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Mitochondrial regulation in skeletal muscle: a role for non-coding RNAs?

Jessica Silver, Glenn Wadley, Séverine Lamon

Institute for Physical Activity and Nutrition (IPAN), Deakin University, Geelong, Australia

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Correspondence: Dr Séverine Lamon, Institute for Physical Activity and Nutrition (IPAN), School of Exercise and Nutrition Sciences, Deakin University, Geelong, Australia. Email: severine.lamon@deakin.edu.au

New Findings

- What is the topic of this review?

  This article draws evidence from the current literature to support the hypothesis that non-coding RNA-mediated gene regulation takes place in the mitochondria. An emphasis is put on skeletal muscle.

- What advances does it highlight?

  This review highlights the potential role of miRNAs and lncRNAs in mitochondrial gene regulation. The discovery of a new level of skeletal muscle mitochondria-controlled gene regulation presents exciting perspectives for our understanding of skeletal muscle physiology in health and disease.

Abstract

Skeletal muscle is a highly metabolic tissue characterised by high mitochondrial abundance. As such, skeletal muscle homeostasis relies on the tight control of mitochondrial gene expression to ensure efficient mitochondrial function. Mitochondria retain a conserved genome from prokaryotic ancestors, and mitochondrial gene regulation relies on communication between mitochondrial- and nuclear-encoded transcripts. Small and long non-coding RNAs (ncRNAs) have regulatory roles in the modulation of gene expression. Emerging evidence demonstrates that regulatory ncRNAs, particularly microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), localise within the mitochondria across diverse physiological and pathological states. These molecules present intriguing possibilities for the regulation of mitochondrial gene expression. Current research suggests that all known miRNAs are encoded by the nuclear genome but can target mitochondrial genes. Initial investigations demonstrate direct interactions between the muscle-enriched miR-1 and miR-181c and mitochondrial transcripts, suggesting advanced roles of miRNAs in mitochondrial gene regulation. This review will draw evidence from the current literature to discuss the hypothesis that a level of ncRNA-mediated gene regulation, in particular miRNA-mediated gene regulation, takes

place in the mitochondria. Whilst ncRNA-mediated regulation of the mitochondrial genome is a relatively unexplored field, it presents exciting possibilities to further our understanding of mitochondrial metabolism and human muscle physiology.

Introduction

Mitochondria are the primary regulators of energy metabolism in the cell. As such, they play a central role in cellular homeostasis (Herst, Rowe, Carson, & Berridge, 2017; Russell, Foletta, Snow, & Wadley, 2014). In mammalian cells, mitochondria form a dynamic system that relies on the balance between mitochondrial biogenesis and mitochondrial degradation, termed mitophagy (Herst et al., 2017; Yan, Lira, & Greene, 2012). Mitochondria constantly join and separate this reticular network through processes referred to as mitochondrial fusion and mitochondrial fission (Herst et al., 2017). While most eukaryotic tissues contain mitochondria, differences in mitochondrial abundance reflect tissue-specific energy demands (Little, Safdar, Benton, & Wright, 2011).

Skeletal muscle is a highly metabolic tissue (Zurlo, Larson, Bogardus, & Ravussin, 1990) that is characterized by high-energy needs and high mitochondrial content (Fernández-Vizarra, Enríquez, Pérez-Martos, Montoya, & Fernández-Silva, 2011; Ogata & Yamasaki, 1997). Skeletal muscle mitochondrial content and activity are key determinants of whole-body metabolism and exercise performance (Groennebaek & Vissing, 2017; Nunnari & Suomalainen, 2012). Numerous diseases and conditions, including myopathies, amyotrophic lateral sclerosis, type 2 diabetes and ageing are associated with impaired mitochondrial content and function in skeletal muscle (Russell et al., 2014).

Mitochondria retain a unique conserved genome from prokaryotic ancestors, referred to as mitogenome or mtDNA (Anderson et al., 1981; Groennebaek & Vissing, 2017). The human mitogenome is organized as a circular, covalently closed, double-stranded DNA molecule made of 16,569 base pairs (Anderson et al., 1981; Andrews et al., 1999). It contains 37 genes, out of which 13 encode subunits of enzyme complexes of the oxidative phosphorylation system, 22 encode transfer RNAs (tRNAs) and 2 encode ribosomal RNAs (rRNAs) (Mercer et al., 2011; Taanman, 1999). However, close to 1,500 polypeptides are located in the mitochondria and the vast majority of them, including 85% of the respiratory complex subunits, are encoded in the nuclear genome (Mercer et al., 2011; Taanman, 1999). Mitochondrial regulation therefore relies on the complex coordination of mitochondrial- and nuclear-encoded transcripts.

Non-coding RNAs (ncRNAs) are RNA molecules that are not translated into a protein. NcRNAs include the long-known tRNAs and rRNAs, which are key to production of functional proteins in the

cytoplasm and the mitochondria (Hombach & Kretz, 2016). In addition, several classes of small (< 200nt), regulatory ncRNAs have been described over the last decades, including small interfering (siRNAs), piwi-associated RNAs (piRNAs), microRNAs (miRNAs), small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs). These short RNA species play a role in the modulation of gene expression (C. Zhang, 2009) or in the processing of other RNA species (Matera, Terns, & Terns, 2007). On the other hand, long (> 200nt) non-coding RNAs (lncRNAs) can have multiple functions in the cell, including promoting or repressing gene transcription (Djebali et al., 2012). While tRNA and rRNA are encoded by both the nuclear and the mitogenome, the idea of subcellular localisation of other ncRNAs, particularly within mammalian tissue, is a more recent concept. In 2006, an early study generated cDNA libraries from size-selected murine mitochondrial RNA and cloned a series of putative mitochondrial-encoded small ncRNA species. Presumably owing to low expression levels, their presence could not be confirmed by northern blot analysis (Lung et al., 2006). RNA sequencing performed on mouse testis, mouse small intestine and human small intestine tissue later confirmed that the human and murine mitogenome potentially encodes thousands of small ncRNAs (Ro et al., 2013). In addition, we now know that lncRNAs can also be encoded by the mitochondrial genome (Gao et al., 2018; Landerer et al., 2011; Rackham et al., 2011) and selectively localise in different cell compartments, including the nucleus, the cytoplasm (Djebali et al., 2012), and the mitochondria.

The localisation of regulatory ncRNA species within the mitochondria presents exciting possibilities for mitochondrial gene regulation and opens new avenues to expand our understanding of human muscle physiology (Figure 1). This review will draw evidence from the recent literature to discuss the hypothesis that a level of ncRNA-mediated gene regulation takes place in the mitochondria, with a unique focus on the possible implications for skeletal muscle physiology.

MiRNAs in the mitochondria

Of particular interest is the widely studied class of miRNAs that modulate gene expression (Guo, Ingolia, Weissman, & Bartel, 2010; Humphreys, Westman, Martin, & Preiss, 2005; Olsen & Ambros, 1999). MiRNA-mediated control of gene expression was first suggested in 1993 (R. C. Lee, Feinbaum, & Ambros, 1993; Reinhart et al., 2000) and has added an extra level of complexity to our understanding of transcriptional and translational regulation (Bartel, 2004). MiRNAs are 20-22nt single stranded RNA molecules (Rosalind C. Lee & Ambros, 2001; R. C. Lee et al., 1993) that act by binding to specific target mRNA molecules. Their primary role is to silence protein expression, which can be achieved by either of two posttranscriptional mechanisms: degrading target mRNA molecules

or preventing the translation of the corresponding protein (Guo et al., 2010; Humphreys et al., 2005; Olsen & Ambros, 1999). In some cases, miRNAs can also upregulate protein translation by stabilizing their target mRNA molecule (Vasudevan, Tong, & Steitz, 2007; X. Zhang et al., 2014). The principles underlying miRNAs/mRNAs recognition are not fully understood. It was first established that miRNAs bind to specific sequences of the 3'UTR or the coding region of their target transcript (Pasquinelli, 2012) thanks to a 6-8nt conserved seed sequence localised at their 5' end. This mode of binding can involve perfect or imperfect Watson-Crick recognition (Brennecke, Stark, Russell, & Cohen, 2005) and was long considered the only one. Experimental evidence however demonstrates that close to 60% of miRNAs/mRNAs binding cannot be explained by canonical rules (Chi, Hannon, & Darnell, 2012; Helwak, Kudla, Dudnakova, & Tollervey, 2013), where non-seed motifs or mismatching seed-like motifs are also involved in functional binding. It follows that each miRNA has the potential to target multiple mRNAs through canonical and non-canonical interactions. Approximately 60% of protein-coding genes are regulated by miRNAs (Friedman, Farh, Burge, & Bartel, 2009).

The miRBase database v.22, a searchable database of published miRNA sequences (Griffiths-Jones, Saini, van Dongen, & Enright, 2008; Kozomara & Griffiths-Jones, 2014), retrieves over 4000 entries corresponding to annotated human miRNAs. MiRNAs are encoded in both coding and non-coding parts of the nuclear genome (Bartel, 2004). They are initially transcribed into a primary miRNA molecule (pri-miR) (Bartel, 2004), which is cleaved by the RNase-III type endonuclease Drosha-associated-with-Pasha to become a pre miRNA molecule (pre-miR) (Y. Lee et al., 2003). The pre-miR molecule is then exported from the nucleus to the cytoplasm by Exportin-5 (Lund, Güttinger, Calado, Dahlberg, & Kutay, 2004), where the endoribonuclease Dicer-1 splices it into a single-stranded mature miRNA molecule (Y. Lee et al., 2003). In the cytoplasm, the mature miRNA strand associates with the Argonaute (AGO) proteins to form RNA-induced silencing complexes (RISC) (Bartel, 2004) and direct them to downregulate gene expression. In mammals, the AGO2 protein is necessary to catalyse the cleavage of the target transcript (Chendrimada et al., 2005; Liu et al., 2004; Meister et al., 2004).

MiRNAs typically regulate protein expression within the cytoplasm (Bandiera, Mategot, Girard, Demongeot, & Henrion-Caude, 2013). Extra-cytoplasmic miRNA localisation was first demonstrated in the nucleolus of rat skeletal muscle cells using locked nucleic acid (LNA) probes targeting the mature form of the muscle-enriched miR-206 (Politz, Zhang, & Pederson, 2006). Since then, evidence suggesting further subcellular localisation of miRNAs has emerged (C. Zhang, 2009), including in the nucleus (Salmanidis, Pillman, Goodall, & Bracken, 2014), processing bodies (Gibbings, Ciaudo, Erhardt, & Voinnet, 2009) and the mitochondria (Barrey et al., 2011; Lung et al., 2006), where

miRNAs could regulate the expression of mitochondrial transcripts and subsequent protein translation (Bassi, Heads, Marber, & Clark, 2008; X. Zhang et al., 2014). cDNA libraries generated from size-selected murine liver and kidney mitochondrial RNA (Lung et al., 2006) identified four clones corresponding to nuclear-encoded miRNAs, let-7f-1, let-7g, miR-122a,b and miR-101b. Further observations confirmed the existence of a range of nuclear-encoded miRNAs in highly purified mitochondria isolated from rat liver (Kren et al., 2009) and HeLa cells (Bandiera et al., 2011). It was proposed that the mitochondria might act as a reservoir of miRNAs for the cell, as none of the discovered miRNAs was predicted to target the mitogenome or nuclear transcripts encoding mitochondrial proteins (Kren et al., 2009). A later study however revealed that other mitochondrial miRNAs had several putative nuclear and mitochondrial-encoded targets (Bandiera et al., 2011), suggesting that the newly named "mitomiRs" may reflect another level of crosstalk in the processes underlying mitochondrial regulation.

Current knowledge suggests that all miRNAs are encoded by the nuclear genome (Geiger & Dalgaard, 2017; Ha & Kim, 2014). However, the mapping of a series of pre-miRNA and mature miRNA sequences on the human mitogenome (Bandiera et al., 2011; Barrey et al., 2011; Shinde & Bhadra, 2015) suggest that some miRNAs might originate from the mitochondrial DNA. Whether these miRNAs sequences are actually transcribed from the mitochondrial genome remains speculative.

Overview of miRNA expression within rodent and human mitochondria

Over 100 miRNA species have been identified in mitochondria isolated from rodent tissue, such as rat liver (Kren et al., 2009), rat hippocampus (W. X. Wang et al., 2015), rat heart (Das et al., 2012), mouse liver (Bian et al., 2010) and mouse heart (Bian et al., 2010; Rajaganapathi Jagannathan et al., 2015; X. Zhang et al., 2014). At least 20 miRNA species were observed to be more highly expressed in the mouse liver mitochondria than in whole-liver extracts, with several highly expressed whole-liver miRNAs having low expression in the mitochondria (Bian et al., 2010). The enrichment of miRNAs in mitochondria may therefore be independent of their total cellular abundance.

To date, whilst only a few studies have investigated miRNA expression within human mitochondria, they have identified at least 400 known and 200 purportedly novel miRNAs in mitochondria from muscle, kidney and cancer cells (Bandiera et al., 2011; Barrey et al., 2011; Dasgupta et al., 2015; Lakshmi Sripada et al., 2012). Initially, Barrey et al. used *in situ* hybridisation and isolation of the

mitochondria from human primary cultured myotubes to quantify the expression of 204 miRNAs localised within mitochondria via RT-qPCR (Barrey et al., 2011). Around the same time, 13 nuclear encoded miRNA species enriched in mitochondria from human cervical cancer (HeLa) cells were observed (Bandiera et al., 2011). Additionally, deep sequencing identified an association of 327 known and 13 putative novel miRNAs to mitochondria in HeLa cells, as well as 428 known and 196 putative novel miRNAs to mitochondria in human embryonic kidney (HEK293) cells (Lakshmi Sripada et al., 2012). Other studies have also found miRNA species in isolated human mitochondria from 143B (osteosarcoma) and 206 p0 (derived from 143B) cell lines (Dasgupta et al., 2015; Mercer et al., 2011). Collectively, these studies have established that there are a large number of miRNA species expressed in mitochondria of multiple human cell types.

Mito-myomiRs: potential roles of miRNAs in skeletal muscle mitochondria

Skeletal muscle comprises 40% of human body mass (Rasmussen & Phillips, 2003). As such, skeletal muscle accounts for 20-30% of whole-body energy expenditure at rest (Zurlo et al., 1990), for up to 90% during exercise (Zurlo et al., 1990), and for up to 50% of meal-derived glucose disposal (Radziuk & Inculet, 1983), making it one of the tissues displaying the highest mitochondrial abundance (Fernández-Vizarra et al., 2011). A series of life-threatening and economy-draining conditions, including myopathy, neuro-muscular, neuro-degenerative and metabolic diseases positively associate with impairments in skeletal muscle mitochondrial function (Russell et al., 2014). Expanding our understanding of mitochondrial regulation in muscle health and disease conditions, including by ncRNAs, has therefore the potential to contribute to the identification of novel therapeutic targets.

MicroRNAs regulate skeletal muscle mass, function and metabolism (reviewed in (Zacharewicz, Lamon, & Russell, 2013)). Of particular importance, skeletal muscle displays a series of muscle-enriched miRNAs, termed myomiRs. Canonical myomiRs comprise the miR-1, miR-133 and miR-206 families of miRNAs and originate from three cistrons; (miR-1-2, miR-133a-1), (miR-1-1, miR-133a-2) and (miR-133b, miR-206) (McCarthy, 2011). With time, the myomiR list has expanded to include miR-208a, miR-208b, miR-499, and miR-486 (reviewed in (Kirby & McCarthy, 2013)). MyomiRs undergo high levels of regulation in skeletal muscle development and adaptation in health and disease (Lamon et al., 2017; Zacharewicz et al., 2013). However, muscle specific deletions of individual myomiRs only lead to minor phenotypic effects in mouse models (Kirby & McCarthy, 2013), suggesting possible redundancy and fostering further research in this domain. In addition and

without any formal classification, we and others have identified other muscle-enriched miRNAs, including the miR-23 (Russell, Lamon, et al., 2013; Russell, Wada, et al., 2013), miR-29 (Simona Greco et al., 2009; Russell, Lamon, et al., 2013; Russell, Wada, et al., 2013; H. Wang et al., 2008), miR-31 (Simona Greco et al., 2009; Russell, Lamon, et al., 2013; Russell, Wada, et al., 2013) and miR-181 (Naguibneva et al., 2006; Russell, Lamon, et al., 2013; Russell, Wada, et al., 2013) families of miRNAs. The ubiquitously expressed let-7 family of miRNAs play an anti-proliferative role (Johnson et al., 2007) and can be added to the picture of the miRNAs that are highly expressed in skeletal muscle.

Exercise and disease alter skeletal muscle miRNA expression. For example, in humans, expression levels of the muscle-enriched miR-1, miR-133a, miR-133b and miR-181a are increased (Nielsen et al., 2010; Russell, Lamon, et al., 2013), whereas miR-9, miR-23a, miR-23b and miR-31 are decreased (Russell, Lamon, et al., 2013), following a single bout of submaximal endurance exercise. Furthermore, the type of exercise can have direct impact on the nature of the miRNA response (Drummond, McCarthy, Fry, Esser, & Rasmussen, 2008; Ringholm et al., 2011). When compared to healthy muscle, the same miRNAs are dysegulated in several human myopathies and dystrophies (Eisenberg, Alexander, & Kunkel, 2009; Gambardella et al., 2010; S. Greco et al., 2009), with unloading (Ringholm et al., 2011), as well as in neurodegenerative diseases that are specifically associated to impaired mitochondrial function, such as amyotrophic lateral sclerosis (Russell et al., 2018; Russell, Wada, et al., 2013; Williams et al., 2009).

All known myomiRs are encoded in the nucleus and their pre- and mature sequences do not map the human mitogenome (Barrey et al., 2011). Barrey et al. first demonstrated the presence of mature and immature miRNA forms in the mitochondria isolated from human primary myotubes (Barrey et al., 2011) and RT-qPCR analyses established the presence of multiple miRNA species, amongst which miR-133a, miR-133b, miR-23a and the ubiquitously expressed let-7 family were within the most abundant. Less abundant but still significantly detected in the mitochondria were the myomiRs miR-206 and miR-1. The presence of these miRNAs in muscle mitochondria prompted the question of their potential gene targets. *In silico* analysis identified putative, canonical target sites on several mitochondrial transcripts for miR-133a and members of the let-7 family (Barrey et al., 2011), suggesting that miRNA-mediated regulation of gene expression might occur within the muscle mitochondria.

While few studies have identified a functional role for mitochondrial miRNAs, many authors have speculated on their potential gene and protein targets using target prediction tools. These predictions should, however, be considered with extreme caution. Firstly, target prediction

algorithms do not predict the majority of miRNA/mRNA interactions (Chi et al., 2012; Helwak et al., 2013). Secondly, even when physical binding is confirmed experimentally, for example by the means of a reporter assay, we have frequently observed an absence of regulation of the corresponding gene and/or protein targets *in vitro*, *in vivo* or both (Pinto et al., 2017) (Lamon *et al*., unpublished observations), presumably owning to specific thermodynamic and steric binding conditions. Overall, this indicates that our very partial understanding of miRNA/mRNA binding rules constitutes a major limitation of target predictions. Therefore, if not accompanied by functional validation, *in silico* analysis of the biological pathways that may or may not be influenced by specific miRNAs should be treated as purely speculative. Emerging experimental approaches will likely help to overcome these limitations in the coming years. For example, cross-Linked Immunoprecipitation (CLIP) is a recent technique that identifies the direct RNA binding sites of RNA-binding proteins (Licatalosi et al., 2008). When applied to AGO2, it can be used to identify miRNA binding sites and validate their physical association to RISCs in a physiological environment. AGO2 CLIP can be coupled to high-throughput sequencing or RNA (HITS-CLIP), to high-throughput sequencing of RNA isolated from PhotoActivable-Ribonucleoside (PAR-CLIP) and to individual nucleotide resolution (iCLIP) (Bottini et al., 2017), providing a comprehensive map of AGO2 binding targets in specific biological conditions.

In a very elegant study, Zhang et al. confirmed that miR-1 was localised in the mitochondria of C2C12 cells and that miR-1 quantity increased with myotube differentiation (X. Zhang et al., 2014). More importantly, the functional complex miR-1/AGO2 bound to the mitochondrial transcripts *Cox1, Nd1, Cytb, Cox3* and *Atp8* and enhanced their translation, providing the first available evidence for miRNA-mediated mitochondrial gene regulation in skeletal muscle cells (X. Zhang et al., 2014). Reports of miRNA-mediated translation activation rather than repression are rare but not without precedent (Vasudevan et al., 2007). While the underlying mechanisms can only be speculated, the prokaryotic origin of mitochondria may offer a possible explanation. Firstly, the AGO2 complex play different roles in specific biological contexts and prokaryotic Argonaute proteins possess unique mechanistic features (Hutvagner & Simard, 2008; Shabalina & Koonin, 2008). Secondly, in bacteria, small regulatory RNAs can activate a target mRNA by direct base pairing. A variety of mechanisms, including the 'anti-antisense mechanisms' and the 3'-processing-mediated transcript stabilization act to respectively alleviate the inhibition on a ribosome binding site or to increase the stability of a target transcript (Frohlich & Vogel, 2009). Finally, mitochondrial-encoded mRNAs present polyadenylation patterns inherited from their bacterial ancestors that are different from those found on nuclear-encoded mRNAs (Slomovic, Laufer, Geiger, & Schuster, 2005), including internal

polyadenylation. As such, they may present a possible target for positive regulatory mechanisms mediated by miRNAs in the mitochondria (Nouws & Shadel, 2014; Vasudevan et al., 2007).

Interestingly, miR-1 can simultaneously repress the expression of cytoplasmic gene targets and promote the expression of mitochondrial gene targets (X. Zhang et al., 2014). As such, mitomiRs may coordinate the concurrent translation of nuclear and mitochondrial encoded mitochondrial proteins (Nouws & Shadel, 2014); for example, to control the formation of the respiratory complex. This is especially relevant to skeletal muscle, a tissue that requires constant and rapid adaptation to environmental conditions and changing energy demands. In this regard, the idea of partitioning certain miRNAs between the cytoplasm and the mitochondria to enhance the efficiency of mitochondrial activities in the muscle warrants further investigation. Another example comes from the observation of a functional form of a muscle-enriched miRNA, miR-181, in the mitochondria of cardiac muscle cells (Das et al., 2012). Similar to skeletal muscle, cardiac muscle metabolism primarily relies on substrate oxidation and possesses a high mitochondrial content (Fernández-Vizarra et al., 2011). MiR-181c co-immunoprecipitated with AGO2 complexes extracted from neonatal rat ventricular myocytes. Not only did miR-181c bind to the mitochondrial transcript *Cox1* but it also significantly decreased COX1 protein expression, demonstrating that miR-181c directly regulates COX1 translation in the mitochondria (Das et al., 2012). In addition, overexpressing miR-181c in rat cardiac myocytes impaired mitochondrial function by dysregulating the formation of Complex IV. Overall, these results suggest that, in cardiac muscle, miR-181c might be partitioned in different cellular compartments to fine-tune the formation of the respiratory complexes.

Some miRNA species may also regulate mitochondrial metabolism and function by acting at multiple subcellular locations. A series of nuclear-encoded miRNAs located in the cytoplasm specifically modulate mitochondrial metabolism and function in skeletal muscle (reviewed by our group (Russell et al., 2014; Tsitkanou, Della Gatta, & Russell, 2016) and others (Lima et al., 2017; Massart, Katayama, & Krook, 2016)). Of particular interest are miR-494 (Yamamoto et al., 2012), miR-23a (Russell, Wada, et al., 2013; Wada et al., 2015), miR-696 (Aoi et al., 2010) and miR-542 (Garros et al., 2017). These miRNAs indirectly or directly target key, nuclear-encoded regulators of the mitochondrial function, including mitochondrial transcription factor A (*Tfam*) (Yamamoto et al., 2012), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1α*) (Aoi et al., 2010; Russell, Wada, et al., 2013), cytochrome b and cytochrome c oxidase complex IV (*Cox4*) (Wada et al., 2015), and mitochondrial ribosomal protein MRPS10 (Garros et al., 2017). Of these miRNAs, miR-23a and miR-542 are also expressed in human myotube mitochondria (Barrey et al., 2011), although no putative canonical target site for miR-23a or miR-542 was found in any mitochondrial

transcript (Barrey et al., 2011). It is however, premature to dismiss a regulatory role for these miRNAs within the mitochondria. While the role of miR-23a in the mitochondria is currently unknown, a study demonstrated that miR-23a co-immunoprecipitated with mitochondrial AGO2 complexes isolated from rat brain tissue (W. X. Wang et al., 2015). A direct association between miR-23a and AGO2 may therefore be indicative of functional RISCs within the mitochondria. Bearing in mind the role of miR-23a on the regulation of mitochondrial metabolism in the cytoplasm, the presence of a functional form of miR-23a in the mitochondria warrants further investigation into its potential gene targets and biological effects. A list of miRNAs that are present in muscle mitochondria and have known regulatory functions pertaining to mitochondrial homeostasis is presented in table 1.

Immature forms of miRNA and miRNA-associated proteins are expressed within the mitochondria

Emerging evidence suggests that some level of miRNA biogenesis and processing may occur within the mitochondria itself. This is based on findings that immature forms of miRNA (Barrey et al., 2011; Shinde & Bhadra, 2015), as well as members of the miRNA biogenesis machinery (W. X. Wang et al., 2015) and components of the RISC localize in the mitochondria (Das et al., 2012; Dasgupta et al., 2015; Rajaganapathi Jagannathan et al., 2015; W. X. Wang et al., 2015). Two pre-miR sequences that aligned with the human mitogenome, pre-mir302a and pre-let-7b, were detected within the perinuclear mitochondria of human myoblasts using in-situ hybridisation (Barrey et al., 2011). Further investigation observed the enrichment of the mature form of miR-let-7b, but not miR-302a, in the mitochondrial fraction. This suggests that the biogenesis of pre-miRNAs may occur within the mitochondria. Similarly, pre-miR-338 was observed in the mitochondrial fraction of axons of rat superior cervical ganglion (SCG) neurons (Vargas et al., 2016). The authors proposed that a pool of pre-miR-338 in the mitochondria might act as a reservoir of miRNAs to regulate the translation of nuclear-encoded mitochondrial mRNAs. However, further studies are required to examine the extent to which pre-miRs also undergo processing within the mitochondria. Potential transport mechanisms of mature and immature miRNAs into the mitochondria are discussed in other reviews (Bandiera et al., 2013; Borralho, Rodrigues, & Steer, 2015; Duarte, Palmeira, & Rolo, 2014; Macgregor-Das & Das, 2018; Sripada, Tomar, & Singh, 2012) and suggest a role for AGO2 (Maniataki & Mourelatos, 2005), Polynucleotide Phosphorylase (PNPASE) (Shepherd et al., 2017; G. Wang et al., 2010), P-bodies (Huang et al., 2011) and voltage-gated ion channels (Salinas et al., 2006).

The presence of AGO2 and Dicer in the mitochondria, along with immature forms of miRNAs, reinforces the idea that some level of miRNA-mediated post-transcriptional regulation occurs in the mitochondria. An early seminal study used western blotting to identify the AGO2 protein in purified mouse liver mitochondria (Bian et al., 2010). This observation was confirmed in rat brain tissue, where co-immunoprecipitation of the AGO protein complexes followed by western blotting revealed the presence of AGO2 in both the mitochondrial matrix and outer mitochondrial membrane (W. X. Wang et al., 2015). Studies further confirmed the presence of AGO2 in human mitochondria. AGO2 protein expression was detected in a purified mitochondria fraction of HeLa cells using western blotting, immunofluorescence confocal microscopy and co-immunoprecipitation with the mitochondrial transcript *COX3* (Bandiera et al., 2011), as well as in a purified mitochondria fraction of 143B human bone osteosarcoma-derived cells using western blotting (Dasgupta et al., 2015). With respect to skeletal muscle, tandem mass-spectrometry analysis confirmed the presence of AGO2, but not AGO1 and AGO3, in highly purified mitochondria and mitoplasts fractions extracted from C2C12 cells. The number of AGO2 molecules present in the mitochondria increased with C2C12 cell differentiation, suggesting that more AGO2 may be imported into the mitochondria upon myotube differentiation (X. Zhang et al., 2014). These observations were extended to the DICER protein, which was detected in the mitochondrial matrix and outer mitochondrial membrane of rat brain by western blotting (W. X. Wang et al., 2015). Using the same method, DICER was observed in the mitochondria of different fractions of axons of rat superior cervical ganglion (SCG) neurons (Vargas et al., 2016), a result confirmed by co-immunostaining with the mitochondrial marker VDAC1.

The presence of AGO2 and Dicer in the mitochondria suggests that immature forms of miRNAs may originate from the mitogenome. However, AGO2 and Dicer are versatile proteins that are involved in several biological processes that can be miRNA-dependent or independent (Janowski et al., 2006; R. C. Lee et al., 1993). AGO2 localizes in different cellular compartments, including the nucleus (Ohrt et al., 2008), the Golgi apparatus (Kim, Maizel, & Chen, 2014), the endoplasmic reticulum, and processing bodies (Leung, Calabrese, & Sharp, 2006). Depending on its localization, AGO2 regulates different transcriptional and post-transcriptional processes (Janowski et al., 2006). Similarly, Dicer is an endoribonuclease that cleaves both double-stranded RNA and pre-miRs into mature siRNAs (Carthew & Sontheimer, 2009; Tomari & Zamore, 2005) and miRNAs (Bernstein, Caudy, Hammond, & Hannon, 2001), respectively (R. C. Lee et al., 1993). A few studies started to provide insights into whether DICER and AGO2 respectively, process pre-miRs and associate with mature miRNAs in the mitochondria. Zhang and al. provided several lines of evidence that AGO2 was not only present in the mitochondria, but that the miRNA/AGO2 complex interacted with mitochondrial transcripts to

enhance mitochondrial translation (X. Zhang et al., 2014). The underlying mechanisms however remain speculative, partly because GW182, AGO2's functional binding partner, was not present in the mitochondria (X. Zhang et al., 2014). Interestingly, an earlier study by Ro et al. showed that DICER inactivation impaired, but did not suppress, the production of mitochondrial-encoded small ncRNAs in somatic testis cells. The authors made the intriguing suggestion that mitochondrial small ncRNAs may be the product of other, currently non-described mitochondrial ribonucleases (Ro et al., 2013). These early studies demonstrate that a functional form of AGO2 is present in the mitochondria and suggest that DICER may partially process pre-miRNAs in the mitochondria.

lncRNAs in the mitochondria

Long non-coding RNAs (lncRNAs) are a class of ncRNA that have received less attention than their shorter counterparts have, maybe due to the diversity and complexity of their roles in the cell. LncRNAs can be arbitrary defined as all ncRNAs over 200 nucleotides in length. They are usually shorter than 10,000 nucleotides and can be classified by abundance ("high" *versus* "low") or by function (comprehensively reviewed in (Djebali et al., 2012; X. Wang, Song, Glass, & Rosenfeld, 2011)). A conservative estimation is that the human nuclear genome may encode about 17,000 lncRNAs (X. Wang et al., 2011). LncRNAs can be single- or double-stranded, and while the function of a double-stranded RNA molecule in unclear, authors have suggested that these molecules may act as regulators of their single-stranded equivalents (Rackham et al., 2011; X. Wang et al., 2011). These molecules may or may not be poly-adenylated, determining their stability (Wilusz et al., 2012). LncRNAs regulate gene and protein expression in the cell through a variety of molecular processes (Djebali et al., 2012), including promoting or repressing gene transcription, providing a scaffold for regulatory proteins, facilitating histone methylation and regulating protein activities through allosteric modifications. In addition, lncRNAs are also are involved in the regulation of other ncRNA species (Cesana et al., 2011; Djebali et al., 2012; Jarroux, Morillon, & Pinskaya, 2017; Kung, Colognori, & Lee, 2013; Ponting, Oliver, & Reik, 2009). Human lncRNAs are mostly transcribed by RNA polymerase II, with some rare lncRNAs being dependant on RNA polymerase III (Jarroux et al., 2017). As miRNAs, lncRNAs regulate mitochondrial function and are dysregulated in pathological states. For example, lncRNA *Tug1* binds to and positively regulates *Pgc-1α*, increasing mitochondrial content and respiration (Long et al., 2016), while aberrant *Tug1* expression reduces *Pgc-1α* transcripts and contributes to the mitochondrial dysregulation characteristic of diabetic nephropathy in mouse kidney cells.

LncRNAs are also encoded by the mitochondrial genome (Gao et al., 2018; Landerer et al., 2011; Rackham et al., 2011), but our knowledge and understanding of their putative role in the mitochondria is still in its infancy. In 2011, a study used deep-sequencing, Northern blotting and strand-specific qRT–PCR in the mitochondria form HeLa cells to identify three lncRNAs generated from the mitogenome: lncND5, lncND6, and lncCytb RNA (Rackham et al., 2011). The knockdown of five nuclear-encoded proteins involved in RNA processing independently decreased the abundance of these lncRNAs, suggesting a role for these proteins in the regulation of mitochondrial ncRNA expression (Rackham et al., 2011). Of particular interest, all three lncRNAs were found in relatively high abundance in skeletal muscle mitochondria, when compared to other tissues (Rackham et al., 2011). Whether these lncRNAs act in the mitochondria itself, or are exported from the mitochondria to other cellular compartments (Cesana et al., 2011; Landerer et al., 2011) using and/or contributing to the mitochondrial-nuclear communication pathway (Butow & Avadhani, 2004), and what their roles are, is still to be determined. Recently, two novel lncRNAs encoded by the human mitogenome, *MDL1* and *MDL1AS*, were discovered in the MCF-7 breast cancer cell line (Gao et al., 2018). These lncRNAs may be involved in positive and negative feedback systems that increase or stabilize the expression of mitochondrial transcripts (Gao et al., 2018). A set of chimeric lncRNAs also exists in mouse and human mitochondria (Dong, Yoshitomi, Hu, & Cui, 2017), but with no evidence of expression in muscle tissue in both cases. Future research on lncRNAs and their regulatory role in the mitochondria will help expand our understanding of the regulation of skeletal muscle physiology, and mitochondrial biology in general.

Perspective and conclusion

The efficient regulation of mitochondrial metabolism is essential to whole cell homeostasis. NcRNAs, specifically miRNAs, play an increasingly important role in the regulation of gene expression across a variety of tissues. Emerging evidence highlights the subcellular localisation of miRNAs within the mitochondria of brain, heart, liver and, more recently, skeletal muscle. The idea of miRNA-mediated regulation of the mitochondrial genome therefore presents exciting possibilities for future research.

Initial forays into mitochondrial miRNAs suggested that the mitochondria was simply a reservoir for cytosolic miRNAs (Duarte, Palmeira, & Rolo, 2015). However, direct observations of interactions between mitochondrial miRNAs and mitochondrial transcripts indicate that miRNAs have more advanced roles in influencing mitochondrial metabolism and morphology. The presence of pre-miRNAs within human myoblast mitochondria (Barrey et al., 2011; Shinde & Bhadra, 2015) suggests

that the mitochondrial genome might also harbour sequences for a number of miRNAs. The prospect of extra-nuclear, non-canonical miRNA biogenesis pathways is an intriguing avenue for future investigations.

The bulk of current knowledge describing miRNA profiles in pathological, stress and exercise conditions stems from whole cell investigations. The response of miRNAs at the sub-cellular level is however, largely unknown and is limited to a handful of studies. Differential miRNA expression exists in spatially distinct subsarcolemmal and intermyofibrillar mitochondrial subpopulations within rodent cardiac muscle (Rajaganapathi Jagannathan et al., 2015). While expression of miR-378 in whole cardiac muscle was unchanged following diabetic insult, miR-378 expression increased in intermyofibrillar mitochondria, suggesting that miRNAs may be redistributed between mitochondrial subpopulations in pathological states (Rajaganapathi Jagannathan et al., 2015). Of functional significance, miR-378 was also found to bind to mitochondrial-encoded *Atp6,* along with an associated reduction in ATP6 protein abundance and enzyme activity in intermyofibrillar mitochondria (Rajaganapathi Jagannathan et al., 2015). These findings suggest that, independent of whole cell miRNA populations, mitochondrial miRNAs may have advanced regulatory roles in both mitochondrial and whole cell metabolism.

As yet, the extent of miRNA-mediated regulation of mitochondrial metabolism in healthy skeletal muscle is an underdeveloped field. However, it is reasonable to speculate that miRNAs within the mitochondria might play a role in the increased mitochondrial biogenesis observed after exercise. Firstly, observational studies from whole-muscle report the differential expression of miRNAs following acute exercise and exercise training (Nielsen et al., 2010; Russell, Lamon, et al., 2013; Zacharewicz et al., 2014). For example, miR-1 and miR-133a expression increased, whilst miR-206 expression remained unchanged in whole skeletal muscle following an intense bout of endurance exercise (Nielsen *et al.*, 2010; Russell *et al.*, 2013a). MiR-1/AGO2 binds to the mitochondrial transcripts *Cox1*, *Nd1*, *Cytb*, *Cox3* and *Atp8* in murine skeletal muscle cells (X. Zhang et al., 2014), and further *in sillico* analysis suggests that miR-133a and miR-206 might have more putative binding sites within the mitochondrial genome (Das et al., 2012). Secondly, *PGC-1α*, *NRF-1* and *TFAM* mRNA, all components of the mitochondrial biogenesis pathway, are under miRNA-mediated control in human myoblasts (Barrey et al., 2011) and rodent skeletal muscle cells (Nie et al., 2016; Safdar, Abadi, Akhtar, Hettinga, & Tarnopolsky, 2009). Thirdly, some miRNAs that respond to exercise are found in the mitochondria and play a role in mitochondrial regulation. Examples from our own research are miR-23a and miR-31. MiR-23a is reduced in human skeletal muscle following a single bout of endurance exercise (Russell, Lamon, et al., 2013) and binds to PGC-1α 3'UTR *in vitro* (Russell, Wada,

et al., 2013). In addition, overexpressing miR-23a in mice downregulates skeletal muscle protein abundance of PGC-1α, cytochrome b and cytochrome c oxidase subunit IV (Russell, Wada, et al., 2013). Similarly, miR-31 is reduced in human skeletal muscle following a single bout of endurance exercise and targets HDAC4 and NRF1, genes regulating muscle growth and mitochondrial biogenesis, respectively (Russell, Lamon, et al., 2013). Finally, the upregulation in mitochondrial biogenesis following exercise is influenced by factors at the subcellular level. For example, the subcellular localisation of PGC-1α, albeit between the cytosol and nucleus, is essential to the regulation of mitochondrial biogenesis (Wright et al., 2007). For these reasons, studies investigating how miRNAs respond to endurance exercise within skeletal muscle mitochondria are required to examine how miRNAs mediate adaptive responses in healthy skeletal muscle. MiRNA-mediated regulation of the mitochondrial genome will provide greater depth to our understanding of the adaptive responses targeting mitochondrial metabolism following endurance exercise.

In conclusion, the regulatory role of ncRNAs, particularly miRNAs, in mitochondrial metabolism suggests their expression may be altered within the mitochondria in pathological and physiological states. Though research in this area is currently in its infancy, preliminary studies observe interactions between muscle-enriched miRNAs and mitochondrial mRNAs that may directly influence mitochondrial metabolism within skeletal and cardiac muscle tissue *in vitro*. As yet, the physiological significance of miRNAs within human skeletal muscle mitochondria can only be speculated. Co-ordination between subcellular compartments, for example between the nucleus, cytosol and mitochondria, is essential for efficient mitochondrial function. The expression of the components of certain mitochondrial pathways may therefore be mediated by miRNAs at the subcellular level. Such regulation might even extend to simultaneous changes in miRNA expression in multiple subcellular compartments. Our understanding of mitochondrial miRNA biogenesis, physiology and function will only increase as interest in this field continues to grow.

Author contributions

GW and SL provided a conceptual framework for the review. JS and SL searched and interpreted the literature. All authors wrote and revised the manuscript.

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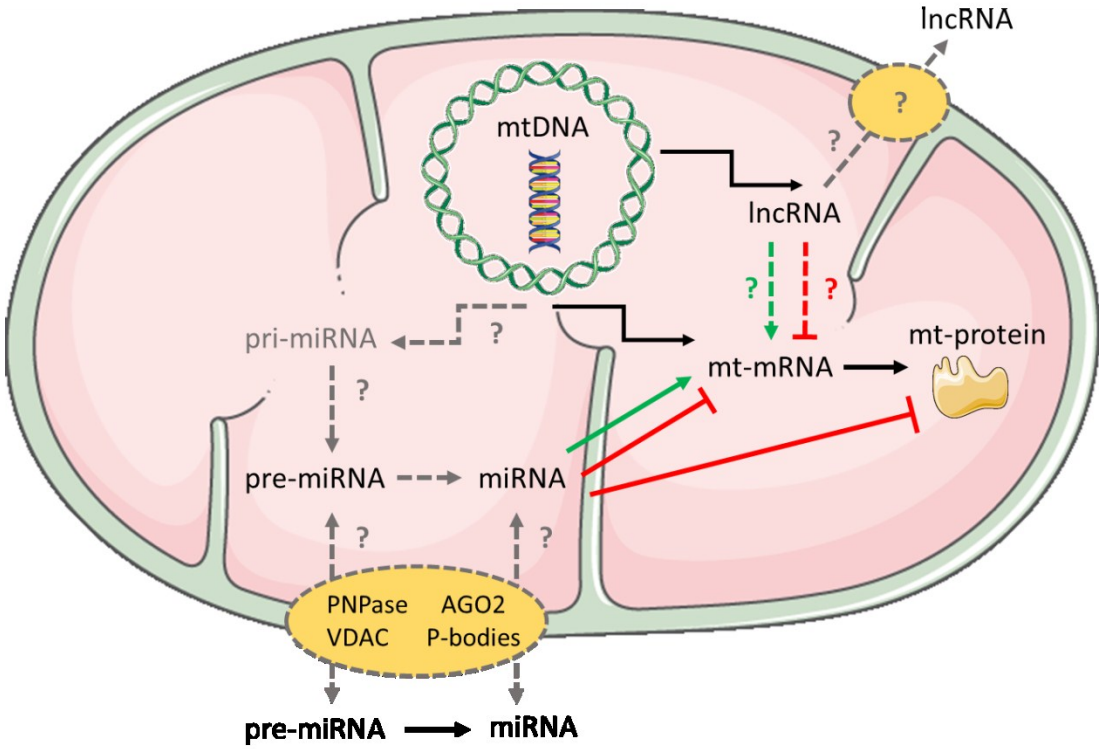
Table 1. Skeletal muscle-related mitochondrial miRNAs were classified as canonical myomiRs, skeletal muscle-enriched miRNAs and cardiac muscle-enriched miRNAs. Their validated gene targets are indicated as well as the level of miRNA-mediated regulation.

miRNA ID	Observed in muscle mitochondria	Experimental Model	Gene targets	Reference
Canonical myomiRs				
miR-1	(Barrey et al., 2011; R. Jagannathan et al., 2015; X. Zhang et al., 2014)	C_2C_{12} myoblasts	mt*Cox1,* mt*Nd1,* mt*Cytb,* mt*Cox3,* mt*Atp8* (transcriptional activation)	(X. Zhang et al., 2014)
miR-133a	(Barrey et al., 2011; R. Jagannathan et al., 2015)	C57Bl/6 mice; miR-133a$^{-/-}$KO	*Pgc-1α*, *Pgc-1β*, *Tfam* (transcriptional repression)	(Nie et al., 2016)
miR-206	(Barrey et al., 2011)	C57Bl/6 mice; miR-206$^{-/-}$KO COS1 cells	HDAC4 (translational repression) *Hdac4* (3'UTR, transcriptional repression)	(Williams et al., 2009)
Muscle-enriched miRNAs				
miR-23a	(Barrey et al., 2011)	Human skeletal muscle HeLa cells miR-23a Tg mice	PGC-1α (transcriptional/translational repression) *Pgc-1α* (3' UTR, transcriptional repression) PGC-1α, COXIV, Cyt-c (translational repression)	(Russell, Wada, et al., 2013) (Wada et al.,

		C57Bl/6 mice	PGC-1α (transcriptional/translational repression), *Cyt-c* (transcriptional repression)	2015) (Safdar et al., 2009)
miR-31	(Barrey et al., 2011)	Human skeletal muscle C_2C_{12} myoblasts	HDAC4, NRF1 (transcriptional/translational repression) *Hdac4*, *Nrf1* (3'UTR, transcriptional repression)	(Russell, Lamon, et al., 2013)
Cardiac miRNAs				
miR-181c	(Barrey et al., 2011; Das et al., 2012)	Neonatal rat primary cardiomyocytes	mt*Cox1* (3'UTR, transcriptional repression) mtCOX1 (translational repression) mtCOX2 (transcriptional/translational repression)	(Das et al., 2012)
miR-378	(Barrey et al., 2011; R. Jagannathan et al., 2015)	HL-1 HEK293	mtATP6 (translational repression) mtATP6 (3'UTR, transcriptional repression)	(R. Jagannathan et al., 2015)

Figure 1. Possible origin and function of mitochondrial miRNAs and lncRNAs. Dashed lines depict relationships that have been hypothesised. Green and red arrows describe "enhancement" and "inhibition", respectively.



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Dr Séverine Lamon graduated from the University of Lausanne, Switzerland, in 2010. She is a former Swiss National Science Foundation (SNSF) and Australian Research Council (ARC) early career fellowship recipient, and currently a senior lecturer at Deakin University, Australia. Her research focuses on understanding the factors that underlie the regulation of skeletal muscle mass in health and disease conditions, with a specific interest in the role of non-coding RNAs in the molecular regulation of skeletal muscle growth. Her research program spans cell, rodent and human models, with the aim to ultimately develop applied interventions to maintain skeletal muscle mass in human subjects.

A/Prof. Glenn Wadley is a muscle physiologist and the associate Head of School (Research) of Deakin University's School of Exercise and Nutrition Sciences (SENS), ranked as the world's top sports science research school for 2016 and 2017 (ARWU University ranking).

Ms Jessica Silver is a PhD student supervised by Dr Lamon and A/Prof. Wadley.