp, annual killifish and leopard frog, concluding that the less well-adapted anoxia-tolerant species appear to rely on large changes in global miRNA expression profiles in their response to anoxia. For example, crucian carp display fewer dynamic changes in their small non-coding RNA profiles in response to anoxia compared with killifish and sharks, a finding that correlates with the bioenergetics activity profiles for each species during oxygen deprivation (i.e. active versus dormant) (Riggs et al., 2018): killifish embryos depress their metabolic profiles to approximately 15% of normoxic rates in order to maintain ATP turnover rates and ionic gradients during anoxic exposures (Podrabsky and Hand, 1999; Podrabsky et al., 2007), whereas crucian carp remain relatively active despite their anoxic and profoundly reduced metabolic state. Similarly, when leopard frogs are compared with more anoxia-tolerant species such as the sharks, the leopard frogs appear to demonstrate more miRNA changes, which may suggest the lack of a robust miRNA response to anoxia (Riggs et al., 2018). Despite the global dissimilarities observed, there were commonalities between a few of the miRNA species. For example, the anoxia-induced decrease in levels of miR-29b in leopard frogs matched miRNA changes reported in anoxic periwinkles (Biggar et al., 2012). The role of miR-29b in these animals has not been characterized, but it could serve anti-apoptotic functions (Chen et al., 2018b). This warrants future investigations, as other studies have reported hypoxia-protective functions when miR-29b is up-regulated (Cai and Li, 2019).

The second multi-species study performed examined the miRNA responses in the brains of six African mole rat species with varying degrees of hypoxia tolerance, an analysis that included the above-mentioned naked mole rat miRNAs (Logan et al., 2020). The greatest amount of differential miRNA expression was observed during hypoxia in the Cape mole rat (*Georychus capensis*) and the Cape dune mole rat (*Bathyergus suillus*), both of which are ecologically adapted to normoxic, not hypoxic, environments. The observation that the least hypoxia tolerant of the six African mole rats demonstrated the most dynamic miRNA regulation suggests, yet again, that instead of an organized response, these species appear to be mounting a 'panic' response, where the animal tries to use whatever molecular tools are at its disposable during oxygen deprivation (Logan et al., 2020).

Conserved miR-210 and HIF-1α targeting

The up-regulation of miR-210 is known to be induced by HIF-1α and regulated by VEGF under hypoxic conditions (Huang et al., 2009). Anoxic crayfish up-regulate miR-210 in the hepatopancreas (English et al., 2018), as seen in anoxic periwinkles (Biggar et al., 2012) and hypoxic high-altitude pigs (Zhang et al., 2015). Indeed, this was even observed in another model of limited oxygen availability that was not discussed in this Review: ischemic and dehydrated *Xenopus laevis* livers, where it probably also contributes to HIF-1α modulation (Wu et al., 2013) (Fig. 2).

Active regulation of HIF-1α has been observed in anoxic crayfish as well as in other organisms undergoing metabolic rate depression, such as the hypoxic sea cucumbers (discussed above) and other hibernators and freeze-tolerant insects that experience hypoxic and ischemic conditions (Morin et al., 2005; Maistrovski et al., 2012). In contrast, levels of miR-210 are down-regulated in hypoxic naked mole rat brains (Logan et al., 2020) (Fig. 2). Reduced expression of this OxymiR has been implicated in the induction of cell cycle arrest and apoptosis (Yang et al., 2012); whether this occurs in hypoxic naked mole rat brains requires additional research. However, it should be noted that when HIF-1α levels were examined in naked mole rat brains, both mRNA and protein levels were found to be up-regulated after hypoxic exposures (Xiao et al., 2017; Hawkins et al., 2019).

OxymiR medical applications and questions for the future

Our investigation of OxymiRs from ocean depths to mountaintops begs the (albeit selfish) question – can we exploit these natural responses for human medical applications? Oxygen deprivation is lethal in humans, and it therefore comes as no surprise that a major challenge of afflictions such as heart attacks, strokes and various pulmonary diseases is the disruption of oxygen uptake and delivery (Dean, 1997; Sarkar et al., 2017). MiRNAs have been implicated in both the pathogenesis of ischemia and the recovery from ischemia/reperfusion injuries. Researchers have explored the use of miRNAs as diagnostic biomarkers and prognostic tools, as well as in the development of therapeutics (Eyileten et al., 2018). How do our miRNA responses to oxygen limitation compare with the established, adaptive OxymiR responses of anoxia/hypoxia-tolerant species? Although we cannot answer this question fully, we can briefly highlight the therapeutic potential of a key OxymiR that we have identified, miR-210.

MiR-210 was identified as being either up- or down-regulated in multiple oxygen-limited models and has been implicated in central hypoxia-inducible processes (Ivan and Huang, 2014). It has been identified as a biomarker for acute cerebral ischemia, and researchers have tested an exosome-mediated targeted delivery of miR-210 to promote angiogenesis and brain tissue repair after cerebral ischemia (Zeng et al., 2011; Zhang et al., 2019). Tests on mice showed that delivery of miR-210 enhances animal survival rates, seemingly supporting the use of this miRNA (in conjunction with other treatments) as a possible angiogenic agent to treat ischemia (Zhang et al., 2019). However, a study using another miR-210 treatment contradicted these findings; it suggested that the positive effects of miR-210 depend on the microenvironment and conditions of the biological system. Indeed, inhibiting miR-210 in mouse models of acute ischemic brain injury decreases cerebral infarctions, ameliorates behavioral deficits and suppresses pro-inflammatory responses (Huang et al., 2018). These two contradictory studies highlight the complexity involved in translating observations made in natural systems to medical applications.

How can we build on these findings to improve our understanding of the mechanisms used to survive extreme stress and to gain new insights into their use as therapeutics? Unfortunately, the generic ending of many review articles also applies here – we need more studies. Only by expanding our work on oxygen-responsive miRNAs and by performing deeper studies, which are now possible due to the reduced cost of large-scale sequencing and advances in bioinformatics pipelines for non-model organisms, can we build a more comprehensive knowledgebase.

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Competing interests

The authors declare no competing or financial interests.

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