Leucine as regulator of mitochondrial biogenesis via modulating PGC-1 α -targeting microRNAs in C2C12 myotubes

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Ph.D. Dissertation

Leucine as regulator of mitochondrial biogenesis via modulating PGC-1α-targeting microRNAs in C2C12 myotubes

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Abbreviations

3’UTR	: 3'-untranslated region
4E-BP1	: eukaryotic initiation factor 4E- binding protein
AATK	: apoptosis-associated tyrosine kinase
AGO	: argonaute
AMPK	: AMP-activated protein kinase
ATF2	: activating transcription factor 2
ATL	: adult T-cell leukemia
ATP6	: ATP synthase membrane subunit 6
BCAA	: branched-chain amino acids
BCAT	: branched-chain aminotransferase
BCKD	: branched-chain α-ketoacid dehydrogenase
Bcl-2	: B-cell lymphoma 2
CaMK	: calmodulin-stimulated protein kinase
CNTFRα	: ciliary neurotrophic factor receptor α
COX1/2/3	: cytochrome c oxidase subunit 1/2/3
CREB	: cAMP-responsive element-binding protein
Ctdsp2	: C-terminal domain small phosphatases
CXCR1	: chemokine receptor
DGCR8	: DiGeorge syndrome critical region gene 8
Dlk1-Dio3	: delta-like homolog 1 - type III iodothyronine deiodinase
DNM2	: dynamin 2
DNMT3B	: DNA methyltransferase 3B
DRP1	: dynamin-related protein 1

eIF-4E : eukaryotic initiation factor 4E

eNOS : endothelial nitric oxide synthase

ER : endoplasmic reticulum

ERK1/2 : extracellular signal-regulated protein kinase 1/2

ERR : estrogen-related receptor

ETC : electron transport chain

$FADH_2$: flavin adenine dinucleotide

FoxJ3 : Forkhead box J3

FXR1 : Fragile X mental retardation syndrome-related protein 1

GATOR : GTPase activator-mTOR interacting protein

GLUD1 : glutamate dehydrogenase 1

GSK3β : glycogen synthase kinase 3β

HDAC1 : histone deacetylase 1

HMB : β-hydroxy β-methyl butyrate

Hsp70 : 70 kDa heat shock protein

IMM : inner mitochondrial membrane

IMS : mitochondrial intermembrane space

IRS1 : insulin receptor substrate 1

KIC : α-ketoisocaproate

KIV : α-ketoisovalerate

KMV : α-keto-β-methylvalerate

lncRNA : long non-coding RNA

Mcl-1 : induced myeloid leukemia cell differentiation protein

MEF2A : myocyte enhancer factor 2A

MFF : mitochondrial fission factor

Mfn 1/2 : mitofusin 1/2

MIB : mitochondrial intermembrane space bridging complex

miRNA : microRNA

miRNPs : micro-ribonucleoproteins

mitomiRs : mitochondrial miRNAs

mtDNA : mitochondrial DNA

mTERF1 : transcription termination factor

MTHFR : methylenetetrahydrofolate reductase

mTOR : mammalian target of rapamycin

MyHC : myosin heavy chain

NADH : nicotinamide adenine dinucleotide

ncRNA : non-coding RNAs

ND1 : NADH-ubiquinone oxidoreductase chain 1

NRF 1/2 : nuclear respiratory factors 1/2

OMM : outer mitochondrial membrane

OPA1 : optic atrophy 1

OXPHOS : oxidative phosphorylation

p38 MAPK : p38 mitogen-activated protein kinase

PAM : pre-sequence translocase-associated motor

PARP-2 : poly (ADP-ribose) polymerase 2

PDH : pyruvate dehydrogenase

PGC-1α : peroxisome proliferator-activated γ - receptor coactivator 1 α

PI3K : phosphoinositide 3-kinase

piRNA : Piwi associated RNAs

P-MK2 : MAPK-activated protein kinase-2

PNPase : polynucleotide phosphorylase

POLRMT : mitochondrial RNA polymerase

PPARα/δ/γ : peroxisome proliferator-activated α/δ/γ

pri-miRNA : primary miRNA

PTEN : phosphatase and tensin homolog

Raptor : regulatory-associated protein of mTOR

RIP140 : receptor-interacting protein 140

RISC : RNA-induced silencing complex

ROS : reactive oxygen species

S6K1/p70S6K : Ribosomal protein S6 kinase 1

SAM : sorting and assembly machinery

siRNA : small interfering RNAs

SIRT1 : protein deacetylase sirtuin 1

SUMO : small ubiquitin-like modifier proteins

TCA : the tricarboxylic acid cycle

TEFM : transcription elongation factor

TF2BM : mitochondrial transcription factor B2

TFAM : mitochondrial transcription factor A

TIM : translocase of the inner membrane

TNF-α : tumor necrosis factor α

TOM : translocase of the outer membrane

TRAP/DRIP : vitamin D3 receptor-interacting proteins/ thyroid hormone receptor-associated proteins

UCP1 : uncoupling protein 1

UQCC2 : ubiquinol-cytochrome c reductase assembly factor 2

VDAC : voltage-dependent anion channel

XPO5 : exportin-5 enzyme

YY1 : Ying Yang 1

This dissertation consists of the results of the following publications and unpublished experimental data.

(1) Pérez-López, C., Shibaguchi, T., Morino, K., Koma, R. and Masuda, K. (2020) Effect of leucine on microRNAs and mitochondrial biogenesis in C2C12 myotubes. *Advances in Exercise and Sports Physiology, under review.*

I. Introduction

Several studies have demonstrated that small-size nutrients are beneficial for skeletal muscle development by modulating glucose metabolism, fatty acid oxidation, protein synthesis, and other cellular processes (Craig et al., 2015; Efeyan et al., 2015). For example, branched-chain amino acid leucine has emerged as a favorite amongst athletes and recreational exercisers to promote protein synthesis and muscle growth (Vaughan et al., 2013; Craig et al., 2015). Moreover, leucine induce mitochondrial biogenesis through diverse mechanisms that converge in the activation of peroxisome proliferator-activated γ (PPARγ)-receptor coactivator 1 α (PGC-1α) (D'Antona et al., 2010; Schnuck et al., 2016), leading to enhanced transcriptional activity and mitochondrial biogenesis.

MicroRNAs (miRNAs) are small non-coding RNA of approximately 22 nucleotides in length that have been reported to regulate ~60% of the protein-coding genes, thereby controlling most cellular processes (Aoi, 2015; Bianchi et al., 2017). They exert their biological function by binding to the 3'-untranslated region (3'UTR) of targeted mRNA, either by inhibiting translation or promoting mRNA degradation (Hussain, 2012). Here we focused on three miRNAs, miR-494-3p, miR-696, and miR-761 that play a part in regulating mitochondrial biogenesis of skeletal muscle, although the exact regulatory mechanism has not been elucidated (Yamamoto et al., 2012; Long et al., 2013; Xu et al., 2015). These miRNAs are downregulated following treadmill running and voluntary wheel exercise thus contributing to skeletal-muscle adaptations in mice (Yamamoto et al., 2012; Xu et al., 2015). Several studies showed that miR-494-3p could be regulated by other conditions such as cold exposure (Lemecha et al., 2018), dietary interventions (Cameron et al., 2016), and the progression and metastasis of diverse types of cancer (Chai et al., 2015; Dutta et al., 2016), whereas modulation of miR-696 and miR-761 has been less studied. The low-expression level of miR-494-3p in

cancer cells promotes invasion and survival of many cell types through the phosphatase and tensin homolog (PTEN) / phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway that prevents caspase-mediated apoptosis (Li et al., 2015). It has been proposed that miR-494-3p, miR-696, and miR-761 regulate mitochondrial biogenesis partially by direct binding to the 3'UTR of PGC-1α gene (Xu et al., 2015; Fang et al., 2016; Lemecha et al., 2018). Initially, miR-696 was identified as a stretch-sensitive miRNA, downregulated by exercise, and upregulated by immobilization (Aoi, 2015). Later, the function of miR-696 in skeletal muscle was found to be implicated in energy metabolism, mitochondrial biogenesis, and the resistin-mediated ectopic deposition of lipids (Wen et al., 2015). Similarly, miR-761 was found to repress the mitochondrial fission program and apoptosis by targeting mitochondrial fission factor (MFF) along with the p38 mitogen-activated protein kinases (p38 MAPK) / activating transcription factor 2 (ATF2) signaling pathway, therefore enhancing the transcription of PGC-1α (Long et al., 2013). Mitochondrial transcription factor A (TFAM), Forkhead box J3 (FoxJ3), PGC-1α, MFF, and members of MAPK pathway are validated targets of these miRNAs (Aoi et al., 2010; Xu et al., 2015; Sun et al., 2016). Among these genes, PGC-1α is of vital importance as a key regulator of cell metabolism, mitochondrial biogenesis, antioxidant defense, and inflammatory response in skeletal muscle (Kang and Ji, 2013; Peng et al., 2017). Also known as the master regulator of mitochondrial biogenesis, it constitutes the only common validated target among the exercise-responsive miRNAs: miR-494-3p, miR-696, and miR-761. Because PGC-1α is highly sensitive to cellular energy status (Khan et al., 2015), we consider it to be an important candidate for the study of nutrients-mediated regulation of mitochondrial biogenesis through miRNAs and address it in the present study.

The accumulated knowledge confirms the miRNAs connection with a wide range of biological processes that demands a strict regulatory mechanism to maintain cellular homeostasis. In the field of skeletal muscle, miRNAs-focused research is expected to identify

biomarkers of athletic performance, physical fatigue, and the incidence risk of disease development (Aoi and Sakuma, 2014). Within the vast family of miRNAs, herein we centered our attention on the study of miR-494-3p, miR-696, and miR-761 referred here as exercise-responsive miRNAs due to their typical response after physical activity. Although discovered separately, they respond to similar stimuli and share target genes, with PGC-1α as one of the most relevant, thus regulating major cellular processes including mitochondrial biogenesis, fatty acid oxidation, and apoptosis (Aoi et al., 2010; Yamamoto et al., 2012; Long et al., 2013; Li et al., 2015; Xu et al., 2015). This might suggest a cooperative mechanism of action among these miRNAs. However, it remains unclear whether the exercise-responsive miRNAs miR-494-3p, miR-696, and miR-761, are regulated individually or coordinately by nutrients stimulation. Therefore, the present study focused mostly on the effect of leucine on their functions and how it affected downstream effectors, and ultimately, mitochondrial biogenesis. We hypothesized that: i) leucine regulates the expression of miR-494-3p, miR-696, and miR-761 in skeletal muscle, ii) the regulation of these miRNAs differs between nutrients, and iii) leucine promotes mitochondrial biogenesis partially through this miRNAs-mediated regulation of PGC-1α.

II. Literature Review

2.1 Mitochondrial Structure and Functions

Mitochondria are dense, double membrane-enclosed organelles present in almost all mammalian cells that provide a host of metabolic functions, including energy production (Spinelli and Haigis, 2018). This complex membrane structure enables ATP generation via oxidative phosphorylation (OXPHOS) through an electrochemical gradient established across the inner membrane. This gradient involves electrons donated from reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) generated by oxidation of acetyl-CoA via the tricarboxylic acid (TCA) cycle. The acetyl-CoA is generated by oxidation of energy substrates, predominantly glucose, fatty acids, and amino acids (Shi and Tu, 2015). The electrons are accepted and transferred across components of the electron transport chain (ETC), which is coupled to the transport of protons through the inner mitochondrial membrane (IMM), establishing the electrochemical gradient that leads to ATP synthesis (Dorn et al., 2015).

Although it is conventionally accepted that the primary function of mitochondria is the generation of ATP, this narrow view overlooks the many other impressive biosynthetic and regulatory capacities of this versatile organelle. Mitochondria, for instance, are essential for the synthesis of pyrimidines and purines, contribute to the synthesis of heme, regulate nitrogen balance through the urea cycle, produce ketone bodies, are necessary for sex hormone production, are involved in the processing of xenobiotics, play a critical role in redox signaling, innate immunity, autophagy, and are key regulators of the apoptotic program in mammals (Dominy et al., 2010; Tilokani et al., 2018).

As ubiquitous, semi-autonomous cellular organelles, mitochondria are separated from the cytoplasm by the outer and inner mitochondrial membrane. The outer membrane (OMM) is

porous and therefore, permeable to ions and small, uncharged molecules through pore-forming membrane proteins (porins), such as the voltage-dependent anion channel (VDAC) (Bayrhuber et al., 2008). Any larger molecules, especially proteins, have to be imported by special translocases. By contrast, the IMM is a tight diffusion barrier to all ions and molecules. These can only get across with the aid of specific membrane transport proteins, each of which is selective for a particular ion or molecule (Kühlbrandt, 2015).

The inner and outer membranes of mitochondria define three compartments within the organelle, each with its distinct role and corresponding protein components. The innermost compartment is the mitochondrial matrix, site of organellar DNA replication, transcription, protein biosynthesis, and numerous enzymatic reactions. The next compartment is the intermembrane space (IMS), a ~20 nm gap between the outer and inner membrane. This area is responsible for maintaining the proton gradient generated by the ETC machinery, which is localized to the IMM and pumps protons from the mitochondrial matrix into the IMS. The IMM forms invaginations, called cristae, that extend deeply into the matrix. The inner membrane is anchored to the outer membrane through the mitochondrial intermembrane space bridging complex (MIB), and this anchor is required to maintain proper cristae structure. The cristae define the third mitochondrial compartment, the crista lumen. The crista membranes contain most, if not all, of the fully assembled complexes of the ETC and the ATP synthase. The mitochondrial cristae are thus the main site of biological energy conversion in all non-photosynthetic eukaryotes (Kühlbrandt, 2015).

As unique organelles, mitochondria comprise its self-replicating genome. The mitochondrial DNA (mtDNA) encodes 13 essential components of the ETC as well as all rRNAs and tRNAs necessary for translation of the mtDNA-encoded proteins (Scarpulla, 2011). However, the vast majority of mitochondrial proteins (>1000) are encoded by the nuclear genome (Lotz et al., 2014). Therefore, the biogenesis of mitochondria requires exquisite

coordination of both mitochondrial and nuclear genomes. It is not surprising that there exist multiple layers of control that enable cells to coordinate mitochondrial activity with fuel sources, biosynthetic demands, proliferation rates, and external stimuli (Dominy and Puigserver, 2013).

2.1.1 Mitochondrial Biogenesis

Mitochondrial biogenesis is influenced by environmental stress such as exercise, caloric restriction, low temperature, and other cellular events including oxidative stress, cell division, renewal, and differentiation (Jornayvaz and Shulman, 2010). This complex process can be defined as the growth and division of pre-existing mitochondria, a phenomenon that is accompanied not only by variations in number but also in size and mass (Jornayvaz and Shulman, 2010). Transcriptional and post-transcriptional mechanisms contribute to the expansion of the mitochondrial reticulum, as reflected by an increase in mitochondrial protein content, volume density, enzymatic activities, and/or mtDNA copy number. These parameters have been presented to support/refute the ability of a regulatory protein or dietary compound to promote mitochondrial biogenesis (Islam et al., 2020).

Mitochondria constantly undergo biogenesis, fusion, and fission, forming dynamic networks that are necessary for the maintenance of organelle fidelity (Edwards et al., 2010). Mitochondrial biogenesis entails replication of mtDNA, transcription, and translation of mtDNA-encoded genes, as well as loading of phospholipids and nuclear-encoded proteins in different mitochondrial subcompartments (Zhu et al., 2013). Mitochondrial transcription starts in the D-loop region through the assembly of a complex composed by TFAM and mitochondrial transcription factor B2 (TF2BM), and the RNA polymerase POLRMT (Dominy and Puigserver, 2013). Among other proteins controlling the process are the transcription elongation factor (TEFM), and transcription termination factor (mTERF1) (Barshad et al.,

2018). Mitochondrial DNA is compacted by TFAM into supramolecular assemblies called nucleoids (Kukat et al., 2015). Individual mRNA, rRNAs, and tRNAs are then released from the polycistronic transcripts by the cleavage of tRNAs, which in humans is performed by endonucleases RNase P complex and ELAC2 (Brzezniak et al., 2011). After release, the rRNAs undergo chemical nucleotide modifications before becoming part of the mitoribosome. Finally, the mature mRNAs, tRNAs, and the assembled mitoribosome come together in the translation apparatus, for the synthesis of 13 essential OXPHOS components required for the function of respiratory complexes I, III and IV, as well as the ATP synthase (D'Souza and Minczuk, 2018).

As mtDNA's coding capacity is very limited, mitochondria are heavily dependent on the import of nuclear-encoded proteins. The majority of proteins are encoded in the nucleus, therefore, a mechanism for targeting, import, and correct assembly exists to ensure correct mitochondrial function and shape. Globally, mRNAs are translated to precursor proteins in the cytosol and imported to different mitochondrial compartments via the TOM (translocase of the outer membrane) (Ploumi et al., 2017). TOM40 forms the import pore of the OMM and is permeable by all mitochondria-targeted precursor proteins. Another component of the TOM complex, TOM20, anchors nuclear-encoded transcripts and stabilizes them in the mitochondrial vicinity, making them available for local translation by resident ribosomes, also participating in their concomitant import (Eliyahu et al., 2010). In the matrix, the pre-sequence is then cleaved by a protease, and often with the aid of molecular chaperones, the imported protein is folded. However, a majority of protein precursors use different import pathways to the other mitochondrial compartments since they do not typically contain the N-terminal targeting signals. Currently, beside the TOM complex, three major membrane protein translocase complexes are known. Upon translocation through TOM, precursors are handed over to the TIM complex of the inner membrane, which sorts matrix-targeted precursors. In addition, PAM (pre-sequence translocase-associated motor) regulates matrix heat shock

protein Hsp70 action and assists TIM-mediated transport into the matrix. Precursors containing β-barrel signal are translocated from TOM to the SAM (sorting and assembly machinery) complex mediating their integration to the OMM. All of these processes are an integral part of mitochondrial biogenesis (Jornayvaz and Shulman, 2010; Ploumi et al., 2017; Wiedemann and Pfanner, 2017).

2.1.2 Mitochondrial Dynamics

Mitochondrial dynamics are the repetitive cycles of fission and fusion to maintain mitochondrial content and structural homeostasis (Valera-Alberni and Canto, 2018). These are highly orchestrated events influenced by a variety of physiological and environmental cues. For instance, nutrient supply: nutrient overload is linked to the fragmentation of the mitochondrial network, while mitochondria elongate under starvation (Gomes et al., 2011). Fission and fusion events act as a quality control mechanism by removing defective mitochondria or by selecting the organelles with the optimal matrix metabolites, intact mtDNA copies, and mitochondrial membrane components (Tilokani et al., 2018). The balance between these activities will determine the architecture of the mitochondrial network and influences multiple functions, including respiratory coupling, calcium buffering, or apoptosis (Valera-Alberni and Canto, 2018). In this regard, the disruption of mitochondrial dynamics can give rise to a wide range of health and metabolic diseases, including diabetes and obesity (W. Wang et al., 2012), neurodegenerative diseases (Fu et al., 2014), and age-related physiological decline (Wang et al., 2011).

Mitochondrial fission is a multi-step process that promotes the fragmentation of mitochondria. The main steps, constriction, and scission, are carried out by the GTPases dynamin-related protein 1 (Drp1) and dynamin 2 (Dnm2), respectively (Kraus and Ryan, 2017). During mitochondrial division, depolarization of mitochondria recruits Drp1 to the OMM

where it forms a ring-like structure around mitochondria leading to the narrowing of the membrane (Fröhlich et al., 2013). Then, GTP hydrolysis enhances this membrane constriction which marks a potential future site of mitochondrial scission (Mears et al., 2011). Finally, Dnm2 is transiently and specifically recruited to the endoplasmic reticulum (ER) and Drp1-induced constriction sites leading to fission (Lee et al., 2016). Once isolated, the damaged mitochondria are then degraded by mitophagy and their components recycled. While the mechanisms regulating OMM constriction are well documented, the events leading to IMM constriction or division are poorly understood. Recent studies have suggested that IMM constriction is Ca^{2+}-dependent and occurs at mitochondria–ER contact sites early during the mitochondrial division process (Chakrabarti et al., 2018).

Mitochondrial fusion is the process involving the joining of the IMM and OMM of either two distinct mitochondria or different regions of the mitochondrial reticulum. This serves as a 'compensatory' mechanism to equilibrate proteins, complexes, and metabolites from a healthy mitochondrion to a second one that might be damaged. Therefore, fusion may recruit dysfunctional mitochondria into the active pool, instead of being eliminated by mitophagy (Drake et al., 2016). Mitochondrial fusion is dependent on the transmembrane GTPases mitofusins 1 and 2 (Mfn 1 and Mfn 2) and optic atrophy 1 (OPA1), which mediate OMM and IMM fusion, respectively (Pernas and Scorrano, 2016). Initially, the dimerization of mitofusins results in the tethering of the outer membranes of adjoining mitochondria. Before fusion, the curving of the outer membranes is promoted by the phospholipase D-dependent hydrolysis of cardiolipin. In the second step, the fusion of the inner membranes requires a motor-like process driven by OPA1 and coordinated by various other proteins, including the prohibitins (Ranieri et al., 2013).

The deregulation of mitochondrial fission and fusion programs results in either a fragmented network characterized by a large number of small round-shape mitochondria or a

hyper fused network with elongated and highly connected mitochondria. Organelle fragmentation is often linked to mitochondrial dysfunction as this morphological state predominates during elevated stress levels and cell death (Zemirli et al., 2018). However, it is also observed in the phase G2/M of the cell cycle and is needed for mitochondrial motility, quality control, and mtDNA inheritance (Pickles et al., 2018). Mitochondrial elongation also protects the organelle from degradation by autophagy during starvation and is mainly associated with cell survival mechanisms (Rambold et al., 2011).

2.1.3 Regulation of Mitochondrial Functions by the PGC-1α-Signaling Cascade

During mitochondrial biogenesis, the crosstalk between mitochondrial and nuclear genes it is mediated primarily by the activation of PGC-1α (Li and Susztak, 2018), nuclear transcriptional coactivator which regulates several important metabolic processes, including mitochondrial biogenesis, adaptive thermogenesis, respiration, insulin secretion and gluconeogenesis (Liu and Lin, 2011). Activated PGC-1α triggers downstream effects in a tissue-specific manner. In the liver, PGC-1α controls gluconeogenesis via increasing TCA cycle flux and inducing the expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase upon caloric restriction or prolonged fasting (Scarpulla, 2011). In brown adipose tissue, PGC-1α induces uncoupling protein 1 (UCP1) expression and coordinately modulates adaptive thermogenesis (Sharma et al., 2014). In skeletal muscle, PGC-1α is a potent regulator of glucose uptake, fatty acid oxidation, and muscle fiber type switching and marked increases in exercise performance (Handschin and Spiegelman, 2011; Kang and Ji, 2012).

PGC-1α enhances transcription by interacting directly with different nuclear receptors via specific LXXLL recognition domains, recruiting molecules that mediate chromatin remodeling via histone acetylation, and interacting with the vitamin D3 receptor-interacting proteins/ Thyroid hormone receptor-associated proteins (TRAP/DRIP) complex to recruit RNA

polymerase II (Bhat et al., 2019). Effector transcription factors within this cascade include members of the PPAR, nuclear respiratory factors (NRF1 and NRF2), estrogen-related receptor (ERR), and transcription factor families (Villena, 2015). These transcription factors control and regulate diverse aspects of mitochondrial energy metabolism, respiration, and biogenesis.

All three PPARs (PPARα, γ, and δ) play essential roles in lipid and fatty acid metabolism by directly binding to and modulating genes involved in fat metabolism. PPARδ plays a role in controlling oxidative metabolism and fuel preference, mainly fatty acid oxidation, mitochondrial OXPHOS, and glucose utilization (Liu et al., 2011). Besides, PPARδ also increases the proportion of oxidative fibers that are rich in mitochondria, thus dramatically boosting mitochondrial oxidative metabolism in skeletal muscle (Corona and Duchen, 2016).

Nuclear factors NRF1 and NRF2 regulate expression of the ETC subunits encoded by the nuclear genome (Satoh et al., 2013). Specifically, NRF1 controls the expression of TFAM and TFB2M (Gleyzer et al., 2005). TFAM plays a critical role in maintaining copy number and structure of mtDNA, while TFB2M acts as a specific dissociation factor that provides interaction between POLRMT and TFAM. Therefore, regulation of these factors is crucial for efficient transcription of mtDNA genes. Studies reveal that NRFs regulates multiple target genes, encoding proteins of the mitochondrial protein import machinery (TOM20, TOM34), the detoxification response (i.e. components of glutathione biosynthesis pathway), the heme biosynthetic pathway, among others (Satoh et al., 2013; Yang et al., 2014).

The ERR family of nuclear receptors serves a central function in the PGC-1α regulatory circuitry. In cooperation with PGC-1α, ERRα has been shown to regulate genes in virtually every pathway of mitochondrial energy transduction and ATP synthesis, including fatty acid oxidation, the TCA cycle, and the ETC/OXPHOS (Singh et al., 2018). DNA-binding sites for ERRα have been mapped in a large number of nuclear-encoded mitochondrial genes, including

those involved in oxidative phosphorylation, fatty acid oxidation, TCA cycle, and factors regulating mitochondrial fusion/fission (Dominy and Puigserver, 2013).

2.1.4 Regulation of PGC-1α

PGC-1α is typically expressed in mitochondria-enriched tissues with high energy demands (i.e. heart, skeletal muscle, brown fat, kidney, liver, and brain) (Correia et al., 2015). Essentially, PGC-1α receives inputs from multiple pathways to control its expression and activity under diverse developmental and physiological contexts (Dorn et al., 2015). These include the activation of p38 MAPK, AMP-activated protein kinase (AMPK), Ca^{2+}-mediated pathways involving calmodulin-stimulated protein kinases (CaMKs), as well as reactive oxygen species (ROS)-induced signaling (Figure II-1). All have been implicated in the regulation of PGC-1α promoter activity. For example, activation of the cAMP-responsive element-binding protein (CREB) and ATF2 via either Ca^{2+}/CaMKs or p38 MAPK, respectively, stimulate binding of CREB to the cAMP-responsive element (CRE) in the promoter of PGC-1α (Yan et al., 2007). Interestingly, PGC-1α can self-regulate its transcription through Ying Yang 1 (YY1), in a direct interaction that requires the presence of the mammalian target of rapamycin (mTOR), thus stimulating mitochondrial activity (Cunningham et al., 2007). Conversely, nuclear regulators receptor-interacting protein 140 (RIP140), DNA methyltransferase 3B (DNMT3B), adult T-cell leukemia (ATL), and p53 are known negative transcriptional regulators of PGC-1α (Wenz, 2013).

The PGC-1α activity is also regulated by multiple post-transcriptional modifications, including, acetylation, phosphorylation, and ubiquitination. Specifically, histone acetyltransferase GCN5 directly acetylates PGC-1α at multiple lysine residues and negatively regulates its transcriptional activity, while the NAD^{+}-dependent protein deacetylase sirtuin 1 (SIRT1) activates PGC-1 through lysine deacetylation (Dominy et al., 2010). Furthermore

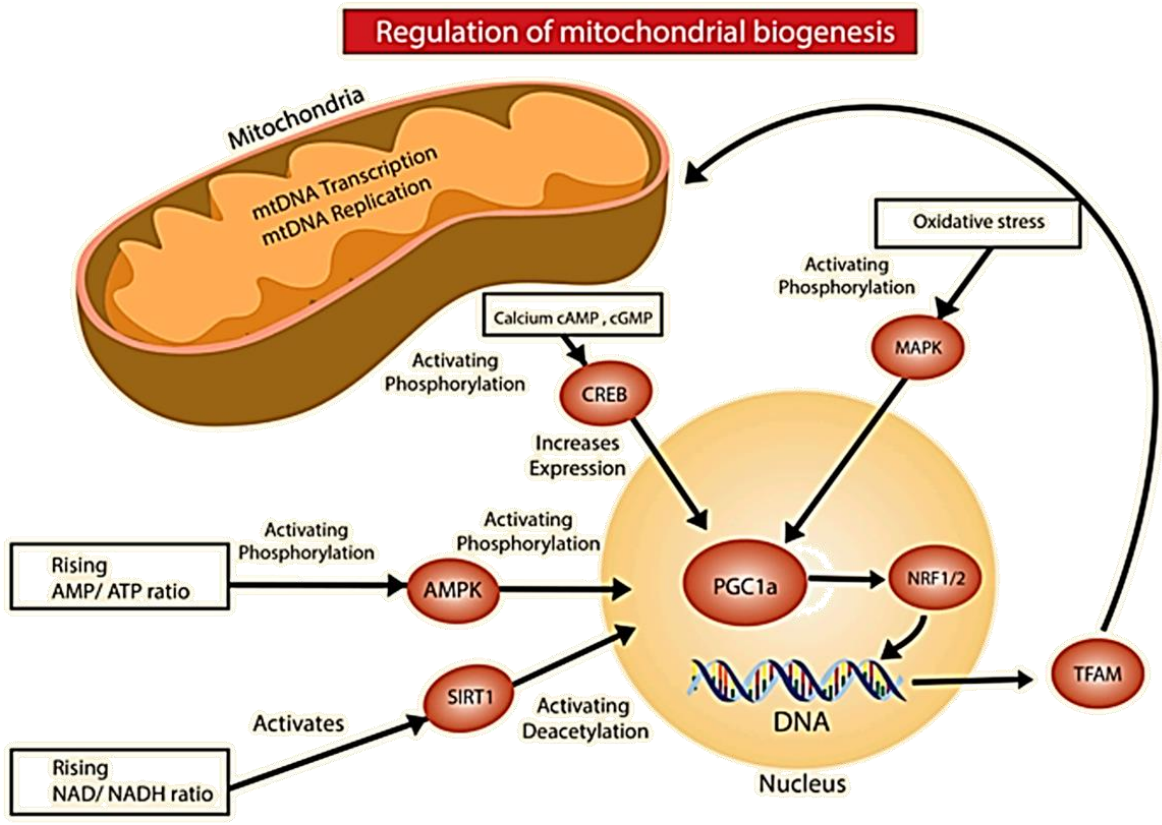

Figure II-1. Regulation of mitochondrial biogenesis. Activation of different signaling pathways, such as AMPK, SIRT1, CREB, MAPK has been associated with mitochondria biogenesis by increasing PGC-1α gene transcription. PGC-1α represents the major co-transcriptional factor that regulates mitochondria biogenesis by activating the nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF2) which leads to increase expression of mitochondrial transcription factor (TFAM), driving transcription and replication of mtDNA (Roque et al., 2020).

PGC-1α may be subjected to other modifications such as phosphorylation and methylation, as well as interaction with other proteins including co-repressors (Drake et al., 2016). p38 MAPK, AMPK, Akt, and glycogen synthase kinase 3β (GSK3β) are the best-characterized kinases that regulate PGC-1α by phosphorylation. Unlike p38 MAPK and AMPK, the kinase activity of Akt and GSK3β is associated with the inhibition of PGC-1α by promoting its proteasomal degradation (Fernandez-Marcos and Auwerx, 2011).

In response to metabolic stress, mitochondrial biogenesis is stimulated through AMPK, which acts as a sensor for the AMP/ATP ratio (Ross et al., 2016). There is some evidence that phosphorylation by AMPK activates PGC-1α, providing a link between cellular energy status and mitochondrial biogenesis (Wan et al., 2014). Interestingly, AMPK not only influences the activity of PGC-1α but of another key regulator of cellular metabolism and mitochondrial function, SIRT1 (Cantó and Auwerx, 2009). SIRT1, in turn, can deacetylate and activate PGC-1α as well, leading to its activation (Gurd, 2011).

In addition to acetylation, lysine residues on PGC-1α can also be modified by small ubiquitin-like modifier (SUMO) proteins. The sumoylation of proteins has many functions that can affect protein subcellular localization, stability, activity, and capacity to interact with other proteins and activity. PGC-1α can be sumoylated on lysine residue 183 attenuating its transcriptional activity and enhancing the sensitivity of PGC-1α to the repressive actions of RIP140 (Rytinki and Palvimo, 2009).

2.2 MicroRNAs

Historically, RNAs have been classified according to their protein-coding potential (Dinger et al., 2008). Although up to date, protein-coding regions encompass only ~2% of the human genome, detected RNAs not translated into proteins are still commonly termed non-coding RNAs (ncRNAs) (Gusic and Prokisch, 2020). Unlike housekeeping ncRNAs (e.g.

tRNAs, rRNAs, and snRNAs), regulatory ncRNAs are still poorly understood and a very heterogeneous group with diverse functions, from gene expression regulation to modulation of protein and RNA distribution within cells (Szymański et al., 2003). Based on their length, they are divided into short (< 200 nt) and long (> 200 nt, lncRNAs) RNAs. Short ncRNAs consist of microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi associated RNAs (piRNAs) (Brosnan and Voinnet, 2009).

miRNAs are endogenous, single-stranded, ~22nt in length RNAs that can bind to a target mRNA with a complementary sequence to induce its cleavage, degradation, or interfere with translation (Gusic and Prokisch, 2020). There are over 2,700 known human mature miRNAs (miRBase release 22.1, 2018) and each one can have hundreds of mRNA targets making miRNAs powerful regulators of gene expression (Kozomara et al., 2019). Evidence showed that miRNAs are sensitive to internal and external environments, possessing the ability to modulate a broad spectrum of developmental and cellular processes including tissue development, cell proliferation, cell division, cell differentiation, neuronal asymmetry, metabolism, stem cell properties, apoptosis, protein secretion, and viral infection, among many others (Bhaskaran and Mohan, 2014; Orang et al., 2014). Unsurprisingly, miRNAs are aberrantly expressed in cardiovascular disease (Romaine et al., 2015), cancer (Jansson and Lund, 2012), obesity and insulin resistance (Arner and Kulyté, 2015), and other numerous pathologies. Certainly, disease-associated miRNAs are a valuable tool for the development of miRNA-based novel therapies or as diagnostic/prognostic biomarkers (Denham and Prestes, 2016).

2.2.1 The miRNA Biogenesis Pathway

The biogenesis and biological functions of miRNAs have been widely studied in eukaryotic cells (Bartel, 2009). The initial process of miRNA biogenesis occurs in the nucleus with the transcription of a primary transcript (pri-miRNA) through RNA polymerase II (Figure II-4). The pri-miRNA sequences contain different hairpin structures that are processed by a complex mechanism involving a ribonuclease III endonuclease Drosha in association with the DiGeorge syndrome critical region gene 8 (DGCR8) RNA binding protein, into stem-loop structures of approximately 60–70 nucleotides named pre-miRNAs. Subsequently, the exportin-5 enzyme (XPO5), along with GTP-binding nuclear protein Ran, exports the pre-miRNA from the nucleus to the cytoplasm (Mendell and Olson, 2012; Borralho et al., 2015). In the cytoplasm, another RNase III endonuclease, named DICER1 in humans, processes the pre-miRNA to produce a 22nt double-stranded RNA molecule. The pre-miRNA hairpin is cleaved by DICER to generate miRNA-5p and miRNA-3p. Among them, the 5p strand is present in the forward (5'-3') position, while the 3p strand is located in the reverse position, defining a passenger and a guide strand (Guo et al., 2015). In the next step, the "passenger strand" undergoes RNA degradation while the remaining "guide strand" associates with argonaute 2 (AGO2) and the RNA-binding protein Tarbp2, becoming part of the RNA-induced silencing complex (RISC) (Borralho et al., 2014). Then, the mature miRNA allows the RISC complex to recognize the 3'UTR of the target mRNA, through complementary binding based on Watson–Crick base pairing from nucleotide 2-8 in the mature miRNA, defined as the seed region (Lima et al., 2017).

Genomic analyses have demonstrated that miRNAs can be found in clusters and transcribed as polycistronic primary transcripts, or as independent units, including intergenic regions, exon sequences of non-coding transcripts, or intronic sequences of either protein-coding or non-coding transcripts (Ebert and Sharp, 2012; Horak et al., 2016). If miRNAs are

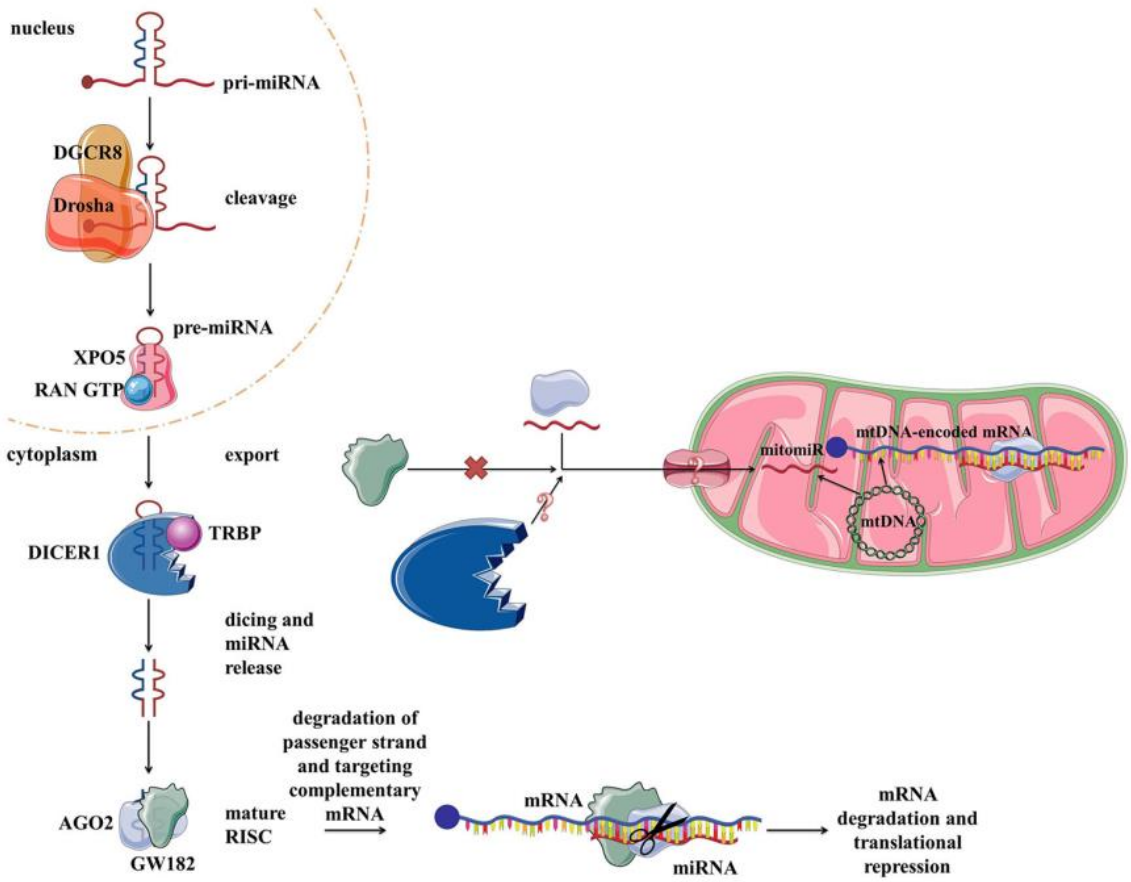

Figure II-2. miRNA biogenesis, function in the cytoplasm within RISC, and proposed transport/presence in mitochondria. RISC: RNA-induced silencing complex (Gusic and Prokisch, 2020).

located in an intronic region and have the same direction as the host genes, they are generally transcribed simultaneously and excised by the splicing machinery (Rodriguez et al., 2004). One example is miR-338-3p, which inhibits the transcription of its host gene apoptosis-associated tyrosine kinase (AATK) by binding to its 3'UTR (Kos et al., 2012). Another example is miR-26b, which controls neuronal differentiation by repressing its host transcript, C-terminal domain small phosphatases (ctdsp2) (Dill et al., 2012). Besides direct regulation, a miRNA can negatively regulate its host gene function by targeting a gene whose product is a downstream effector of the host gene product. Such regulation could be termed second-order negative feedback (Liu et al., 2019). Certainly, transcribing intronic miRNAs may represent an efficient mechanism for a protein-coding gene to regulate itself but also the expression of other proteins (van Rooij et al., 2009).

miRNAs are estimated to be responsible for the regulation of about 60% of human genes (Friedman et al., 2009). A single miRNA can target dozens of mRNAs, whereas individual mRNAs can be targeted by multiple miRNAs, allowing for additional levels of complexity in gene expression regulation. The miRNA seed region is crucial for target recognition. Generally, the seed region completely pairs at the 3'UTR of the target mRNAs, although miRNAs can also bind to the 5'UTRs of the open reading frames of targeted mRNA (Forman and Coller, 2010; Moretti et al., 2010). The 3'-sequence downstream from the seed sequence of a miRNA, however, often forms imperfect base-pairings containing mismatches, GU wobbles, and bulges with the target. These imperfections in complementation have been routinely identified among miRNA-target complexes, suggesting that perfect base-pairings are not necessary for miRNA function (Bartel, 2009; Brodersen and Voinnet, 2009). Moreover, some miRNA-target interactions do not occur through the seed regions, but via non-canonical sites, such as direct transcription and chromatin state regulation in the nucleus, and even translational promotion (Seok et al., 2016; Yao et al., 2019).

Transcription is one of the major regulatory steps involved in the biosynthesis of miRNAs. The features of miRNA gene promoters are similar to the promoters of the protein-coding genes and the transcription of miRNA is known to be regulated by similar kinds of transcription factors as those of protein-coding genes. In addition to the involvement of various canonical transcription factors, other epigenetic mechanisms, such as DNA methylation and histone modification, are also known to regulate the expression of miRNA genes. About half of the miRNA genes encompass CpG islands or are regulated by DNA methylation in tumors (Bianchi et al., 2017). Similarly, the steps involved in the biogenesis and the maturation of miRNAs are also targets of regulation. These include the processing of pri-miRNA to pre-miRNA by the RNase III enzyme, Drosha, the transport of the miRNA complex from the nucleus to the cytosol, and the conversion of pre-miRNA to mature miRNA by the action of the DICER-associated protein complex (Davis-Dusenbery and Hata, 2010).

2.2.2 Mechanism of miRNA-Mediated Regulation of Gene Expression

In animals, miRNA mediated translational repression has been proposed to occur in distinct ways, which vary according to the degree of complementarity between a miRNA and its target (Hussain, 2012). These include: (1) the inhibition of translation at the initiation stage, (2) the inhibition of translation at the post-initiation stage, and (3) the repression of translation by deadenylation and/or subsequent degradation of target mRNA (Huntzinger and Izaurralde, 2011). The evidence supporting the existence of these distinct pathways is presented below:

(1) Inhibition at initiation stage: mRNAs that lack a functional 5'-cap structure or whose translation is cap-independent are reported to be refractory to miRNA mediated translational repression (Humphreys et al., 2005; Pillai, 2005). Based on these studies, the miRNA silencing machinery is thought to interfere with either eIF4E (which recognizes cap structure) function or eIF4E recruitment to the 5'-cap structure of target mRNAs. In animal cells, miRNAs

predominantly regulate gene expression via translational inhibition, either by interfering with the ribosome assembly or by inducing its early dissociation (Huntzinger and Izaurralde, 2011; Hussain, 2012).

(2) Inhibition at the post-initiation stage: In addition to repressing the initiation steps, various studies indicate that miRNA-mediated translational repression can occur at post-initiation steps. This is based on the finding that several miRNAs, and AGO proteins, have been reported to be associated with the polysomal fractions in both mammalian (Kim et al., 2004) and plant (Lanet et al., 2009) cells.

(3) Inhibition by deadenylation and/or degradation: miRNAs with partial complementarity to their target mRNAs promote mRNA degradation via deadenylation, followed by removing of the poly-A tail and/or capping region and the subsequent activation of 5'-3' miR exonuclease activity (Hussain, 2012; Masi et al., 2016).

Although less reported, miRNAs are also thought to activate translation under certain conditions. Recent studies suggest that this mechanism is associated with specific spatial circumstances within the RISC complex and is not dependent on full base pairings with the target (Saraiya et al., 2013). Observations indicate that AGO2 is involved in gene expression activation when their targeted mRNA lacks poly(A) tail (Turchinovich and Burwinkel, 2012). Moreover, AGO2-RISC binds to eIF4E and is capable of forming a “closed-loop” even in the absence of poly(A) tail and associated proteins and, hence, could activate translation directly (Iwasaki and Tomari, 2009). Besides, the expression of target mRNA could be activated by the direct action of micro-ribonucleoproteins (miRNPs) and/or could be indirectly relieved from miRNA-mediated repression by abrogating the action of repressive miRNPs (Orang et al., 2014).

2.2.3 Regulation of Mitochondrial Functions by miRNAs

miRNAs are highly conserved in sequence between distantly related organisms, indicating their participation in essential biological processes, including growth and development, energy metabolism, insulin secretion, immune function, myocardium remodeling, proliferation, differentiation, survival, and cell death (Hussain, 2012; Masi et al., 2016). Focusing on mitochondria and based on their localization and genetic origin, three different classes of mitochondria-related miRNAs can be distinguished (1) cytoplasmic, nuclear-encoded miRNAs targeting mitochondria-related transcripts; (2) mitochondrial, nuclear-encoded miRNAs; and (3) mitochondrial, mtDNA-encoded miRNAs (Bandiera et al., 2011). The two latter groups, termed mitomiRs, are yet to be functionally deciphered. Each group is discussed in detail below.

2.2.3.1 Nuclear-Encoded miRNAs Targeting Mitochondria

By downregulating transcripts encoding for proteins involved in a variety of mitochondrial processes, miRNAs can indirectly influence mitochondrial biology and homeostasis. A summary of miRNAs targeting nuclear-encoded mitochondrial transcripts is discussed below.

For example, miR-26a targets subunit X of pyruvate dehydrogenase (PDH). As PDH catalyzes a crucial reaction before acetyl-CoA enters the TCA cycle, its repression leads to the decreased levels of acetyl-CoA and the accumulation of pyruvate (Chen et al., 2014). Also, it has been shown that miR-663 positively regulates the OXPHOS subunit by direct stabilization of ubiquinol-cytochrome c reductase (complex III) assembly factor 2 (UQCC2) (Carden et al., 2017). In breast cancer cell lines, mitochondrial dysfunction downregulates miR-663 through hypermethylation of its promoter, which leads to decreasing OXPHOS proteins levels and enzymatic activity, promoting tumorigenesis (Carden et al., 2017). Another example, PPARGC1B encodes for PGC-1β, a transcriptional coactivator that promotes mitochondrial

biogenesis and fatty acid metabolism. Interestingly, this locus can also encode for miR-378 and miR-378*, which counterbalance the effect of PGC-1β by targeting carnitine-O-acetyltransferase (Carrer et al., 2012). Moreover, several miRNAs have been connected to nucleotide and one-carbon metabolism since part of it occurs in mitochondria. For example, miR-149, miR-125, and miR-22 have been found to target methylenetetrahydrofolate reductase (MTHFR), the rate-limiting enzyme in the methyl cycle (Stone et al., 2011).

Several miRNAs are involved in the regulation of mitochondrial dynamics by directly or indirectly targeting biogenesis, fission, and fusion. For example, miR-149 indirectly promotes mitochondrial biogenesis by inhibiting the poly (ADP-ribose) polymerase PARP-2, which increases the NAD^+ levels and SIRT-1 activity, finally leading to increased activity of PGC-1α, the master regulator of mitochondrial biogenesis. Also, miR-499 by directly targeting Fnip1, a negative regulator of AMPK and known activator of PGC-1α, triggers a mitochondrial oxidative metabolism program in skeletal muscle (Liu et al., 2016). The miR-30 family, highly expressed in heart, was reported to regulate mitochondria fission and apoptosis by directly targeting p53, a transcriptional activator of Drp1 (Li et al., 2010). Also, Drp1 is indirectly regulated by miR-499, which targets Drp1 activator calcineurin (Wang et al., 2011).

Unsurprisingly, miRNAs are also involved in the mitochondria-mediated apoptosis. Moreover, they are frequently dysregulated in human cancers, where they may function as potent oncogenes or tumor suppressors (Peng and Croce, 2016). Since mitochondrial dysfunction is one of the hallmarks of cancer, miRNAs targeting apoptosis-related transcripts could be important in the development of cancer therapies. miR-101, miR-30a, miR-15a, and miR-16 have been reported to target oncogenic B-cell lymphoma 2 (Bcl-2) and myeloid leukemia cell differentiation protein (Mcl-1), and are frequently deleted or decreased in chronic lymphocytic leukemia. miR-21 levels are significantly increased, leading to reduced expression of PTEN in human lung and hepatocellular carcinomas (Gusic and Prokisch, 2020).

2.2.3.2 MitomiRs

As mentioned earlier, mitomiRs are defined as miRNAs with mitochondrial localization (Bandiera et al., 2011). The majority of mitomiRs were suggested to originate from the nuclear genome, but there have been also reports of mtDNA-encoded miRNAs. Diverse experimental approaches across mammalian tissues and cell lines indicated the mitochondrial presence of miRNAs, but also proteins involved in miRNAs biogenesis and function, suggesting miRNAs import, transcription, and/or processing and function within mitochondria themselves (Gusic and Prokisch, 2020). Intriguingly, mitomiRs have some unique features which distinguish them from conventional cytosolic miRNAs. Most of the nuclear-encoded mitomiRs loci are located within mitochondrial gene clusters or close to mitochondrial genes, and their transcriptions are often coregulated (Bandiera et al., 2011). They contain short 3' overhangs, stem-loop secondary structures, and unique thermodynamic features (Vendramin et al., 2017). They lack 5' cap and most were predicted in silico to target multiple mtDNA sites. It has thus been speculated that at least some of these features could present a signal for mitochondria import (Bandiera et al., 2011; Barrey et al., 2011).

The presence of miRNA-associated proteins in the mitochondria remains controversial. Some studies reported the presence of DICER in mitochondria isolated from the brain (Wang et al., 2015), but it was absent in the mitochondria isolated from the heart (Jagannathan et al., 2015). If indeed true, the presence of DICER could indicate that mature miRNAs are formed from precursors in the mitochondria, from where they could directly affect mitochondrial transcripts or even be exported to act in the cytosol (Bienertova-Vasku et al., 2013). Other studies have documented the presence of RNA-interference components, most notably AGO2, in the mitochondria (Das et al., 2012). Besides, Fragile X mental retardation syndrome-related protein 1 (FXR1), a postulated RISC subunit, has been found together with AGO2 in the mitochondrial matrix of mouse cardiomyocytes (Jagannathan et al., 2015). However, an

important factor for miRNA-mediated translational repression GW182 has not been detected in any studies (Ro et al., 2013; Zhang et al., 2014).

Although protein transport across mitochondrial membranes is well described, the translocases for RNA transport across mitochondrial membranes remain speculative. Several mechanisms of miRNAs transport into the mitochondria have been proposed. The potential players are AGO2, processing bodies (P-bodies), polynucleotide phosphorylase (PNPase) and VDAC. AGO2 has been proposed as an important factor in the subcellular localization of miRNAs. Zhang et al. (2014) proposed that loss of GW182 alone or in combination with changes in the target mRNA within RISC facilitates the transport of AGO2/miRNA into mitochondria. Still, it remains unclear if AGO2 and miRNA translocate together as a complex or separately into the mitochondria and by which mechanism. Another hypothesis involves P-bodies, as they interact with mitochondria and can regulate mRNA decay, mRNA storage, and possibly miRNA import into different cellular compartments (Bandiera et al., 2011; Huang et al., 2011; Luo et al., 2018). Activation of several pathways and the phosphorylation of AGO2 has been shown to separate the AGO2/miRNA complex from the RISC and activate its intake into the P-body (Huang et al., 2011; McKenzie et al., 2016). As another candidate, PNPase has been postulated to recognize specific structures of the housekeeping ncRNAs and help RNA fold properly to migrate through the mitochondrial membranes and return to its original conformation when they arrive in the mitochondrial matrix (Wang et al., 2010; Wang et al., 2012). PNPase levels were reported to affect mitomiRs mitochondrial localization and coimmunoprecipitation showed association with AGO2, suggesting that PNPase can bind to the miRNA within the complex with AGO2 (Shepherd et al., 2017). Transport across mitochondrial membranes could occur via TOM/TIM complexes, although additional studies are needed to prove this theory. Finally, it has been demonstrated that VDAC could help

transport tRNAs across the OMM in plant cells (Salinas et al., 2006). This mechanism is yet to be tested in the animal systems.

Although many have been detected, very few mitomiRs were functionally described to impact mitochondria (Baradan et al., 2017). Das et al. (2012) found miR-181c, AGO2, and cytochrome c oxidase 1 (COX1) coimmunoprecipitate in mitochondria, suggesting that mature miR-181c could translocate to mitochondria and together with AGO2 repress the translation of this mitochondrial transcript. Overexpression of miR-181c seems to lead to a loss of COX1 and an increase of COX2 and COX3, resulting in complex IV remodeling. Another example, miR-378 has been proposed to bind ATP6 in mitochondria in the presence of AGO2 and FXR1, leading to a decrease of ATP synthase membrane subunit 6 (ATP6) in mouse type 1 diabetic heart (Jagannathan et al., 2015). miR-1, specifically induced during myogenesis, can promote translation of COX1 and NADH-ubiquinone oxidoreductase chain 1 (ND1) within the AGO2/miRNA complex in mitochondria, while, on the contrary, suppressing its target transcripts in the cytosol (Zhang et al., 2014). Finally, a recent report reveals the role of mitomiRs in mitochondrial transcriptional regulation. mitomiR-2392, together with AGO2, was reported to recognize target sequences in the H-strand and partially inhibit polycistronic mtDNA transcription in tongue squamous cell carcinoma cells, leading to downregulation of oxidative phosphorylation and upregulation of glycolysis (Fan et al., 2019).

2.2.4 PGC-1α Targeting miRNAs

Endogenous miRNAs constitute promising candidates to regulate the process of mitochondrial biogenesis in the skeletal muscle. Moreover, miRNAs have been reported to play a role in regulating numerous diseases, as its expression profile is substantially modified during mitochondrial dysfunction (Mendell and Olson, 2012; Rottiers and Näär, 2012). Diverse

studies reveal the presence of several miRNAs that directly target PGC-1α coactivator and thus, regulate mitochondrial biogenesis.

A previous report showed that miR-494-3p reduced the protein expression of PGC-1α in adipocytes and attenuated mitochondrial biogenesis and oxygen consumption by directly binding to the 3'UTR of PGC-1α (Lemecha et al., 2018). Several studies observed the presence of miR-494-3p in mitochondria, regulating the mtDNA genome (Bandiera et al., 2011; Jagannathan et al., 2015). Among the validated targets of miR-494-3p, can be found TFAM, FoxJ3, and CREB1, suggesting that miR-494-3p serves as a fine-tuner of mitochondrial biogenesis (Yamamoto et al., 2012; Wen et al., 2017). The other two potential miRNAs that have been related to the mitochondrial oxidative capacity and biogenesis are miR-696 and miR-761. These two miRNAs are markedly reduced by endurance training, resulting in elevated PGC-1α expression, mitochondrial oxidative capacity, and biogenesis (Aoi et al., 2010; Xu et al., 2015). The involvement of miR-494-3p, miR-696, and miR-761 in the mitochondrial function and biogenesis has been confirmed by in vitro experiments, using C2C12 myoblast cells with down or overexpression of these miRNAs (Aoi et al., 2010; Yamamoto et al., 2012; Xu et al., 2015).

2.2.4.1 miR-494-3p

The 14q32 locus (chromosome 12F1 in mice) contains one of the largest miRNA clusters (>50 miRNAs) in the human genome, located within the delta-like homolog 1 gene and the type III iodothyronine deiodinase gene (Dlk1-Dio3) region (Benetatos et al., 2013). The fact that the cluster is so specifically conserved in mammals and that it is located in a strictly regulated, imprinted region of the genome hint at its biological importance. A recent study showed that the cluster is under transcriptional control of a binding site for the myocyte enhancer factor 2A (MEF2A), a transcription factor known to influence vascular remodeling

(Snyder et al., 2013; Welten et al., 2014). Among the miRNAs located in this region, miR-494-3p has emerged as a mitomiR due to its import to the mitochondria. In Figure II-3 are summarized some of the main functions of miR-494-3p discovered to date. For example, gene analysis has revealed its involvement in regulating translation in mitochondria (Bandiera et al., 2011; Figure II-3A). Besides, miR-494-3p regulates mitochondrial biogenesis by downregulating TFAM and Foxj3 during myocyte differentiation and skeletal muscle adaptation to physical exercise (Yamamoto et al., 2012; Sun et al., 2016; Figure II-3B). Moreover, decreased miR-494-3p expression has also been associated with increased PGC-1α mRNA following acute swimming exercise in mice (Yamamoto et al., 2012; Figure II-3C), while overexpression of miR-494-3p in adipocytes, reduced the protein expression of PGC1-α and decreased oxygen consumption in both Ucp1-dependent and Ucp1-independent manners (Lemecha et al., 2018; Figure II-3D). Other findings indicate that tumor necrosis factor α (TNF-α)-mediated inflammation upregulates miR-494-3p expression and exacerbates insulin resistance in C2C12 muscle cells (Lee et al., 2013).

Interestingly, miR-494-3p has been found implicated in senescence (Comegna et al., 2014), cell cycle arrest (Yamanaka et al., 2012), and apoptosis (Kim et al., 2011) in some cancer cell types. For example, the expression of miR-494-3p is increased in colorectal cancer and glioblastomas, promoting cell migration and invasion via targeting and inhibiting PTEN (Sun et al., 2014; Li et al., 2015). Also, miR-494-3p was overexpressed in human hepatocellular carcinoma cells and supported transformation by regulating the G1/S cell cycle (Lim et al., 2014), while in breast cancer is associated with metastasis and a poor prognosis (Marino et al., 2014). Similarly, it has been observed a marked increase in miR-494-3p, associated with fragmented mitochondria in 769-P renal cancer cells, suggesting that autophagosome protein LC3B may be a novel downstream target of miR-494-3p (Dutta et al., 2016).

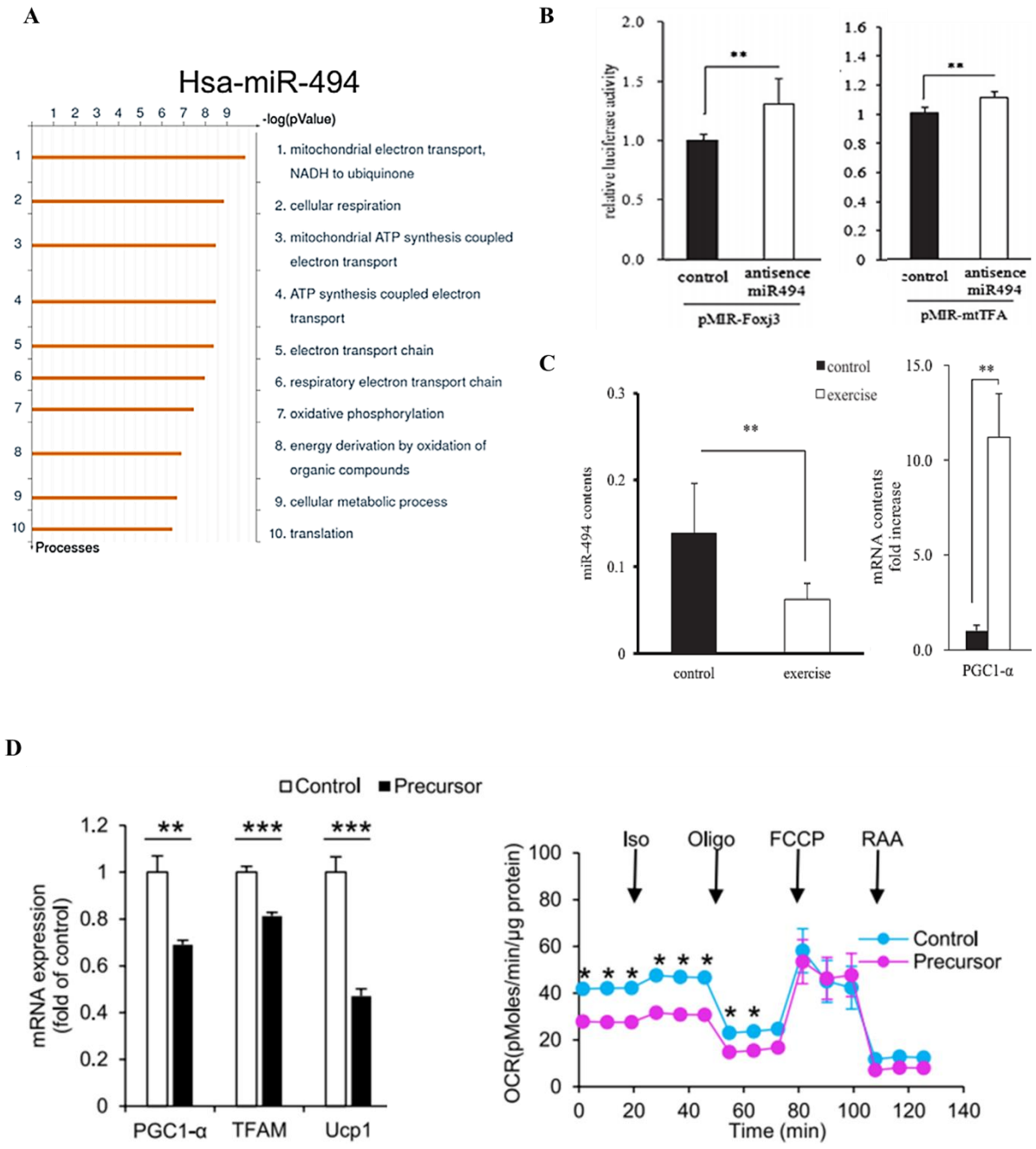

Figure II-3. Main discovered functions of miR-494-3p in skeletal muscle and adipocytes. (A) Ontology enrichment analysis for target genes of miR-494-3p. Ten most significantly enriched processes for the genes targeted by human miR-494-3p were scored and ranked according to the obtained p-values (Bandiera et al., 2011). (B) miR-494-3p modulates mitochondrial biogenesis through post-transcriptionally upregulation of TFAM (or mtTFA) and FoxJ3 expression in C2C12 cells (Yamamoto et al., 2012). (C) Endurance exercise stimulates mitochondrial biogenesis in murine skeletal muscle along with a significant decrease in miR-494-3p expression and an increase in expression of its target gene PGC1-α (Yamamoto et al., 2012). (D) Overexpression of miR-494-3p ("Precursor") downregulates the browning and thermogenic gene program in beige adipocytes, negatively impacting mitochondrial biogenesis markers and oxygen consumption rate (OCR) (Lemecha et al., 2018).

2.2.4.2 miR-696

MiR-696 is a miRNA expressed in different mouse tissues (heart, liver, spleen, lung, kidney, stomach, adipose tissue, and skeletal muscle) (Wang et al., 2017). The function of miR-696 in skeletal muscle has been implicated in energy metabolism, mitochondrial synthesis, and resistin-induced ectopic deposition of lipids (Wen et al., 2015; Figure II-4 A, B). In Figure II-4 are represented some of the major discoveries related to miR-696 functions. The high expression of miR-696 in skeletal muscle myogenesis suggests that it may play a role in this process. In this direction, some studies have shown that inhibiting miR-696 expression leads to a modest increase in slow myosin heavy chain (MyHC) (Truskey et al., 2013; Cheng et al., 2016; Figure II-4C). A proposed mechanism miR-696 may suppress the proliferation of C2C12 myoblasts is by blocking the ciliary neurotrophic factor receptor α (CNTFRα) complex-mediated PI3K/AKT and extracellular signal-regulated protein kinase (ERK1/2) / MAPK pathway activation (Wang et al., 2017).

Similarly, miR-696 is expressed in gastrocnemius under exercise and immobilization intervention by targeting PGC-1α, which promotes aerobic metabolism and mitochondrial function in skeletal muscle (Aoi et al., 2010). When overexpressed, miR-696 inhibits fatty acid oxidation, reduces mtDNA copy number and attenuates ATP production, whereas inhibiting miR-696 has the opposite effect (Wen et al., 2015). In the liver, miR-696 plays a role in hepatic gluconeogenesis by directly targeting PGC-1α, as observed in ob/ob mice, an animal model of insulin function deficiency (Fang et al., 2016; Figure II-4D). Previous reports showed that inhibition of miR-696 transcription in insulin-resistant cells re-established the insulin response, PGC-1α transcription, and mitochondrial function suggesting that miR-696 contributes to insulin resistance reducing mitochondrial biogenesis and mitochondrial function in skeletal muscle cells (Queiroz, 2017). Although miR-696 is only expressed in mice, a similar

mechanism of action might exist via a different miRNA that is operative in human skeletal muscle.

2.2.4.3 miR-761

Similar to miR-494-3p and miR-696, the expression of miR-761 in skeletal muscle is downregulated by exercise training (Xu et al., 2015; Figure II-5A). Reported in liver-derived mitochondria (Kren et al., 2009), miR-761 plays a crucial role in mitochondrial biogenesis in response to physical activity via regulating the expression of PGC-1α and the p38 MAPK signaling pathways (Xu et al., 2015). In Figure II-5 are shown some of the known functions of miR-761 in skeletal muscle and liver. The overexpression of miR-761 significantly downregulated the phosphorylation of MAPK-activated protein kinase-2 (P-MK2), a downstream kinase of p38 MAPK, and ATF2. Furthermore, transfection with miR-761 mimics decreased mitochondrial number, mtDNA, and PGC-1α protein expression (Xu et al., 2015; Figure II-5B). Moreover, it has been reported that ATF2 plays a functional role in linking the activation of the p38 MAPK pathway to enhanced transcription of the PGC-1α gene (Akimoto et al., 2005). Evidence shows that miR-761 can directly inhibit the expression of MFF by base-pairing with the 3'UTR of MFF in cardiomyocytes, protecting cardiomyocytes from hydrogen peroxide and ischemia/reperfusion-induced apoptosis and myocardial infarction (Long et al., 2013).

Besides the role in mitochondrial biogenesis, miR-761 was reported to play important roles in human cancer, including breast and ovarian cancer, hepatocellular carcinoma, non-small cell lung cancer, glioma, and colorectal cancer, acting as an oncogene or tumor suppressor, depending on the tissue in which it is expressed (Guo et al., 2017; Ren et al., 2018). Extensive genes have been identified as the target of miR-761 in numerous cancers, including histone deacetylase 1 (HDAC1), Ras-related protein Rab-3D, chemokine receptor CXCR1, Tripartite

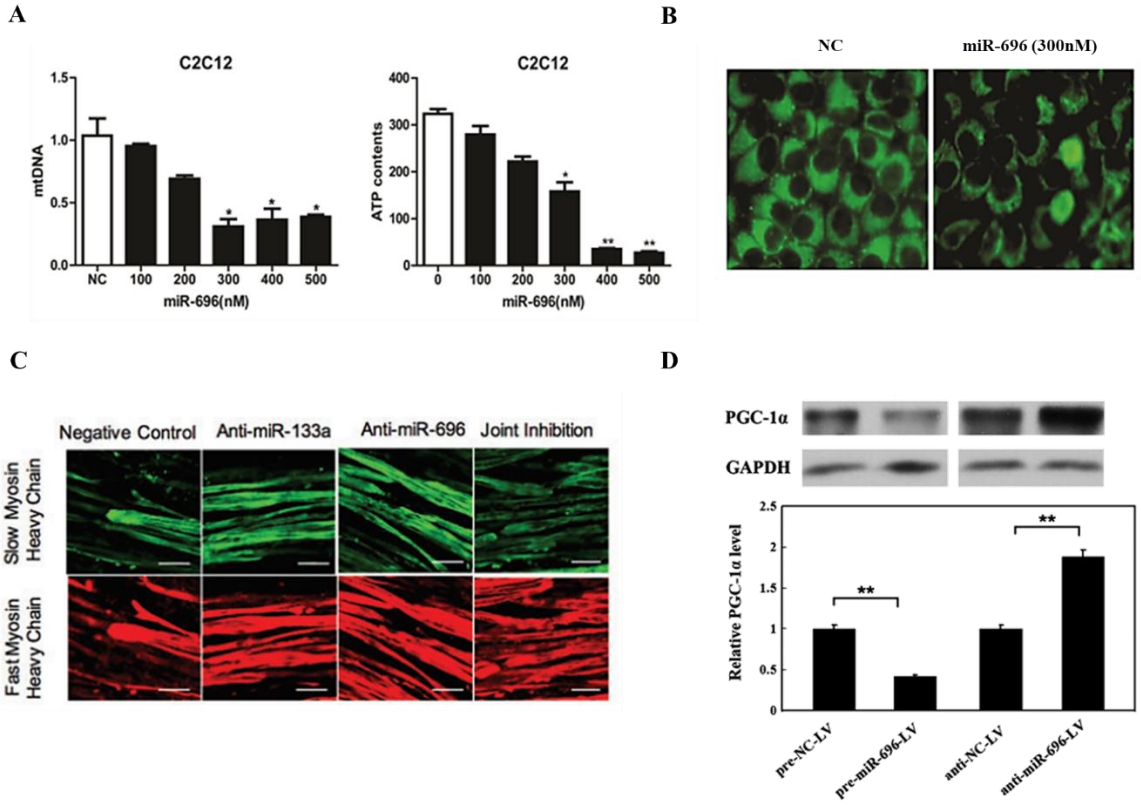

Figure II-4. Main reported functions of miR-696 in skeletal muscle and hepatocytes. (A, B) C2C12 cells were cultured and transfected with different doses of miR-696 mimics. Overexpression of miR-696 downregulated mitochondrial content and impaired mitochondrial function analyzed as mtDNA copy numbers, ATP production, and mitochondrial staining (Wen et al., 2015). (C) Immunofluorescence for slow and fast MHC was analyzed for pixel intensity. Images for slow myosin showed increased intensity in anti-miR-133a and anti-miR-696 samples. Images for fast myosin showed increased intensity for joint inhibition (Cheng et al., 2016). (D) Identification of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) as a target of miR-696 in primary hepatocytes. Western blot analysis of PGC-1α protein expression in primary hepatocytes following infection with pre-miR-696-LV or anti-miR-696-LV vector (Fang et al., 2016).

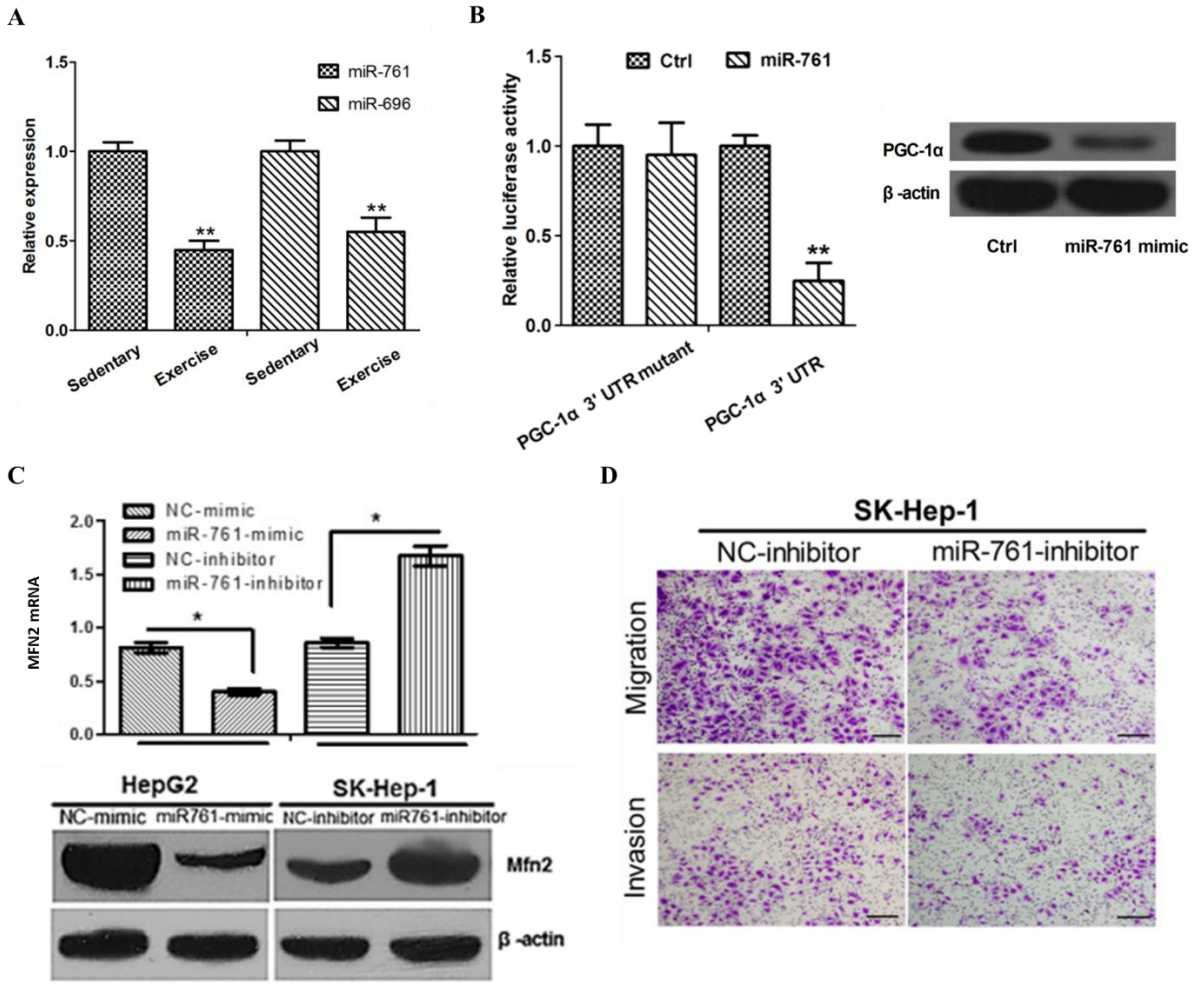

Figure II-5. Role of miR-761 in skeletal muscle and liver. (A) Expression of miR-761 and miR-696 in response to exercise (Xu et al., 2015). (B) PGC-1α is a target of miR-761 in C2C12 cells. The wild-type or mutated PGC-1α 3'-UTR containing binding sites for miR-761 was co-transfected with miR-761 mimic or control (Crtl), and PGC-1α mRNA and protein expression were analyzed in C2C12 cells (Xu et al., 2015). (C) Mfn2 is a direct target of miR-761. HepG2 cells were transfected with NC or miR-761mimics, SK-Hep-1 cells were transfected with NC or miR-761 inhibitors respectively, and MFN2 mRNA and protein expression were determined (Zhou et al., 2016). (D) MiR-761 increases migration and invasion of hepatocellular carcinoma cells in vitro. Representative images of migration and invasion assay after inhibiting miR-761 expression in SK-Hep-1 cells (Scale bar: 100 μm) (Zhou et al., 2016).

Motif 29, and GSK3β (Guo et al., 2017; Ren et al., 2018; Sun et al., 2018; Xiong et al., 2019). Also, it has been confirmed that inhibition of miR-761 expression can damage the mitochondrial function and cause apoptosis by upregulating Mfn2 in hepatocellular carcinoma, mediating the expression of MFF and Mfn2 (Figure II-5C, D). As both factors control two opposing activities, miR-761 might be crucial for maintaining the balance between mitochondrial fission and fusion as well as cellular homeostasis (Zhou et al., 2016).

2.3 Leucine

2.3.1 BCAAs Overview and Metabolism

The branched-chain amino acids (BCAAs), valine, leucine, and isoleucine are essential amino acids that have unique properties with diverse physiological and metabolic roles other than simple nutrition. BCAAs function includes regulating protein translation initiation, modulating insulin-PI3K signaling cascade, serving as a major nitrogen donor for alanine and glutamine, and fuel for oxidation to generate ATP (Monirujjaman and Ferdouse, 2014). Most of the effects of BCAAs (especially that of leucine) on protein synthesis are coordinated through the activation of mTOR (Dodd and Tee, 2012). Interestingly, leucine also appears to induce mitochondrial biogenesis in various cell types possibly through the activation of AMPK and upregulation of PGC-1α (Liang et al., 2014; Schnuck et al., 2016; Sato et al., 2018).

Unlike most amino acids, BCAAs are not degraded directly in the liver but are available for metabolism in skeletal muscle and other tissues due to low hepatic activity of branched-chain aminotransferase (BCAT), the first enzyme in the BCAAs catabolism pathway. Therefore, BCAAs increase rapidly in systemic circulation after protein intake and are readily available to extrahepatic tissues. This phenomenon gives a unique advantage to the BCAAs-based nutritional formulas compared with others, especially those targeted on muscles and brain (Holeček, 2018). First, the BCAT reaction involves the reversible transfer of the BCAAs

amino group to α-ketoglutarate to form glutamate and the corresponding branched-chain keto acids, α-ketoisocaproate (KIC, ketoleucine), α-keto-β-methylvalerate (KMV, ketoisoleucine), and α-ketoisovalerate (KIV, ketovaline). Subsequently, branched-chain α-ketoacids are decarboxylated by the enzyme branched-chain α-ketoacid dehydrogenase (BCKD). Beyond the BCKD reaction, the metabolism of the BCAAs diverges into separate pathways. Catabolism of KIC leads to acetyl-CoA and acetoacetate, KIV is catabolized to succinyl-CoA, and KMV to acetyl-CoA and succinyl-CoA, which enter the TCA cycle (Zhang et al., 2017).

2.3.2 Leucine as a Modulator of Muscle Protein Metabolism

BCAA supplementation (particularly leucine) has become popular amongst athletes and recreational exercisers due to its established role in promoting muscle protein synthesis and positive changes in body composition (Craig et al., 2015). However, leucine is not only a structural component of protein, but is also involved in glucose uptake, insulin secretion, and muscle protein metabolism (She et al., 2013; Liu et al., 2014; Zhang et al., 2014). The mechanisms involved in the regulatory effect of leucine on protein metabolism may include: 1) increasing substrate availability; 2) increasing the secretion of anabolic hormones such as insulin; 3) directly modulating anabolic signaling pathways in skeletal muscle and; 4) potential secondary effects of metabolites such as β-hydroxy β-methyl butyrate (HMB) (Ham et al., 2014).

The mTOR, a conserved Ser/Thr kinase, is considered the major mediator for leucine-induced protein synthesis, controlling skeletal muscle hypertrophy and atrophy (Yoon, 2017). mTOR integrates signals from nutrients (e.g. amino acids), growth factors (e.g. insulin & IGF-1), energy status (ATP), and stress to drive cell growth or activate energy sparing processes (Bond, 2016). It also interacts with several other regulatory proteins to form two distinct complexes named mTOR complex 1 and 2 (mTORC1 and mTORC2) (Laplante and Sabatini,

2012). Compared to mTORC2, studies have focused more on mTORC1 signaling and functions. The mTORC1 comprises mTOR, Raptor (regulatory-associated protein of mTOR), mTOR-associated protein, LST8 homolog, proline-rich Akt substrate of 40 kDa, and DEP domain-containing mTOR-interacting protein (Laplante and Sabatini, 2012). Upon activation, mTORC1 phosphorylates and activates two parallel signaling pathways involved in the control of protein translation initiation (Figure II-6). Ribosomal protein S6 kinase 1 (S6K1, also known as p70S6K) phosphorylation leads to activation of the ribosomal protein S6, while phosphorylation of the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) releases its inhibition of the translation initiation factor eIF-4E, allowing initiation of translation and the synthesis of new proteins (Ham et al., 2014; Liang, 2014).

However, the mechanisms of leucine activating mTOR signaling are poorly understood. Some studies indicate that leucine activates mTOR signaling partially through AMPK inhibition by reducing intracellular AMP/ATP ratio (Du et al., 2007), while others suggest a direct interaction between leucine and mTORC1. Recently, Sestrin2, a GATOR2 (GTPase activator)-interacting protein that inhibits mTORC1, was reported as an intracellular leucine sensor for the mTOR pathway in HEK293T cells (Wolfson et al., 2016). Other sensing mechanisms include leucyl-tRNA synthetase (LARS) (Han et al., 2012; He et al., 2018) and glutamate dehydrogenase (GLUD1) (Lorin et al., 2013). Finally, some discoveries showed that leucine signaling to mTORC1 does not necessarily require a “sensor” in some cell lines and primary cells, as acetyl-CoA positively regulates mTORC1 via Raptor acetylation (Son et al., 2019).

When consumed in very large doses, leucine stimulates insulin secretion from the pancreas by allosterically activating glutamate dehydrogenase in the β-cells and via oxidative decarboxylation (Yang et al., 2010; Leenders and van Loon, 2011). However, studies have linked the development of insulin resistance to increased BCAA catabolism in obese humans

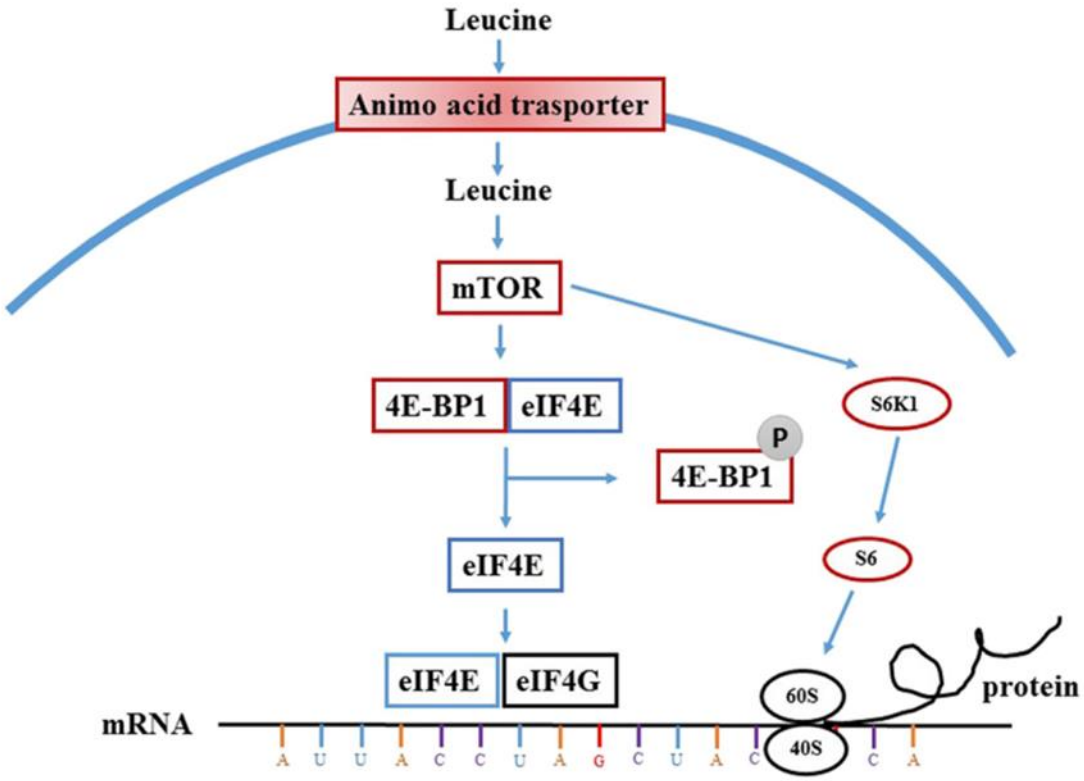

Figure II-6. Leucine increases protein synthesis by activation of the mTOR signaling pathway. Leucine enhanced muscle synthesis via the mammalian target of rapamycin (mTOR) pathway leading to phosphorylation of its downstream target proteins, eukaryotic initiation factor 4E-binding protein (4E-BP1) and p70 ribosomal S6 kinase 1 (S6K1). Under unphosphorylated conditions, 4E-BP1 tightly binds to eIF4E, forming the inactive eIF4E·4E-BP1 complex. During anabolic conditions, mTORC1 induces the phosphorylation of 4E-BP1, resulting in the dissociation of eIF4E from the inactive complex and allowing eIF4E to form an active complex with eIF4G. The process of association of eIF4E with eIF4G is obligatory for the binding of the 43S pre-initiation complex with mRNA. S6K1 is another mTORC1 substrate that participates in the regulation of mRNA translation. This kinase plays an important role in the regulation of terminal oligopyrimidine mRNA which is responsible for the translation of proteins involved in the protein synthetic apparatus (Zhang et al., 2017).

and chronic BCAA supplementation in rats fed a high-fat diet. This group showed that a BCAA-induced chronic activation of mTORC1 led to impaired insulin signaling through a negative feedback loop involving the inhibition of insulin receptor substrate 1 (IRS1) by S6K1 (Newgard et al., 2009). As such, despite potential short-term benefits, long term supplementation with high doses of leucine may negatively impact insulin signaling in some populations.

The effect of leucine on cancer cell growth and metabolism have been evaluated. When studied the effects of leucine supplementation on pancreatic cancer growth in lean and overweight mice, it was found that leucine increased tumor growth (Liu et al., 2014). In another study, the effects of leucine deprivation in breast cancer, both *in vitro* and *in vivo* models, showed that the withdrawal of this amino acid regulated apoptosis in the selected breast cancer models by inducing autophagy and lysosomal-dependent proteolysis (Xiao et al., 2016).

Due to their anabolic effects and ability to promote muscle recovery following injury and in some muscle diseases, the dietary supplementation of leucine or its metabolites is considered an attractive therapeutic agent for reducing muscle loss in aging populations (Alway et al., 2013; Pereira et al., 2014).

2.3.3 Leucine and Mitochondrial Biogenesis

A considerable body of evidence supports the use of protein and amino acids for skeletal muscle adaptation to resistance training programs aimed at increasing muscle mass (Atherton and Smith, 2012). However, in comparison, the role of amino acids in mitochondrial biogenesis/fat oxidation is underrepresented in the literature (Craig et al., 2015).

Given leucine's ability to activate both mTOR and protein synthesis, it has been proposed that many of leucine effects are driven in an mTOR-dependent fashion (including mitochondrial biogenesis). In mice, it was shown that BCAA ingestion increased mitochondrial

biogenesis and SIRT1 expression in skeletal muscle, which consequently increased lifespan in middle-aged mice (D'Antona et al., 2010). In the case of leucine supplementation, several studies have shown activation of AMPK, PGC-1α, and sirtuins (Banerjee et al., 2016; Chen et al., 2019), as well as PPARβ/δ (Schnuck et al., 2016), as result of the treatment. Figure II-7 summarizes the effect of leucine on mitochondrial content, mitochondrial-related genes, oxidative metabolism, and lipid accumulation.

Other studies reported that leucine (0.5 mM) administration to C2C12 cells and 3T3-L1 adipocytes exhibited increased mitochondrial mass, stimulated PGC-1α and SIRT1 gene expression, with increased cell respiration (Sun and Zemel, 2009). Besides, dietary leucine supplementation increased hepatic ATP production of weanling piglets, concomitantly with increased in mtDNA content, upregulated mRNA abundance of hepatic genes involved in mitochondrial biogenesis, and improved activities of citrate synthase, α-ketoglutarate dehydrogenase, malate dehydrogenase, and complex V (Su et al., 2017). Similarly, muscle cells treated with serum from overweight subjects fed a high-dairy diet for 28 days resulted in increased SIRT1 and PGC-1α expression in vitro (Bruckbauer and Zemel, 2011). Recent data indicate that leucine can directly activate SIRT1, resulting in elevated mitochondrial biogenesis and fatty acid oxidation in adipocytes (Zemel and Bruckbauer, 2012). Also, Vaughan et al. (2013) found that leucine in the 0.1 mM-0.5 mM range induces a dose-dependent expression of PGC-1α, leading to significant increases of mitochondrial density and oxidative capacity in skeletal muscle cells.

Leucine stimulation of muscle protein synthesis causes an increased energy need and elevated PPARβ/δ expression, which is necessary to activate oxidative metabolism and recover the synthetic energy deficit (Schnuck et al., 2016). Fu et al. (2015) showed elevated mitochondrial biogenesis and nitric oxide (NO) production in C2C12 cells treated with phosphodiesterase inhibitors. These effects were enhanced following additional leucine

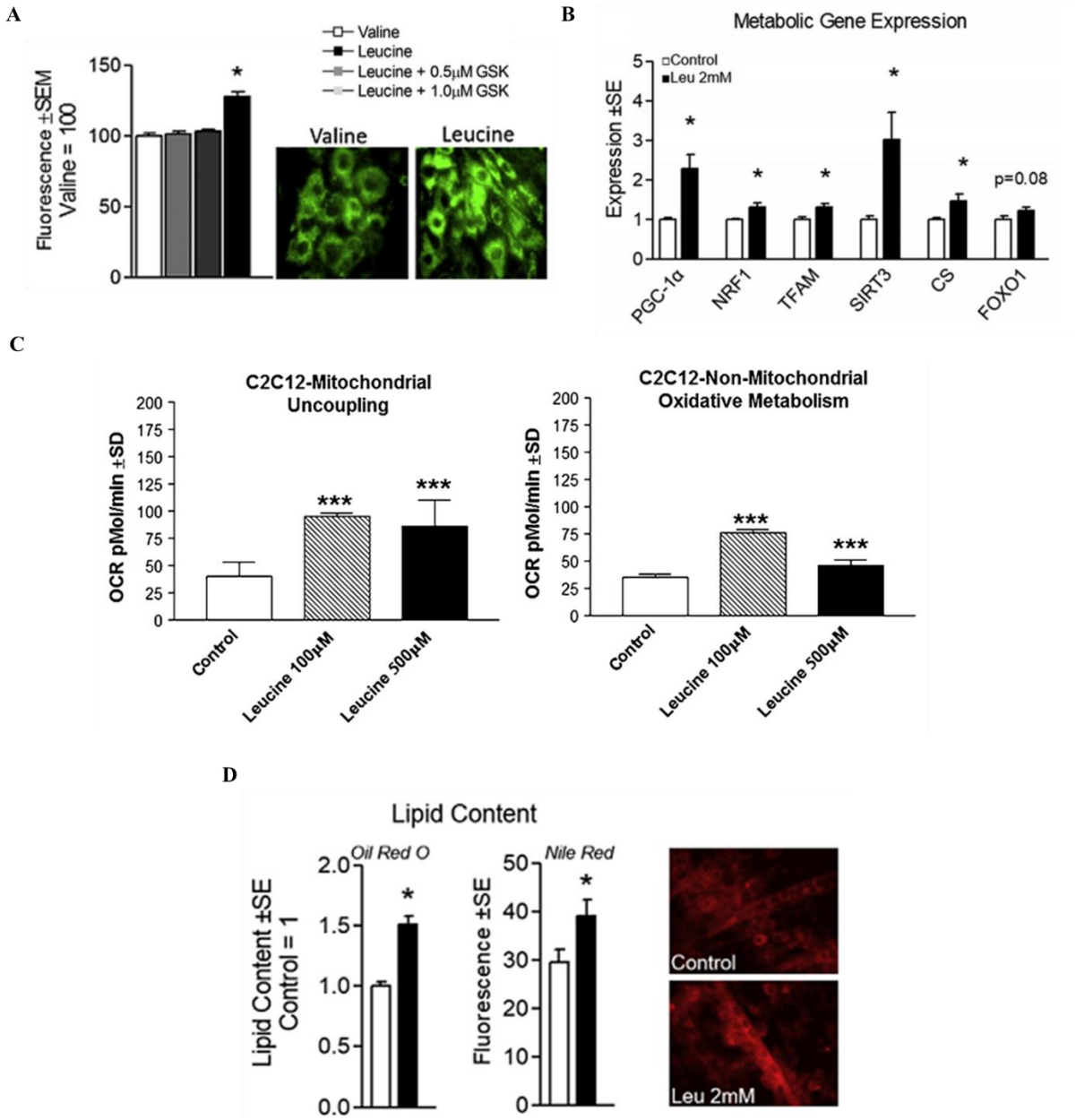

Figure II-7. Leucine increases mitochondrial biogenesis. (A) Mitochondrial content indicated by Mitotracker Green of C2C12 myotubes treated with either valine (control) or leucine at 2 mM for 24 h and leucine treated cells with and without the selective PPARβ/δ inhibitor GSK3787 (GSK). Representative live-cell images taken at 40X are displayed on the right (Schnuck et al., 2016). (B) Effect of leucine at 2 mM for 24 h on myotube mRNA expression of PGC-1α, NRF1, TFAM, NAD^+-dependent deacetylase sirtuin 3 (SIRT3), citrate synthase (CS), and FOXO1 (Rivera et al., 2020). (C) Mitochondrial proton leak (uncoupling) expressed as oxygen consumption rate (OCR) of C2C12 cells treated with either 100 or 500 μM for 24 h following addition of oligomycin and non-mitochondrial oxygen consumption of C2C12 cells treated as described above for 24 h following addition of rotenone (Vaughan et al., 2013). (D) Lipid content following treatment with and without leucine at 2 mM for 24 h indicated by Oil Red O extraction (left) or Nile Red fluorescent microscopy indicated by relative fluorescent units (RFU) at (right) (Rivera et al., 2020).

treatment (at 0.5 mM), which supports other observations that leucine may enhance mitochondrial content in-part through the activation of the eNOS (endothelial NO synthase)/NO/cGMP/SIRT pathway (D'Antona et al., 2010). Another evidence, the expression of leucine degradation enzymes is regulated by PPARα and PGC-1α, and it has been suggested that increased mitochondrial/enzyme content is necessary to support BCAA clearance (Hatazawa et al., 2014). Altogether, these observations suggest that leucine promotes nutrient/energy uptake thereby stimulating a compensatory response by the cells to dissipate increased energy through a variety of means such as substrate oxidation (PGC-1α, PPARβ/δ, and PPARα), structural/ contractile components (mTOR), and de novo lipogenesis (PPARγ).

III. Research Design and Structure

Among ncRNAs, miRNAs have emerged as an important class of post-transcriptional regulators of gene expression in all fundamental cellular processes, hence its significance for the maintenance of cellular homeostasis. In almost three decades after their discovery, the mechanism of miRNA-mediated gene regulation has been well established, but many aspects of the complex mechanisms that direct the activity of these tiny regulators remain to be clarified. Due to its small size, presence in body fluids, transport through circulation, and resistance to RNase activity, miRNAs denote potential biomarkers and prognostic molecules in the treatment of numerous diseases, metabolic conditions, or nutritional status.

A growing number of studies have highlighted the beneficial role of leucine supplementation in skeletal muscle functions, as maintenance of muscle mass, and therapeutic effects for muscle regeneration and strength recovery. Additionally, amino acids and especially leucine in C2C12 cells, have been found to regulate miRNA profile, sometimes by affecting the biogenesis of the miRNAs (Chen et al., 2013). Therefore, leucine presents a good candidate to study the relationship between nutrients and miRNAs in skeletal muscle in the context of mitochondrial biogenesis.

This research aimed to elucidate the effect of leucine treatment on the expression of 3 miRNAs and the impact on mitochondrial biogenesis. We hypothesized that: i) leucine regulates the expression of miR-494-3p, miR-696, and miR-761 in skeletal muscle, ii) the regulation of these miRNAs differs between nutrients (leucine and caffeine), and iii) leucine promotes mitochondrial biogenesis partially through the regulation of PGC-1α mediated by these miRNAs. A schematic representation of the experimental design is shown in Figure III-1. The work presented here was approached through the following 2 specific aims:

1. **<u>Specific Aim I:</u> Effect of leucine treatment on miRNAs (miR-494-3p, miR-696, and miR-761) expression and mitochondrial biogenesis in C2C12 myotubes (Experiment I).**

   Among nutrients, leucine has been widely known for its role in promoting not only skeletal muscle protein synthesis but also mitochondrial biogenesis through SIRT1 activation of PGC-1α. However, the effect of leucine treatment on PGC-1α-targeting miRNA has not been yet elucidated. In this regard, the Experiment I focused on the regulation of miR-494-3p, miR-696, and miR-761 and mitochondrial biogenesis by leucine treatment. Several specific tasks guided the work to validate the leucine treatment and our culture conditions. Multiple markers were used to assess the effect of leucine on skeletal muscle functions, ranging from the detection of mTOR pathway proteins to mitochondrial markers, including OXPHOS, COXIV, VDAC1, and cytochrome c protein expression. Mitochondrial DNA copy number was also assessed.

2. **<u>Specific Aim II:</u> Relationship between leucine, miRNA levels, and mitochondrial biogenesis in skeletal muscle (Experiment II).**

   Because of the molecular redundancy of miRNAs and its overlapping targets and functions, target perturbation by the miRNA network is very modest, sometimes <20%, making it difficult to analyze results. Therefore, to elucidate how leucine-induced regulation of miRNAs impacts mitochondrial biogenesis, gain- and loss-of-miRNA function analysis was performed on Experiment II. Transfection with precursor miRNA or anti-miRNA specific sequence, and treatment with leucine afterward, were the main procedures conducted in this section. To evaluate the molecular outcome of both transfection protocols on mitochondrial biogenesis, the levels of the miRNAs of interest, as well as mtDNA copy number, mRNA and protein expression of nuclear regulators (PGC-1α and TFAM) were detected.

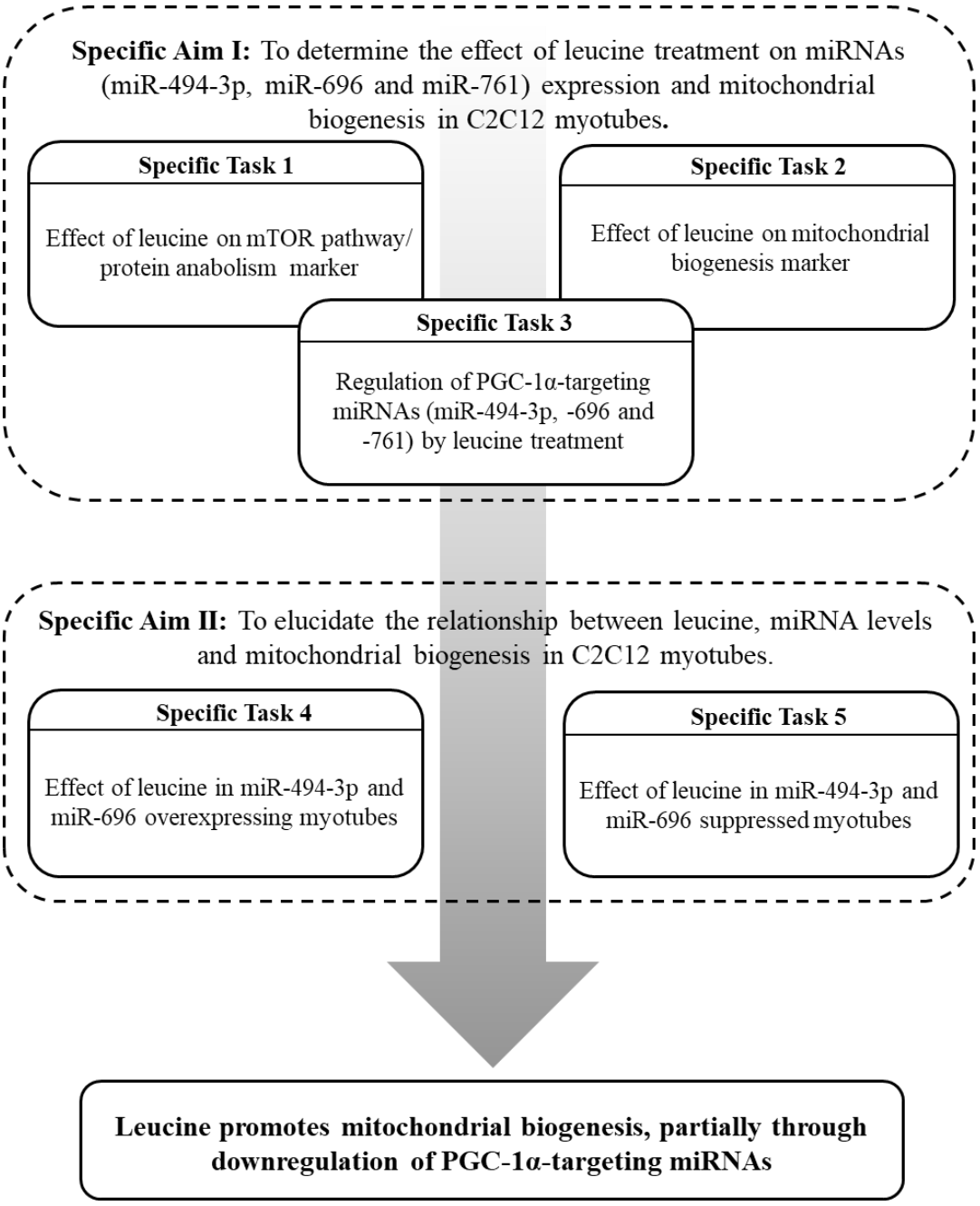

Figure III-1. Schematic representation of the content of the present study.

IV. Effect of Leucine Treatment on miRNAs (miR-494-3p, miR-696, and miR-761) Expression and Mitochondrial Biogenesis in C2C12 Myotubes (Experiment I)

4.1 Purpose

It has been well established that nutrients can regulate the expression of protein-coding genes. However, growing evidence has accumulated supporting a role for nutrients in the regulation of miRNA. Compared with other nutrients, the leucine effect on miRNA regulation is underrepresented in the literature. Some studies have reported that administration of a mix of essential amino acids containing histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine can induce miRNA expression in human muscle biopsies (Drummond et al., 2009). Another study, reported the increase in the myostatin targeting miRNA, miR-27a, after leucine treatment contributing to leucine-induced proliferation in C2C12 cells (Chen et al., 2013). The study of the relationship between nutrition and RNA-based regulation is necessary for the look of new therapeutic agents and diagnostic strategies in the future.

Here we focused on three miRNAs, miR-494-3p, miR-696, and miR-761, which are downregulated by exercise but also play a part in regulating mitochondrial biogenesis of skeletal muscle, although the exact regulatory mechanism has been rarely reported (Yamamoto et al., 2012; Long et al., 2013; Xu et al., 2015). They exert their regulatory effect through a wide range of genes, such as PTEN, MFF, p38 MAPK, FoxJ3, CNTFRα, TFAM and PGC-1α (Aoi et al., 2010; Yamamoto et al., 2012; Long et al., 2013; Li et al., 2015). Among these genes, PGC-1α consists of vital importance as a key regulator of cell metabolism, mitochondrial biogenesis, antioxidant defense, and inflammatory response in skeletal muscle (Kang and Ji, 2013; Peng et al., 2017). In the past decades, PGC-1α has emerged as a vital transcriptional coactivator, providing mechanistic insight into how nuclear regulatory

pathways are coupled to the metabolic regulation of mitochondria in skeletal muscle (Peng et al., 2017). Also known as the master regulator of mitochondrial biogenesis, it constitutes the only common validated target among the exercise responsive miRNAs, miR-494-3p, miR-696, and miR-761. Because PGC-1α is highly sensitive to cellular energy status (Khan et al., 2015), along with leucine, we consider them as important candidates for the study of nutrients-mediated regulation of mitochondrial biogenesis through miRNAs.

The purpose of this study was to elucidate the effect of leucine treatment on the expression of miR-494-3p, miR-696, and miR-761 and the impact on mitochondrial biogenesis since the effect of leucine treatment on PGC-1α-targeting miRNA has not been yet elucidated. Although leucine treatment has been extensively described in the literature, due to the wide variation in administration regimes, routes, doses, nutrient composition and experimental model used to study the potential therapeutic benefits of leucine on skeletal muscle, we began the study by validating the leucine treatment through morphological analysis of myotubes (myotubes width), followed by evaluation of protein synthesis and mitochondrial markers on C2C12 cells. Concomitantly, we supplemented caffeine to another batch of cells, to determine if the effect on miRNAs was similar to leucine treatment. Finally, we detected the miRNA levels after treatment with both leucine and caffeine, observing a leucine-specific response. This is the first report of nutrients, especially leucine, regulating the exercise-responsive miRNAs, miR-494-3p, and miR-696 in skeletal muscle.

4.2 Materials and Methods

4.2.1 Cell Culture

This experiment was approved by the Gene Recombination Experiment Safety Committee of Kanazawa University (approval ID: Kindai 6-2145). Mouse C2C12 myoblasts were obtained from American Type Culture Collection (ATCC, VA, USA) and cultured in Dulbecco's

modified Eagle's medium (high glucose DMEM; Gibco, MA, USA) containing 10% fetal bovine serum, 1% penicillin-streptomycin, at 37 °C in 5% CO_2 air atmosphere. At 80% of confluence (defined as day 0), cells were cultured in differentiation medium, DMEM containing 2% calf serum, 1% penicillin-streptomycin, and 1% non-essential amino acids to induce myotubes formation. All experiments were performed using cells of up to 9 passages.

4.2.2 Leucine Treatment

After 5 days of differentiation of C2C12 myoblasts, the myotubes were starved in Leu-free custom medium (IFP, Yamagata, Japan) without serum for 24-h before treatment with Leu (1 or 3 mM; Nacalai Tesque, Kyoto, Japan) or Caff (3 mM; Wako, Osaka, Japan) for further 24-h (Figure IV-1). The starvation condition before treatment has been widely accepted to determine the effects of nutrients and other compounds *in vitro* (D'Antona et al., 2010; Camera et al., 2016; Sun et al., 2016; Lemecha et al., 2018). As a control, 2% H_2O was added to the culture medium. In order to determine if the observed effect on miRNA levels was leucine-specific, another batch of cells was treated with caffeine (3 mM; Machino et al., 2017). Following each treatment, cells were harvested and then, DNA, RNA, and whole-protein fractions were isolated for immunoblotting and qPCR analyses.

4.2.3 Myotube Diameter

During nutrient exposure, differentiated cells were micrographed, using an Olympus CKX41 inverted microscope coupled to a DP71 digital camera (Tokyo, Japan). Before and after nutrient treatment, 8 micrographs from randomly selected fields in 8 different wells were obtained. From each micrograph, 5 myotubes were measured (n = 40/group, total n = 120). Image analysis was performed using specialized software (Image J, version 1.52p; NIH, MD, USA).

4.2.4 Mitochondrial DNA Copy Number

Cells were collected with (D)-PBS, centrifuged at 600 g for 5 min and the cell pellets were immediately frozen in liquid nitrogen before being stored at -80°C. Genomic DNA was extracted using the spin-column based DNeasy Blood & Tissue Kit (QIAGEN, Mississauga, ON, Canada) and diluted in Tris-EDTA (pH 8.0) for long-term storage. Real-time PCR was performed using ABI 7300 system with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). The following primers were used: 5' - GCCGACTAAATCAAGCAACA - 3' (forward) and 5' - CAATGGGCATAAAGCTATGG - 3' (reverse) specific for cytochrome c oxidase subunit II (COXII, mitochondrial), and 5' - GCGACCAGCCCATTGTAGA - 3' (forward) and 5' - GCGTTCTGGGTAC CATCCTAAC - 3' (reverse) specific for mitochondrial uncoupling protein 2 (UCP2, nuclear) genes (Eurofins Genomics, Tokyo, Japan). The PCR reaction was set up as follows: UDG incubation for 2 min at 50°C, initial denaturation step at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. The relative copy numbers of mitochondrial to nuclear DNA were determined by the comparative method $2^{-\Delta\Delta Ct}$ using UCP2 as an internal control.

4.2.5 miRNA Expression

Cells were prepared using the same conditions as for DNA extraction. Total RNA was isolated using the ReliaPrep miRNA Cell and Tissue Miniprep System (Promega, WI, USA) according to the manufacturer's instructions and quantified with Denovix Spectrophotometer (DS-11; Wilmington, DE, USA). Then, 20-60 ng of total RNA was reverse transcribed into cDNA with the TaqMan MiRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For miRNA expression detection TaqMan miRNA assay kits were used (mmu-miR-494-3p: 002365, mmu-miR-696: 001628, mmu-miR-761: 002030 and snU6: 001973,

Thermo Fisher, MA, USA); and real-time qPCR was performed according to the manufacturer's instructions using TaqMan Universal PCR Master Mix, no AmpErase^{TM} UNG, (Thermo Fisher). The PCR reaction was set up as follows: initial denaturation step at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. Expression levels of individual miRNAs were determined by the ΔΔCt approach relative to the average Ct of normalization controls (snU6).

4.2.6 Sample Preparation and Immunoblotting

Cell lysates were homogenized in RIPA buffer (20 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) containing a protease- and phosphatase-inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) using a pestle homogenizer (Nippi, Tokyo, Japan). Homogenates were then sonicated for 20 s at power 5 with an Ultrasonic Disruptor (UR-21P; Tomy Seiko, Tokyo Japan) and incubated for 30 min at 4°C in a rotator. Finally, lysates were centrifuged at 10,000 g for 20 min at 4°C and the supernatant was used for immunoblotting (Manabe et al., 2012; Yamada et al., 2016). The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Whole protein fraction samples were solubilized in equal volume of 2x SDS sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.002% bromophenol blue), adjusted to the final concentration of 1.5 µg protein /µl with 1x SDS sample buffer, and then incubated at 95°C for 5 min. Normalized samples were separated by SDS-PAGE, and then transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated with blocking buffer followed by incubation with primary antibodies specific for p-mTOR (S2448) (1:1,000; 2971S, Cell Signaling Technology, Beverly, MA, USA), mTOR (1:1,000; 2972S, Cell Signaling Technology), p-p70S6K (T389) (1:1,000; 9234S, Cell Signaling Technology), p70S6K (1:1,000; 9202S, Cell Signaling Technology), p-4E-BP1 (T37/46) (1:1,000; 2855S, Cell

Signaling Technology), 4E-BP1 (1:1,000; 9452S, Cell Signaling Technology), oxidative phosphorylation complexes antibody cocktail (OXPHOS, 1:100; ab110413, Abcam, Cambridge, UK), voltage-dependent anion channel 1 (VDAC1, 1:2,500; 55259-1-AP, Proteintech, Rosemont, IL, USA), cytochrome c oxidase subunit 4 (COXIV, 1:5,000; 11242-1-AP, Proteintech), cytochrome c (1:1,000; 10993-1-AP, Proteintech), α-tubulin (1:2,000; 3873S, Cell Signaling Technology) and GAPDH (1:1,000; ab8245, Abcam) at 4°C overnight. After exposure to a secondary antibody, reaction products were visualized using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). Band densities were quantified with ImageJ software (version 1.52p; NIH, MD, USA).

4.2.7 Statistical Analysis

Data were analyzed by one-way ANOVA followed by Dunnett's post-hoc test for differences between leucine treatment and control group. The Pearson product-moment correlation coefficient was computed to assess the relationship between variables. All statistical analyses were performed using IBM SPSS Statistics 25 (IBM Japan, Tokyo, Japan). The results are expressed as mean ± SEM with a significance level of $p < 0.05$, while a trend was defined as $p < 0.1$.

4.3 Results

4.3.1 Leucine Stimulates Downstream Effectors of the mTOR Pathway and Induces Myotube Hypertrophy

Figure IV-1 represents the experimental conditions of this study; C2C12 myotubes were preconditioned with the leucine-free medium without serum at day 5 of differentiation before 24-h treatment with leucine.

Treatment with leucine at different concentrations (1 and 3 mM) to fully differentiated myotubes resulted in significant hypertrophy in vitro (Figure IV-2). After nutrient treatment, protein levels and phosphorylation rate of mTOR pathway members were detected by immunoblotting. As shown in Figure IV-3, phosphorylation of mTOR at Ser2448 was unchanged after 24-h of treatment with leucine. On the contrary, phosphorylation of p70S6K, a downstream effector of mTORC1, showed a tendency to increase at 1 mM of leucine concentration ($p < 0.1$). Additionally, showed a tendency to increase after treatment with leucine 3 mM ($p < 0.1$).

4.3.2 Leucine Treatment Increases Mitochondrial Biogenesis Markers in C2C12 Myotubes

Treatment of fully differentiated C2C12 myotubes with 3 mM Leu tended to increase the expression of mtDNA copy number determined by the COXII/UCP2 ratio ($p = 0.058$, Figure IV-4A). On the contrary, no significant changes in the expression of the oxidative phosphorylation (OXPHOS) Complexes I-V was observed (Figure IV-4). Similarly, leucine treatment did not affect the expression levels of mitochondrial proteins VDAC1, COXIV, and cytochrome c (Figure IV-5).

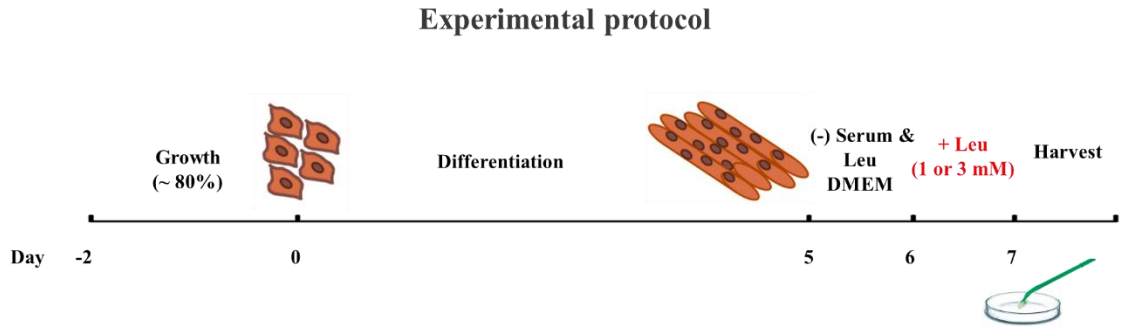

Figure IV-1. Experimental design, treatment, and sample collection. When the cells reached 80% confluence, the medium was exchanged to induce myotube differentiation for 5 days. Next, cells were starved with leucine-free medium without serum for 24-h followed by the addition of leucine (Leu; 1 or 3 mM) to the medium and incubation for another 24-h. At the end of the treatment, cells were harvested for downstream analyses.

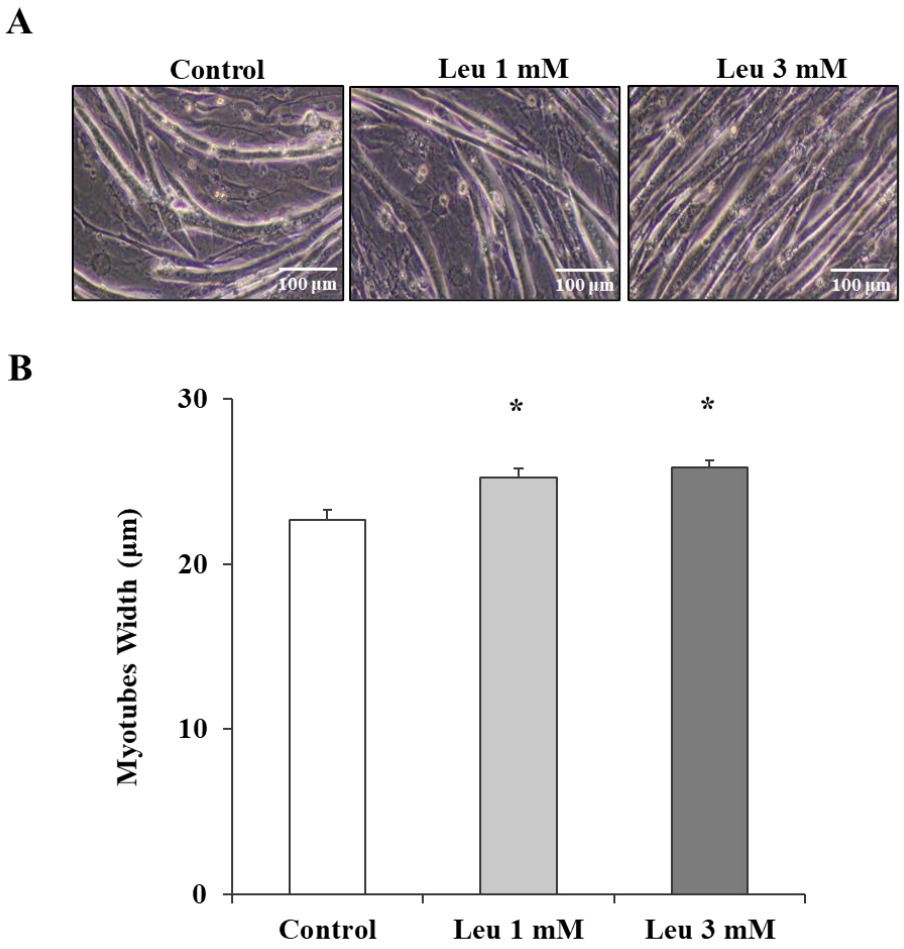

Figure IV-2. Effect of leucine treatment on C2C12 myotubes. (A) Micrographs were taken after 24-h of treatment with Leu (1 or 3 mM), magnification 100x, scale bar 100 µm (n = 120, 40 myotubes/group). (B) Myotubes width after 24-h of treatment with Leu (1 or 3 mM) measured with ImageJ software. Values are presented as mean ± SEM (n = 8 dishes / group). * $p < 0.05$, different from control by one-way ANOVA with Dunnett's post-hoc test.

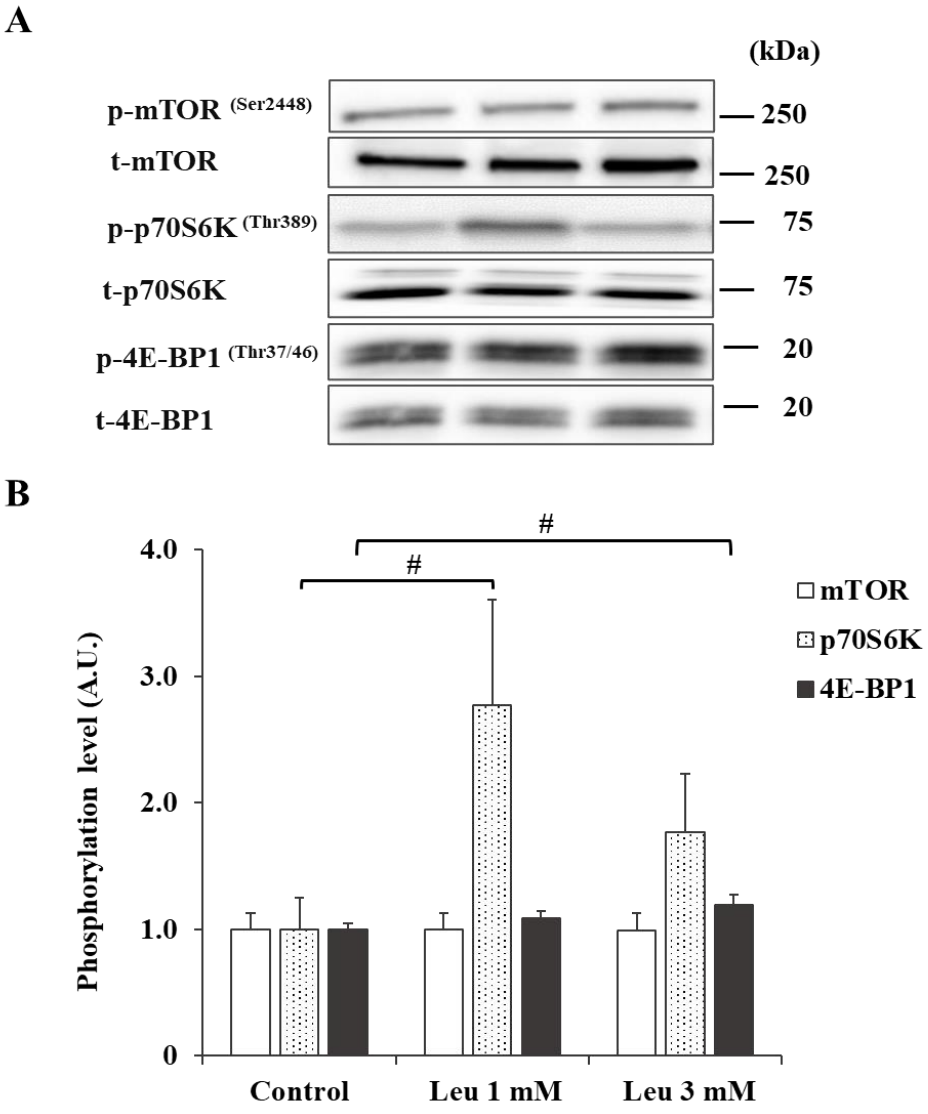

Figure IV-3. Phosphorylation levels of mTOR pathway proteins in C2C12 myotubes after 24-h treatment with leucine. (A) Immunoblot images of mTOR and downstream effector proteins, p70S6K and 4E-BP1. (B) Phosphorylation levels of mTOR, p70S6K and 4E-BP1. Data of each group are expressed as the ratio relative to the average value of the control group (1.0). Values are presented as mean ± SEM (n = 8/group). # $p < 0.1$, tendency as compared with the control condition by one-way ANOVA with Dunnett's post-hoc test.

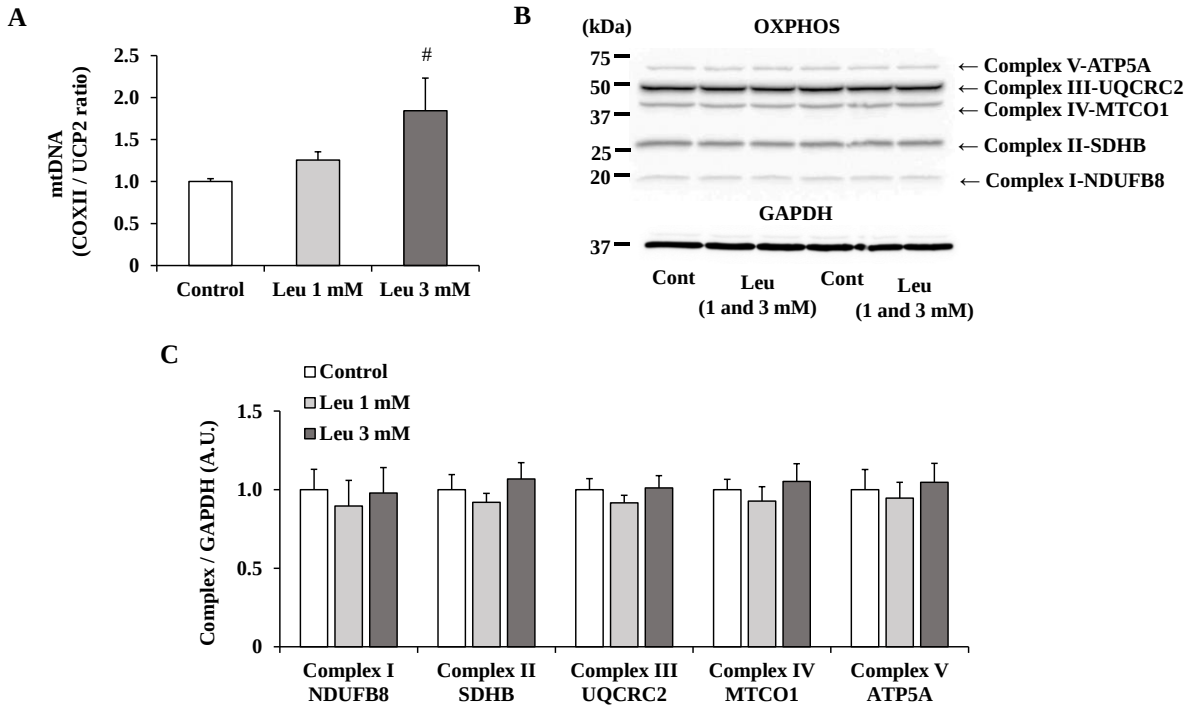

Figure IV-4. Effect of leucine treatment on markers of mitochondrial biogenesis. (A) Mitochondrial DNA copy number (mtDNA) after 24-h of treatment with Leu (1 or 3 mM). The relative copy number of mitochondrial to nuclear DNA were determined by the comparative method $2^{-\Delta\Delta Ct}$ using UCP2 as an internal control. (B) Immunoblot images of oxidative phosphorylation (OXPHOS) proteins and the loading control GAPDH. (C) Expression levels of OXPHOS complexes I-V after 24-h of treatment with Leu. Data of each group are expressed as the ratio relative to the average value of the control group (1.0). Values are presented as mean ± SEM (n = 5-8/group). #: $p < 0.1$, tendency as compared with the control condition by one-way ANOVA with Dunnett's post-hoc test.

4.3.3 Leucine Downregulates miR-494-3p and miR-696 expression in Myotubes

Regarding the miRNA's expression profile, miR-494-3p levels tended to be lower in both 1- and 3-mM leucine-treated groups ($p = 0.096$ and 0.084, respectively; Figure IV-6A). A similar trend was also observed in the miR-696 level, but the miR-696 expression significantly decreased in the 3 mM leucine group ($p < 0.05$). Overall, there was a strong, positive linear correlation between miR-494-3p and miR-696 levels in C2C12 myotubes ($r = 0.503$, $p < 0.05$, $n = 24$; Figure IV-6B). The results summarized in Figure 3B might indicate a cooperative effect between these two miRNAs, independent of the treatment applied. On the contrary, miR-761 levels were not affected by leucine treatment. When caffeine treatment was evaluated, we observed no effect on these miRNAs' levels (Figure IV-6C), which might suggest the involvement of a leucine-specific regulatory mechanism.

4.4 Discussion

Nutrients constitute an important factor in regulating skeletal muscle functions. In the present study, we aimed to determine the effect of leucine treatment on exercise-responsive miRNAs. Before testing our hypothesis about nutrient regulation of miR-494-3p, miR-696, and miR-761 expression, we verified the effect of leucine on skeletal muscle functions and mitochondrial biogenesis in C2C12 cells. First, we observed a dose-dependent increase in myotubes width following treatment with 1 and 3 mM of leucine, which is indicative of skeletal muscle hypertrophy (Figure IV-2A and 2B). Similar to this finding, it was reported that leucine concentrations of 0.1, 0.5, and 2 mM for 3 days promote cell growth in a dose-dependent manner (Dai et al., 2015).

Amino acids and in this case, leucine, stimulate mTORC1 inducing phosphorylation of its two main targets, p70S6K and 4E-BP1 (Zheng et al., 2016). However, our experiments only measured the phosphorylation of mTOR at residue Ser2448 which cannot be considered as a

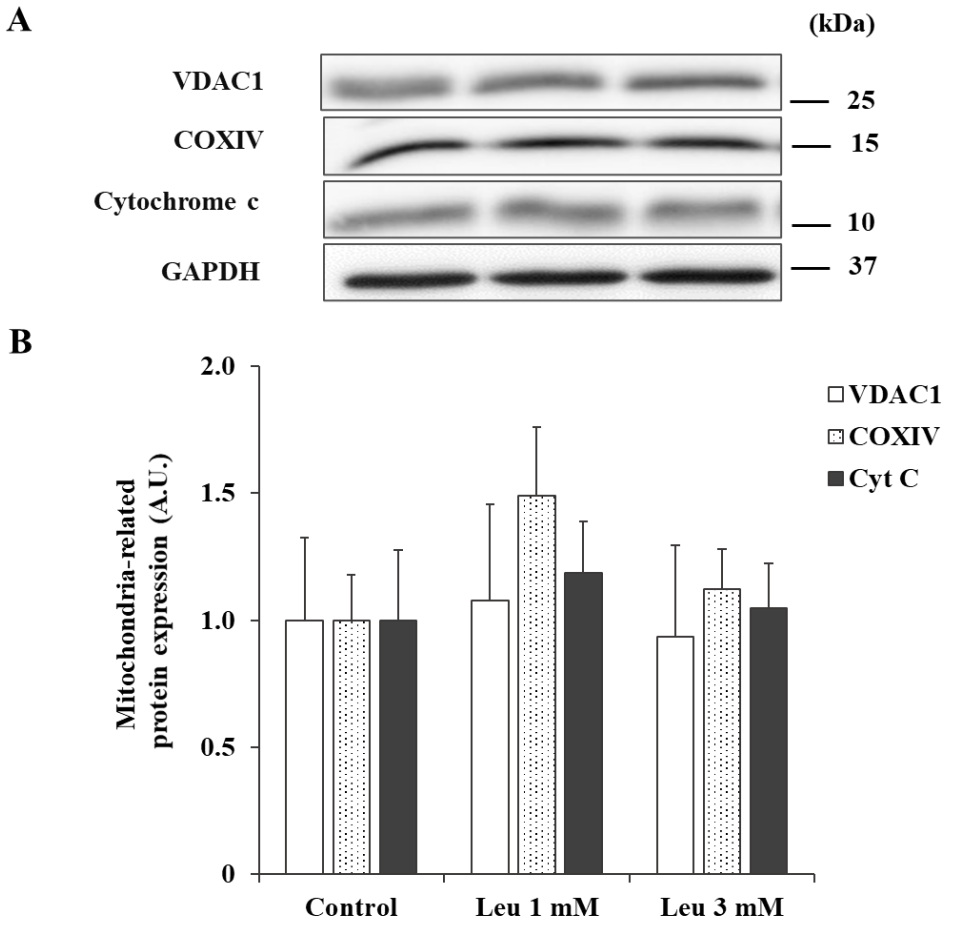

Figure IV-5. Expression levels of mitochondria-related proteins after 24-h treatment with leucine. (A) Immunoblot images of mitochondrial proteins VDAC1, COXIV and Cytochrome c, and the loading control GAPDH. (B) Expression levels of VDAC1, COXIV and Cytochrome c after 24-h with Leu (1 or 3 mM). Data of each group are expressed as the ratio relative to the average value of the control group (1.0). Values are presented as mean ± SEM (n = 6-8/group).

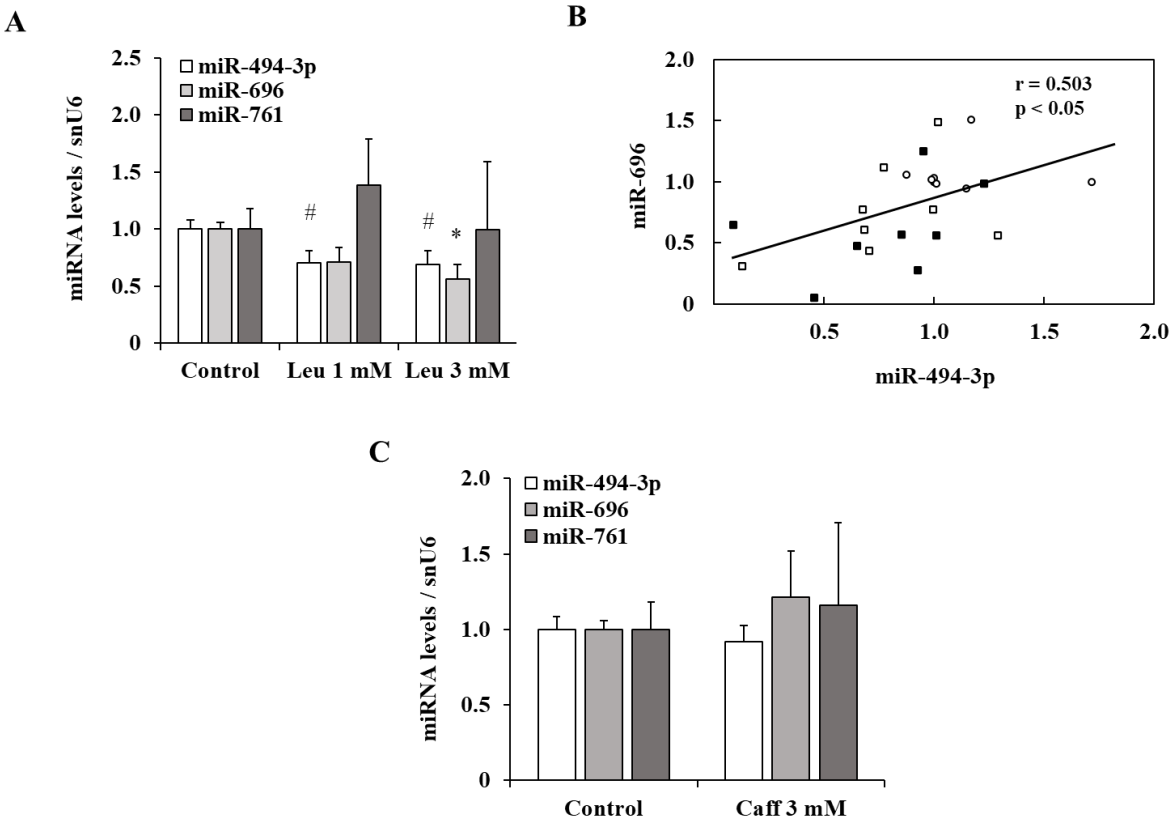

Figure IV-6. Fold change in miRNAs after 24-h treatment with leucine or caffeine. (A) Relative expression levels of miR-494-3p, miR-696 and miR-761 after treatment with Leu (1 or 3 mM). Expression levels of these miRNAs were determined by the comparative method $2^{-\Delta\Delta Ct}$ using snU6 as an internal control and expressed as the ratio against control levels. (B) Correlation between miR-494-3p and miR-696 expression levels in Control (○), 1 mM Leu (□), 3 mM Leu (■) (n = 8/group, total n = 24). Pearson's correlation coefficient was obtained across all the data, independent of the Leu treatment applied ($r = 0.503$ and $p < 0.05$). (C) Relative expression levels of miR-494-3p, miR-696 and miR-761 after treatment with Caff (3 mM). Values in (A) and (C) are expressed as mean ± SEM. *: $p < 0.05$, different from the control group, #: $p < 0.1$, tendency as compared with the control condition by one-way ANOVA with Dunnett's post-hoc test.

specific marker for mTORC1 activation (Manning and Toker, 2017). In contrast, p70S6K appeared tended to be upregulated at 1 mM of leucine. Opposite to p70S6K, the phosphorylation rate of 4E-BP1 tended to be augmented at 3 mM of leucine concentration. The activation of both proteins is indicative of protein synthesis stimulation.

Regarding mitochondrial biogenesis regulation, we observed that 3 mM leucine increased mtDNA copy numbers (Figure IV-4A). In correspondence with our observations, a study reported that 0.5 mM leucine was sufficient to increase mitochondrial content and mitochondrial biogenesis-related gene expression of sirtuin 3 (SIRT3), PGC-1α, COXII and COXV in C2C12 myotubes after 48-h treatment (Liang et al., 2014). Furthermore, high fat diet-induced mitochondrial damage was partially restored by leucine supplementation, and was accompanied by increases in the mtDNA content, TFAM expression, and ATP production, confirming the protective role of leucine in skeletal muscle (D'Antona et al., 2010; Li et al., 2012; Liu et al., 2017).

In contrast, leucine treatment did not affect the expression of both nuclear- and mitochondrial-encoded proteins detected by OXPHOS (Figure IV-4B and 4C). This discrepancy may be partially explained by the interactions of miRNAs with mitochondrial-biogenesis genes. Another possibility might be the leucine-treatment period. Liang et al. (2014) reported only a 5% increase in mtDNA after 24-h treatment with 0.5 mM Leu (in normal DMEM, final concentration 1.3 mM), with this effect being 2-fold higher at 48-h. Therefore, 24-h Leu treatment may be too short to induce large changes in mitochondrial biogenesis and the level of mitochondrial-related proteins.

Since skeletal muscle is highly sensitive to nutrient availability, the miRNA profile in this tissue might exhibit a nutrient-specific response as well. In the present study, consistent with our hypothesis, miR-494-3p and miR-696 were downregulated by leucine treatment. Furthermore, we found a positive correlation between miR-494-3p and miR-696 expression

across all the study groups that was independent of the treatment applied (Figure IV-6B). This could suggest that coordination between these miRNAs is involved to exert their effect in skeletal muscle, independently of the nutritional status. A possible explanation for this might involve the insulin-like growth factor 1 (IGF-1)/PI3K/Akt signaling pathway since all experiments were conducted in a high-glucose DMEM. The IGF-1/PI3K axis is known to promote cell differentiation and proliferation, but also constitutes a survival signal in most cancer types (Denduluri et al., 2015). Some observations indicate that the cluster 14q32 (where miR-494-3p is located) is silenced in most tumors, although treatment with demethylating agents and histone deacetylases (HDAC) inhibitors restored the activity of the miRNAs in the cluster targeting IGF-1, which led to reduced cell migration (Zehavi et al., 2012). One of the observed mechanisms of miRNA control involves their targets and/or upstream/downstream members from the same cascade; it has been reported that IGF-1 alters miRNA expression through MAPK (Martin et al., 2012), the family in which both of our miRNAs of interest have regulatory targets (Li et al., 2015; Wang et al., 2017). Further experiments should be performed to clarify the relationship between these miRNAs' functions and different nutritional conditions.

In summary, 24-h of leucine supplementation induced a mild increase in mitochondrial biogenesis in C2C12 myotubes, potentially via downregulation of miR-494-3p and/or miR-696. These observations suggest a leucine-specific mechanism to regulate miRNAs in skeletal muscle. A positive correlation between the expression of miR-494-3p and miR-696 was found, indicating possible coordination between these miRNAs. Furthermore, the data presented constitute the first report of a nutrient, especially leucine, regulating the exercise-responsive miRNAs, miR-494-3p, and miR-696. Prospective studies should focus on modulating miRNAs levels in skeletal muscle, to discover novel therapeutic approaches for the prevention of sarcopenia, treatment of muscular diseases, and other possible indications.

V. Relationship Between Leucine, miRNA Levels and Mitochondrial Biogenesis in C2C12 myotubes (Experiment II)

5.1 Purpose

Undoubtedly, miRNAs are key important factors in the response to environmental stress as they help to restore homeostasis in cases of sudden extracellular changes or if the stress condition is severe and of long duration (Leung and Sharp, 2010). It has been estimated that nearly 60% of all mammalian mRNAs are targets of miRNA regulation, being essential for gene expression reprogramming of cells during adaptation to external stimuli (Friedman et al., 2009). Since skeletal muscle is highly sensitive to nutrient availability, the miRNA profile in this tissue might exhibit a nutrient-specific response as well. Therefore, it would be of great interest to determine the effect of nutrients, especially leucine, on the expression and functions of some miRNAs.

Because of the molecular redundancy of miRNAs and its overlapping targets and functions, target perturbation by the miRNA network is very modest, sometimes <20%. This might lead to some difficulties to analyze the results since a clear phenotype is not obtained. To date, the most widely used approaches for investigating the impact of miRNAs in various biological conditions are systemic and organ-specific knockdowns/transgenic strategies, gain-of-function strategies, and loss-of-function strategies (Stenvang et al., 2012). Gain-of-function strategies represent the transfection of miRNA or synthetic miRNA mimics into tissue or cells (Deiuliis, 2016). Alternatively, loss-of-function strategies most commonly include the application of anti-miRNAs (miRNA specific oligonucleotides), that specifically bind and inhibit a target miRNA, which then leads to the downregulation of that miRNA (Oliveira-Carvalho et al., 2012).

We previously reported that treatment with leucine (1 and 3 mM) reduced the expression of the exercise-responsive miRNAs miR-494-3p and miR-696 in C2C12 myotubes. In the current study, we tested the hypothesis that leucine promotes mitochondrial biogenesis partially through these miRNAs-mediated regulations of PGC-1α. To achieve this, we transfected myotubes with precursor miRNAs or anti-miRNAs specific sequence, followed by treatment with leucine 3 mM, as used in the former experiment. The outcome of both transfection protocols on PGC-1α signaling and mitochondrial biogenesis was evaluated through the detection of mtDNA copy number, gene and protein expression of PGC-1α downstream target TFAM and mitochondrial proteins levels.

5.2 Materials and Methods

5.2.1 Cell Culture

Mouse C2C12 myoblasts were obtained from American Type Culture Collection (ATCC, VA, USA) and cultured in Dulbecco's modified Eagle's medium (high glucose DMEM; Gibco, MA, USA) containing 10% fetal bovine serum, 1% penicillin-streptomycin, at 37 °C in 5% CO_2 air atmosphere. When the cells reached 80% confluence (defined as day 0), the medium was changed to DMEM supplemented with 2% calf serum, 1% penicillin-streptomycin, and 1% non-essential amino acids to induce myotubes formation. For the time-course of miRNAs, after 5 days of differentiation, cells were treated with Leu-free custom medium (IFP, Yamagata, Japan) without serum for the next 48-h, similar to the treatment phase described in Figure IV-1. The cells were then harvested at several time points as shown in Figure V-1 (upper panel) to perform downstream analysis. All experiments were performed using cells of up to 9 passages.

5.2.2 Myotubes Transient Transfection

As shown in Figure V-1 (lower panel), after 2 days of differentiation, myotubes were double transfected with 30pmol of mmu-miR-494-3p (#AM17100, ID: PM12409) and mmu-miR-696 (#AM17100, ID: PM11434) precursor sequence or mmu-miR-494-3p (#AM17100, ID: AM12409) and mmu-miR-696 inhibitor (#AM17100, ID: AM11434), using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA), according to the protocol provided by the manufacturer. For the control groups, cells were transfected with the precursor control, Pre-miR Negative Control #1 (AM17110) or the inhibitor control, anti-miR Negative Control #1 (AM17010), both from Ambion by Life Technologies (Carlsbad, CA, USA). Briefly, precursor or inhibitor miRNAs and Lipofectamine RNAiMAX reagent were diluted in Opti-MEM I Reduced Serum Medium (Gibco, MA, USA). Next, the diluted precursor or inhibitor miRNAs and Lipofectamine reagent were mixed (1:1 ratio) and incubated at room temperature for 10 min, before adding it to cells in differentiation medium without serum. The transfection mix was removed 24-h later and cells were grown in differentiation medium for a similar period. Forty-eight hours after the transfection, the cells were supplemented with or without leucine (3 mM) for another 24-h. Cells were then harvested for western blot and qPCR analyses.

5.2.3 Mitochondrial DNA Copy Number

Cells were collected with (D)-PBS, centrifuged at 600 *g* for 5 min and the cell pellets were immediately frozen in liquid nitrogen before being stored at -80°C. Genomic DNA was extracted using the spin-column based DNeasy Blood & Tissue Kit (QIAGEN, Mississauga, ON, Canada) and diluted in Tris-EDTA (pH 8.0) for long-term storage. Real-time PCR was performed using ABI 7300 system and software (Applied Biosystems, USA) and THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). The following primers were used: 5'- GCCGACTAAATCAAGCAACA - 3' (forward) and 5' -

CAATGGGCATAAAGCTATGG - 3' (reverse) specific for cytochrome c oxidase subunit II (COXII, mitochondrial), and 5'- GCGACCAGCCCATTGTAGA - 3' (forward) and 5'- GCGTTCTGGGTAC CATCCTAAC - 3' (reverse) specific for mitochondrial uncoupling protein 2 (UCP2, nuclear) genes (Eurofins Genomics, Tokyo, Japan). The PCR reaction was set up as follows: UDG incubation for 2 min at 50°C, initial denaturation step at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. The relative copy numbers of mitochondrial to nuclear DNA were determined by the comparative method $2^{-\Delta\Delta Ct}$ using UCP2 as an internal control.

5.2.4 miRNA Expression

Cells were prepared in the same conditions as for DNA extraction. Total RNA was isolated using the ReliaPrepTM miRNA Cell and Tissue Miniprep System (Promega, WI, USA) according to the manufacturer's instructions and quantified with Denovix Spectrophotometer (DS-11; Wilmington, DE, USA). Then, 20-60 ng of total RNA was reverse transcribed into cDNA with the TaqMan MiRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For miRNA expression detection TaqMan miRNA assay kits were used (mmu-miR-494-3p: 002365, mmu-miR-696: 001628, mmu-miR-761: 002030 and snU6: 001973, Thermo Fisher, MA, USA) and real-time qPCR was performed according to the manufacturer's instructions with TaqMan Universal PCR Master Mix, no AmpEraseTM UNG, (Thermo Fisher). The PCR reaction was set up as follows: initial denaturation step at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. All samples were run in duplicate and expression levels of individual miRNAs were determined by the $2^{-\Delta\Delta Ct}$ method using snU6 as the internal control.

5.2.5 Quantification of mRNA Expression

Real-time PCR was performed using total RNA samples. Double-stranded cDNA was synthesized from 25 ng of total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The mRNA expression of PGC-1α and TFAM was quantified with ABI 7300 system and software (Applied Biosystems, USA), following the manufacturer's instructions using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). Forward and reverse primers for the target genes are listed in Table V-1 (Eurofins Genomics, Tokyo, Japan). Conditions for amplification were 60 s at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 60°C for primer annealing and elongation. All samples were run in duplicate and data were analyzed according to the $2^{-\Delta\Delta Ct}$ method using GAPDH as the control housekeeping gene. The purity of the amplified product was checked through analysis of the melting curve carried out at the end of amplification.

5.2.6 Sample Preparation and Immunoblotting

Cell lysates were homogenized in RIPA buffer (20 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) containing protease and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), using a pestle homogenizer (Nippi, Tokyo, Japan). Homogenates were then sonicated for 20 s at power 5 with an Ultrasonic Disruptor (UR-21P; Tomy Seiko, Tokyo Japan) and incubated for 30 min at 4°C in a rotator. Finally, lysates were centrifuged at 10,000 g for 20 min at 4°C and the supernatant was used for immunoblotting (Manabe et al., 2012; Yamada et al., 2016). The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Whole protein fraction samples were solubilized in equal volume of 2x SDS sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.002% bromophenol blue), adjusted to the

Table V-1. Primer sequences for real-time PCR.

PGC-1α	forward	AAG TGT GGA ACT CTC TGG AAC TG
	reverse	GGG TTA TCT TGG TTG GCT TTA TG
TFAM	forward	CAT TTA TGT ATC TGA AAG CTT CC
	reverse	CTC TTC CCA AGA CTT CAT TTC
GAPDH	forward	TGT GTC CGT CGT GGA TCT GA
	reverse	TTG CTG TTG AAG TCG CAG GAG

final concentration of 1.5 µg protein /µl with 1x SDS sample buffer, and then incubated at 95°C for 5 min. Normalized samples were separated by SDS-PAGE, and then transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated with blocking buffer followed by incubation with primary antibodies specific for Myosin Heavy Chain (MyHC, 1:2,000; A4.1025, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), PGC-1α (1:1,000; ab54481, Abcam, Cambridge, UK), TFAM (1:1,000; 22586-1-AP, Proteintech, Rosemont, IL, USA), α-tubulin (1:2,000; 3873S, Cell Signaling Technology, Beverly, MA, USA) and GAPDH (1:1,000; ab8245, Abcam) at 4°C overnight. After exposure to a secondary antibody, reaction products were visualized using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). Band densities were quantified with ImageJ software (version 1.52p; NIH, MD, USA).

5.2.7 Statistical Analysis

Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test in case of multiple comparisons. A two-way ANOVA was performed to assess main vs. interaction effects of miRNAs overexpression/inhibition and leucine treatment (transfection and treatment). All statistical analyses were performed using IBM SPSS Statistics 25 (IBM Japan, Tokyo, Japan). The results are expressed as mean ± SEM with a significance level of $p < 0.05$, while a trend was defined as $p < 0.1$.

5.3 Results

5.3.1 Time-Course of miRNA Expression During Myogenesis

To examine the expression profile of miR-494-3p, miR-696 and miR-761 during myotubes differentiation (day 0 to 5) and the starvation/treatment period (day 5 to 7), RNA was purified at indicated time points after myogenic induction and miRNA content was quantified by

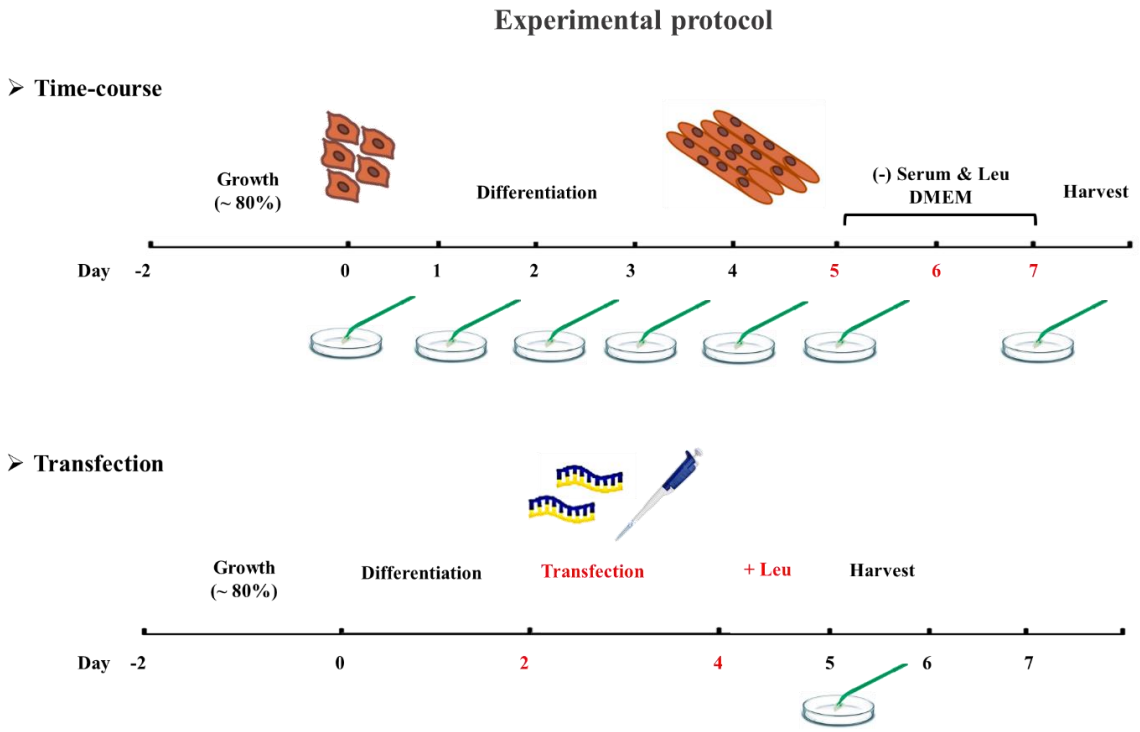

Figure V-1. Experimental design, treatment, and sample collection. When the cells reached 80% confluence, the medium was exchanged to induce myotube differentiation for 5 days. (Upper panel) For the time-course experiment, cells were starved with leucine-free medium without serum for 48-h to mimic our previous treatment conditions. Cells were harvested at different time points to examine miRNA levels during skeletal myogenesis. (Lower panel) A schematic protocol of double transfection of C2C12 cells with miR-494-3p and miR-696 precursor/inhibitor at day 2, using lipofection with RNAiMAX, followed by 24-h of treatment with leucine (3 mM) at day 4. Cells were harvested at day 5 for downstream analyses.

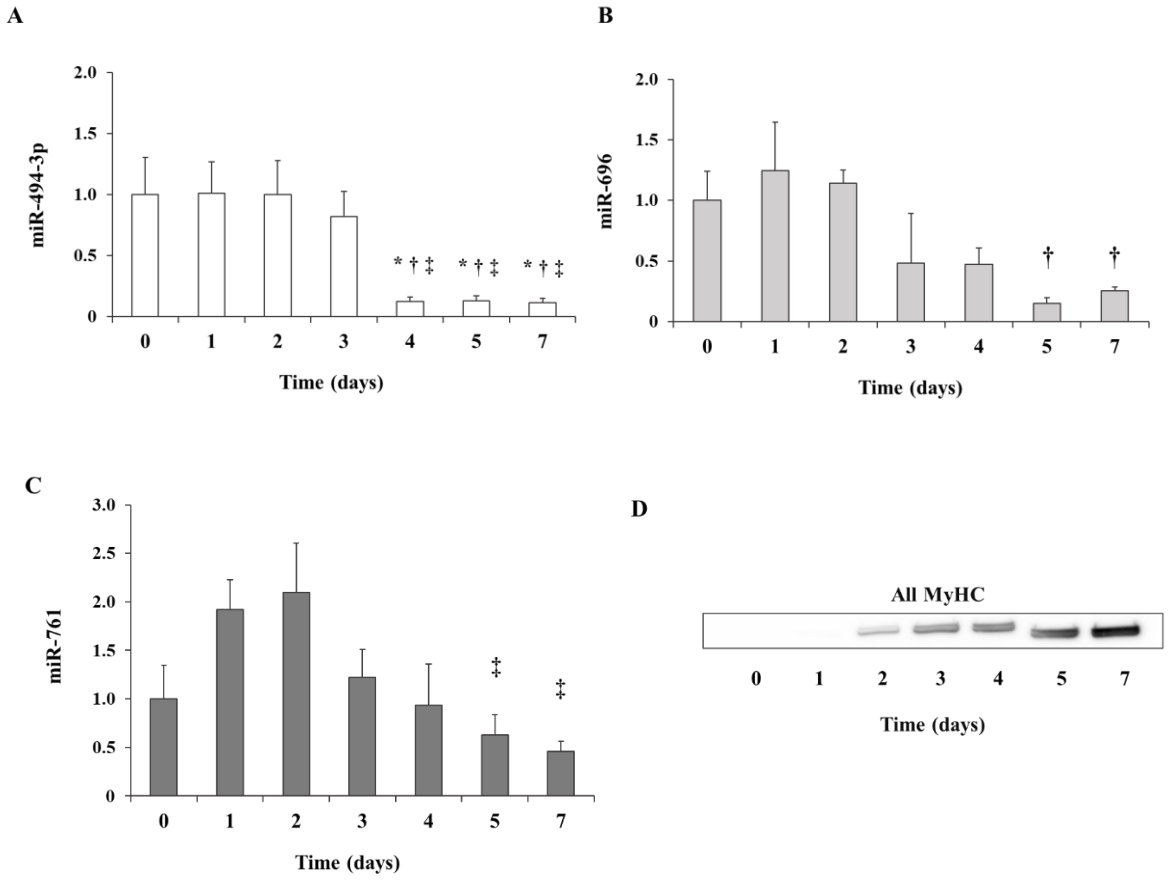

Figure V-2. Changes in miRNA expression during differentiation of C2C12 myoblast. Time-course of miR-494-3p (A); miR-696 (B); and miR-761 (C) expression in C2C12 cells after myogenic induction. The data are normalized against the endogenous control snU6 and each point represents the mean ± SEM (n = 7/day). (D) Immunoblotting image of MyHC during myogenesis progression. * different from day 0, † different from day 1, ‡ different from day 2 by one-way ANOVA with Tukey's honestly significant difference test ($p < 0.05$).

TaqMan RT-qPCR assay (Figure V-1, upper panel). As shown in Figure V-2A, miR-494-3p levels relative to snU6, remained upregulated until day 3 of the myogenic process, while a subsequent decrease was observed at day 4 until the end of the experiment ($p < 0.05$). When analyzing miR-696 levels, it did not change throughout the differentiation period, although it drastically decreased on days 5 and 7 compared to day 2 (Figure V-2B; $p < 0.05$). Similarly, miR-761 did not show significant changes along the study period but it was downregulated at the end of the myogenic phase and the starvation/treatment period (Figure V-2C; $p < 0.05$). The fact that these miRNAs were highly expressed during myogenesis is indicative of their role in skeletal muscle differentiation. Concomitantly, the protein levels of myosin heavy chain (MyHC) were detected (Figure V-2D). The expression of all MyHC increased progressively from day 3 to day 7, during myotubes formation and maturation, reaching the highest level at the later time point, which is indicative of myoblast differentiation.

5.3.2 Effect of miR-494-3p and miR-696 Overexpression on Mitochondrial Biogenesis

Since we previously observed that miR-494-3p and miR-696 were downregulated by leucine treatment, we decided to elucidate their effect on myotubes and PGC-1α-mediated signaling by gain- and loss-of-function experiments. On day 2, cells were transfected with the scrambled control sequence (defined as Control), or concomitantly with miR-494-3p and miR-696 precursor (defined as miRs OE) to keep their levels persistently high during myotubes formation. Also, we chose to perform the transfection on day 2 because fully differentiated myotubes show low transfection efficiency. Forty-eight hours post-transfection, leucine (3 mM) was added to the culture medium and posterior analyses were performed after 24-h of treatment.

Two-way ANOVA indicated a significant main effect of transfection in miR-494-3p and miR-696 expressions ($p < 0.05$, Figure V-3A). The expression level of both miR-494-3p and

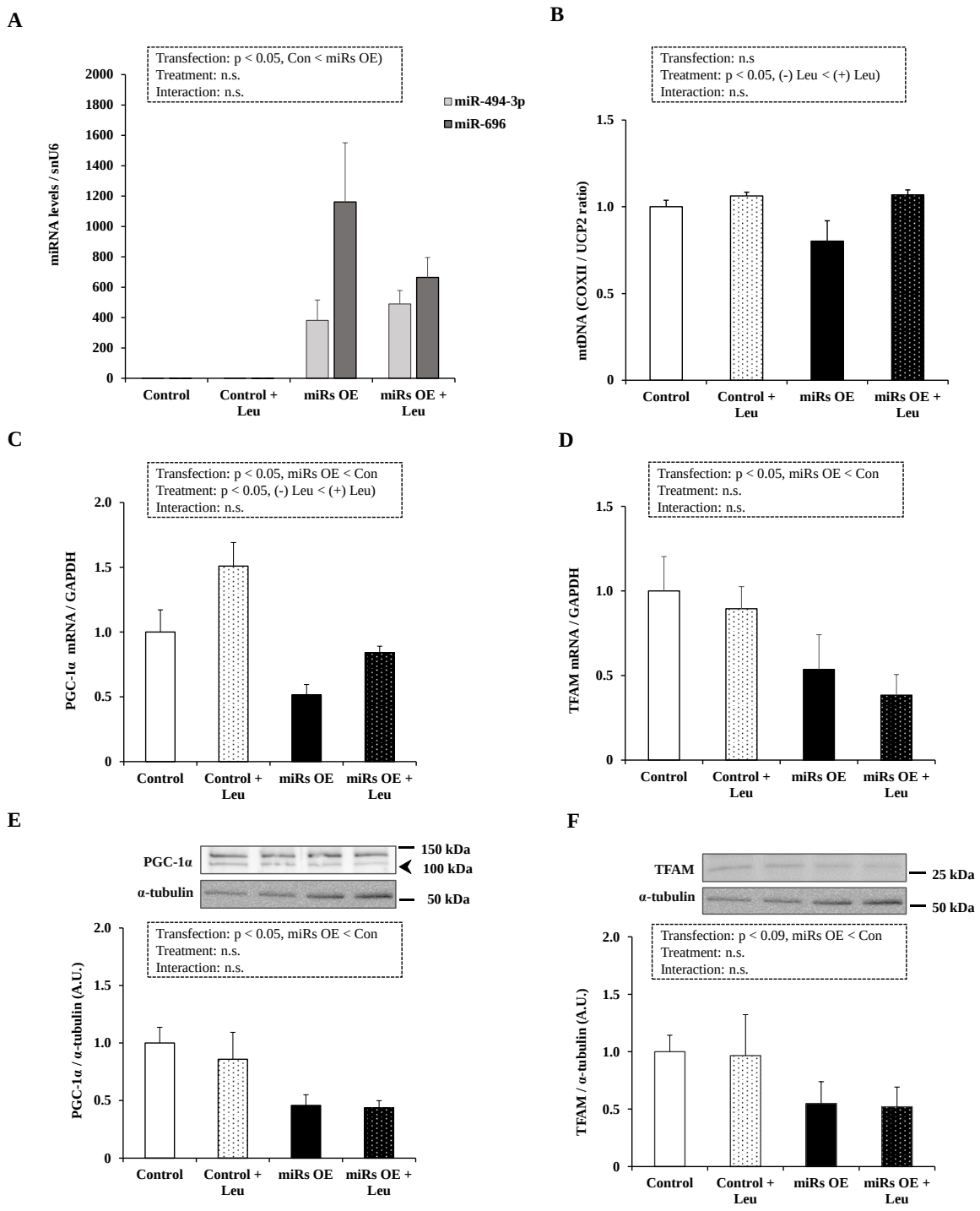

Figure V-3. Effect of miR-494 and miR-696 overexpression on mitochondrial biogenesis. (A) Expression levels of 494-3p and miR-696 were determined by the comparative method $2^{-\Delta\Delta Ct}$ using snU6 as an internal control and expressed as the ratio against control levels. (B) Mitochondrial DNA copy number (mtDNA) expressed as the ratio of mitochondrial to nuclear DNA, was determined in cells double transfected with miRNA precursors, followed by Leu treatment. (C) mRNA, and (E) protein expression levels of PGC-1α in cells double transfected with miRNA precursors, followed by Leu treatment. The arrowhead (➤) indicates the quantified band (105 kDa). (D) mRNA, and (F) protein expression levels of TFAM in cells double transfected with miRNA precursors, followed by Leu treatment. Values are expressed as mean ± SEM (n = 3/group). The main effects of miRNA overexpression (miRs OE) and Leu treatment were determined by two-way ANOVA test.

miR-696 was significantly higher in these miRNAs-transfected cells (abbreviated as miRs OE) than in non-transfected cells ($p < 0.05$). To examine the effect of miR-494-3p and miR-696 overexpression on mitochondrial content, we quantified the mtDNA copy number. There was a significant main effect of treatment on mtDNA copy number ($p < 0.05$, Figure V-3B). Compared to the (-) leucine cells, mtDNA copy number was significantly greater in leucine-treated cells ($p < 0.05$). Moreover, two-way ANOVA revealed significant main effects of transfection and treatment in PGC-1α mRNA expression ($p < 0.05$, Figure V-3C). The mRNA expression of PGC-1α was significantly lower in miRs OE than in control cells, whereas the value in Leu-treated cells was significantly higher than in (-) leucine cells. A similar trend, except for the main effect of treatment, was observed in PGC-1α protein expression and TFAM mRNA and protein expressions (Figure V-3 D-F). These results indicate that miR-494-3p and miR-696 target PGC-1α while presenting a relationship between leucine treatment, miR-494-3p and miR-696, and PGC-1α in skeletal muscle.

5.3.3 Effect of miR-494-3p and miR-696 Suppression on Mitochondrial Biogenesis

Similar to the gain-of-function experiment, at day 2 of myogenic induction cells were transfected with scrambled antisense sequence (defined as Control), or with miR-494-3p and miR-696 antisense oligonucleotides at the same time, indicated as “Inhibitor” group. Forty-eight hours post-transfection, leucine (3 mM) was added to the culture medium. After 24-h of treatment with leucine, cells were harvested for posterior analyses.

Contrary to the overexpression experiment, two-way ANOVA analysis showed no main effects of the transfection with miR-494-3p and miR-696 inhibitors or leucine treatment on miRNA levels (Figure V-4A), PGC-1α mRNA and protein (Figure V-4C and -4E), as well as TFAM mRNA and protein expression (Figure V-4D and -4F). In correspondence with the

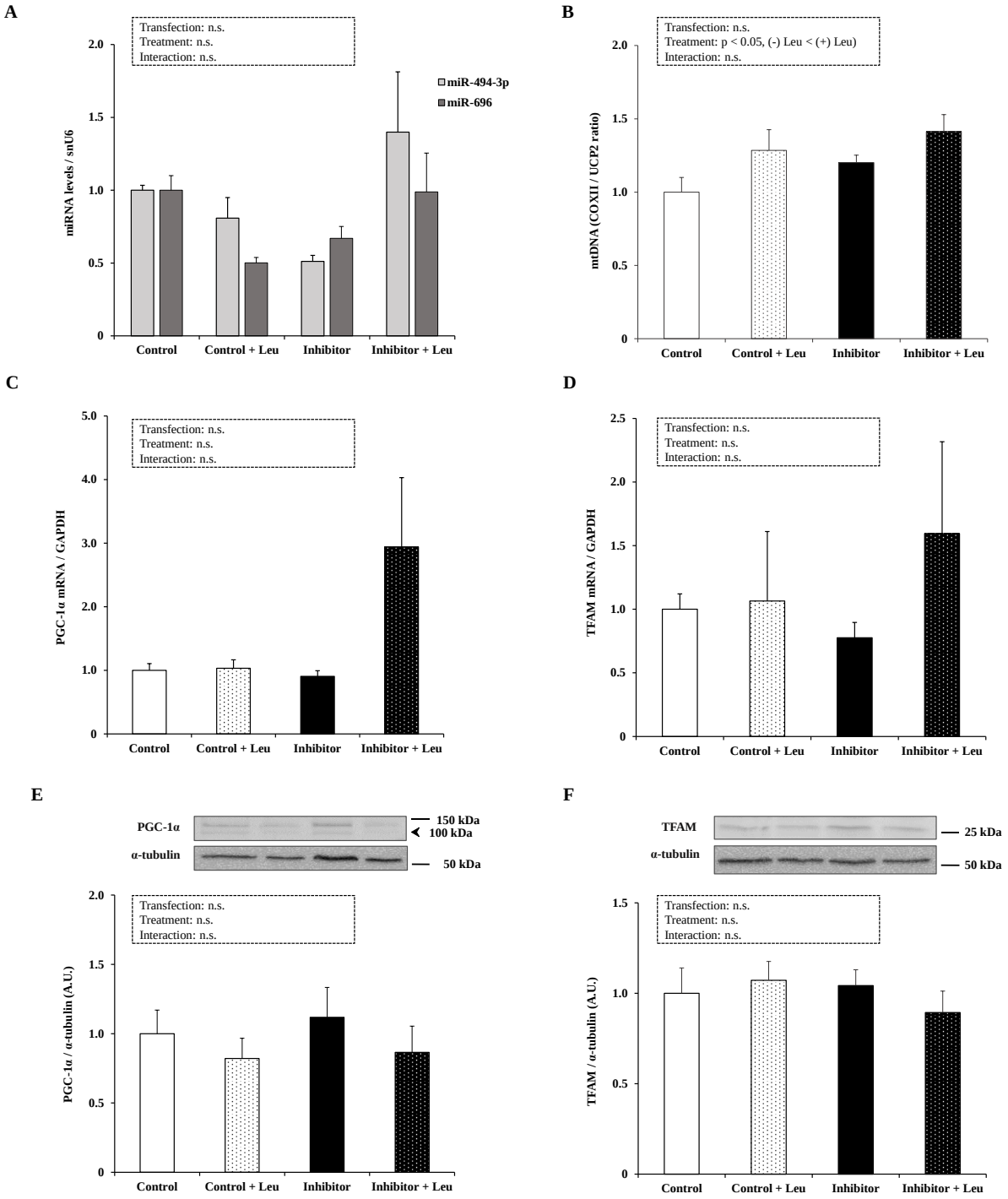

Figure V-4. Effect of miR-494 and miR-696 inhibition on mitochondrial biogenesis. (A) Expression levels of 494-3p and miR-696 were determined by the comparative method $2^{-\Delta\Delta Ct}$ using snU6 as an internal control and expressed as the ratio against control levels. (B) Mitochondrial DNA copy number (mtDNA) expressed as the ratio of mitochondrial to nuclear DNA, was determined in cells double transfected with miRNA inhibitors, followed by Leu treatment. (C) mRNA, and (E) protein expression levels of PGC-1α in cells double transfected with miRNA inhibitors, followed by Leu treatment. The arrowhead (➤) indicates the quantified band (105 kDa). (D) mRNA, and (F) protein expression levels of TFAM in cells double transfected with miRNA inhibitors, followed by Leu treatment. Values are expressed as mean ± SEM (n = 4-5/group). The main effects of miRNA inhibition and Leu treatment were determined by two-way ANOVA test.

overexpression experiment, mtDNA copy number was significantly greater in leucine-treated cells, compared to the (-) leucine cells (Figure V-4B, $p < 0.05$). These results might indicate the presence of a leucine-induced positive effect on mitochondrial biogenesis, with mtDNA as a sensitive marker to leucine treatment.

5.4 Discussion

In this study, we determined the expression of the exercise-responsive miRNAs, miR-494-3p, miR-696, and miR-761 during skeletal muscle differentiation. As a marker of myotubes differentiation, MyHC protein was observed, by us and others (Kim et al., 2015; Sin et al., 2016), to increase gradually during the study period. We found miR-494-3p upregulated up to day 3, while for miR-696, it was increased on day 1, and miR-761 on day 2 of myogenesis (Figure V-2). After that, the three miRNAs were significantly reduced by the end of the myogenic differentiation. Similarly, Lemecha et al. (2018) found miR-494-3p increased at day 2 of adipocytes differentiation, while Iwasaki et al. (2015) reported a peak for this miRNA on day 1 in human iPS cells. Recently, it was discovered that the miR-494-3p gene localizes inside a cluster right under transcriptional control of MEF2, implying the importance of these miRNAs in muscle regeneration (Snyder et al., 2013). Also, Wang et al. (2017) observed that miR-696 levels were elevated on day 2, suggesting that miR-696 might play a negative role in skeletal myogenesis. On the other hand, this is the first report of miR-761 expression profile in skeletal muscle myogenesis, although its role, along with miR-494-3p, in promoting cell proliferation, differentiation, migration, and metastasis in several cancer types have been confirmed (Chai et al., 2015; Zhang et al., 2019). It was observed that the overexpression of miR-494-3p and miR-696 in skeletal muscle reduced myotubes number and size (Iwasaki et al., 2015; Wang et al., 2017) and inhibition of miR-696 exhibited considerable levels of slow myosin heavy chain (Truskey et al., 2013).

We have previously reported a leucine-mediated downregulation of miR-494-3p and miR-696 in C2C12 myotubes. To clarify how leucine-induced regulation of these miRNAs impacts mitochondrial biogenesis, we performed two experimental manipulations: 1) we overexpressed at the same time miR-494-3p and miR-696 transfecting cells with their precursors, and 2) we suppressed the expression of both miRNAs utilizing antisense specific inhibitors. Overall, the overexpression experiment had more significant effects than the suppression of both miRNAs. The transfection with the precursor sequences induced an increase in these miRNA levels greater than 400-fold, while with the inhibitors no effect of the transfection condition was observed (Figure V-3A and -4A, respectively). Interestingly, C2C12 myotubes overexpressing miRNAs showed decreased levels of PGC-1α mRNA, while leucine treatment upregulated its expression, regardless of the strong overexpression of these miRNAs (Figure V-3C). However, when these miRNAs were downregulated, no effect on PGC-1α mRNA was observed in the leucine-treated group (Figure V-4C). Although only miRs OE condition impacted PGC-1α protein levels (Figure V-4E), these results indicate that miR-494-3p and miR-696 target PGC-1α while representing a relationship between leucine supplementation, miR-494-3p and miR-696, and PGC-1α.

To confirm the impact on mitochondrial biogenesis of the leucine/miR-494-3p and -696/ PGC-1α axis, we performed further investigations. We found that mtDNA copy numbers was increased after leucine treatment, independent of the transfection with miRNAs precursors (Figure V-3B) or inhibitors (Figure V-4B). It is known that reversible miRNA alterations represent an adaptive response to treatments (Tomasetti et al., 2019). Therefore, a possible explanation is that leucine supplementation might be able to cancel or somehow surpass the effect of overexpression or inhibition of miRNAs to induce mitochondrial biogenesis. Overall, these findings highlight the relationship between leucine and miRNA levels, despite we did not find an interaction between both treatments. The expression of PGC-1α downstream target,

TFAM, was significantly downregulated in the cells overexpressing miR-494-3p and miR-696, at mRNA and protein levels (Figure V-3D and -3F, respectively). No other effects were observed for the measured markers in the loss-of-function experiment. Altogether, we observed that the loss or reduced functional regulation of miR-494-3p and miR-696 does not always result in alterations in the expression of their target protein.

Similar to our findings, Iwasaki et al. (2015) reported that miR-494-3p knockdown had virtually no effect in human iPS cells differentiated to the myogenic lineage. Similarly, they observed miR-494-3p overexpression did not affect mtDNA copy numbers, protein expression of the succinate dehydrogenase complex subunit A and manganese superoxide dismutase, as well as TFAM and FoxJ3 mRNAs, validated targets of miR-494-3p. These data suggested that TFAM-mediated regulation of mitochondrial biogenesis was intact in cells overexpressing miR-494-3p. Regarding miR-696 gain- and loss-of-function strategies, Aoi et al. (2010) observed a significant decrease in PGC-1α protein and mtDNA content in myotubes overexpressing miR-696. Although our study matches the protocol and cell line reported by Aoi et al. (2010), the latter used low glucose medium to induce myogenic differentiation and the analysis was performed at 24-h post-transfection, while our protocol included high glucose medium and analysis at 72-h post-transfection. Regardless of the aforementioned differences between the two studies, our results reflect Aoi and colleagues' observations.

In summary, the double overexpression of miR-494-3p and miR-696 resulted in decreased PGC-1α and TFAM, at both mRNA and protein levels. In the present study, treatment with leucine effectively upregulated PGC-1α and mtDNA content. These results corroborate that leucine effects on mitochondrial biogenesis partially involve the regulation of PGC-1α-targeting miRNAs.

VI. Overall Discussion

Nutrients constitute an important factor in the regulation of skeletal muscle metabolism. Among the BCAAs, leucine has become a popular dietary supplement and the main component of many ergogenic products (Vaughan et al., 2013). The evidence points towards a key regulatory role of leucine in gene transcription and translation. It is well established that leucine is capable of activating the mRNA translational machinery through the regulation of mTOR, which serves as a convergence point for leucine-mediated effects on mRNA translation initiation and represents an interesting molecular target for the prevention of muscle loss (van Loon, 2012). In the present study, we evaluated mTOR activity through its phosphorylation on residue Ser2448 following 24-h of treatment with two different doses of leucine (1 or 3 mM). Although no differences in mTOR were observed, the phosphorylation levels of the downstream targets p70S6K and 4E-BP1 were augmented, which is in concordance with the observed increase in cell diameter after treatment with leucine (Figure IV-2).

Beyond the role in protein synthesis stimulation, leucine has been associated with activation of SIRT1 signaling, suppression of insulin resistance, and prevention against metabolic disorders in a diet-induced obesity animal model (Li et al., 2012; Zhang et al., 2007). Recently, it was shown that leucine induced a metabolic shift from glycolytic towards OXPHOS in human and murine skeletal muscle cells and also in some models of cancer (Vaughan et al., 2013; Viana et al., 2019). In skeletal muscle, SIRT1 deacetylates PGC-1α to induce the expression of genes controlling mitochondrial biogenesis and fatty acid oxidation to maintain energy homeostasis (Li et al., 2011; Liang et al., 2014). Although no changes in OXPHOS were found after treatment with leucine (Figure IV-4B and 4C), or in other mitochondrial proteins, an increase in mtDNA copy numbers was observed in myotubes treated

with leucine 3 mM. Altogether, these findings suggest a mild effect of leucine treatment on mitochondrial biogenesis.

In the last three decades, a novel regulatory mechanism of gene expression has been discovered. Small non-coding regulatory RNAs termed miRNAs have been found associated with virtually all cellular processes. Recent studies have suggested that several miRNAs could also function as modulators of myogenesis, muscle mass, and nutrient metabolism in skeletal muscle (Small et al., 2010; Gagan et al., 2011; Zhang et al., 2012), thus raising questions regarding the association of miRNAs with exercise-induced physiological changes, muscular pathogenesis, and age-related muscle dysfunction (Aoi, 2015). Understanding the mechanisms by which nutrients, especially leucine, can alter metabolic signaling through the miRNA network will be of great value for the development of effective nutritional and/or pharmacological interventions to prevent and/or treat chronic metabolic diseases.

Diverse dietary components such as amino acids (Sun et al., 2016), carbohydrates (Druz et al., 2012), fatty acids (Parra et al., 2010), and vitamins (Wang et al., 2011) have been found to affect the expression profile of miRNAs or their function. Particularly, leucine and essential amino acids have been shown to regulate miRNA expression in cultured skeletal muscle cells (Chen et al., 2013). In the present study, a downregulation of the exercise-responsive miRNAs miR-494-3p and miR-696 was observed after treatment with leucine (Figure IV-6A). These miRNAs' expressions were also correlated, suggesting coordinated functions in skeletal muscle (Figure IV-6B). Next, the gain-of-function approach confirmed the regulation of the PGC-1α by miR-494-3p and miR-696 in myotubes transfected with these miRNAs' precursors (Figure V-3C and 3E), which impacted the expression of nuclear factor TFAM, acting downstream of PGC-1α (Figure V-3D and 3F). However, we only visualized a partial recovery of PGC-1α gene expression associated with leucine treatment. In both, gain- and loss-of-function experiments, leucine treatment showed increased mtDNA copy numbers (Figure V-

3B and 4B). These facts indicate that leucine might promote mitochondrial biogenesis partially through the regulation of PGC-1α-targeting miRNAs.

Regarding the control of miRNA expression, diverse regulatory levels have been proposed. At the molecular level, beside the mutual control among miRNAs and their mRNA targets, observations indicate the existence of the so-called “miRNA sponges”. These consist of long non-coding RNAs (lncRNAs), pseudogenes, circular RNAs, and mRNAs that can scavenge specific miRNAs at their seed site, preventing the silencing complex (RISC) to bind their target mRNAs, or competing with miRNAs for binding to the specific mRNAs (Ye et al., 2015). Nevertheless, the exact relationship between nutrients and miRNAs biogenesis in skeletal muscle is not well documented. Figure VI-1 summarizes the possible two mechanisms reported until now, along with our findings. Under high-nutrient conditions (amino acid- or glucose-sufficient), activation of mTORC1 increases the levels of ubiquitin E3 ligase Mdm2 that ubiquitinates and targets the miRNA-processing enzyme Drosha for proteasomal-dependent degradation, this leading to a global decrease of miRNA biogenesis (Ye et al., 2015). Herein, we determined the phosphorylation of mTOR and its main downstream effector proteins including p70S6K and 4E-BP1. The two proteins were significantly increased by leucine and treatment, indicating possible activation of the pathway; however, this is not sufficient to explain the observed downregulation. There is another mechanism that is related to the epigenetic regulation of gene expression by nutrients (Keating and El-Osta, 2015). Accordingly, amino acids and other metabolites are catabolized to acetyl-CoA, the essential acetyl group donor to lysine-acetylation reaction (Adeva-Andany et al., 2017). Because histone acetylation is ubiquitously associated with open-chromatin and gene expression, amino acid catabolism provides a metabolic link to epigenetic regulation (Phang et al., 2013). Data shows that miRNAs are not only regulated by multiple epigenetic mechanisms but can also repress the expression of epigenetic factors (Su et al., 2011; Quintanilha et al., 2017). Moreover, DNA

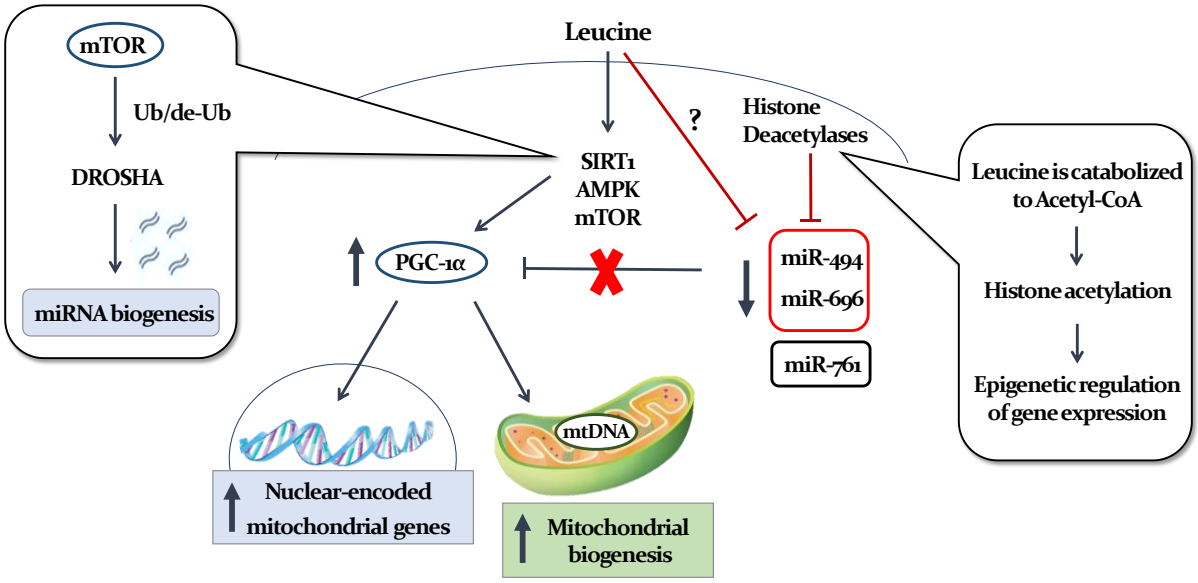

Figure VI-1. Proposed mechanism of leucine-mediated regulation of miR-494-3p and miR-696 in skeletal muscle. After leucine incorporation into the cytosol, several proteins are activated such as SIRT1, AMPK, and mTOR, depending on the metabolic state of the cell. The before mentioned proteins constitute major modulators of PGC-1α, a master regulator of mitochondrial biogenesis. We demonstrated here that leucine downregulates miR-494-3p and miR-696 in skeletal muscle, therefore promoting mitochondrial biogenesis in part through derepressing their target gene: PGC-1α. But what are the key elements for the leucine/miRNA biogenesis crosstalk? Some studies have pointed out mTOR as a direct regulator of miRNA processing machinery, through the Mdm2/Drosha axis. More recently, epigenetic regulation has emerged as a pivotal mechanism of modulating gene expression, in response to cellular stress or disease states, in virtually all cell types. Moreover, DNA methylation and histone modifications often cooperate to regulate miRNA expression, indicating the importance of HDAC in miRNA biogenesis. SIRT1: sirtuin 1; AMPK: 5' AMP-activated protein kinase; mTOR: mammalian target of rapamycin; PPARβ: peroxisome-proliferator-activated receptor β; NRF1: nuclear respiratory factor 1; ERRγβ: estrogen-related receptor γ/β.

methylation and histone modifications often cooperatively regulate miRNA expression (Wang et al., 2013; Bianchi et al., 2017). This leaves open a whole new research field of study to elucidate the regulation of miRNA biogenesis under different nutritional conditions.

There are several limitations of this study that should be addressed in future research. First, the study focused solely on the effect of leucine treatment of the miRNAs of interest to examine the hypothesized underlying regulatory mechanism. Second, the difficulties with determining the significance of miRNA changes need to be taken into consideration since each miRNA has several targets and the resulting changes are often contradictory. Herein, only two target genes, PGC-1α and TFAM were evaluated, therefore other cellular changes might have been missed. Finally, no interactions were found between leucine treatment and miRs OE, possibly due to the small sample size used.

In conclusion, leucine promoted protein synthesis through activation of the mTOR pathway, as well as mitochondrial biogenesis. Furthermore, the expression of miR-494-3p and miR-696 was downregulated after leucine treatment in C2C12 myotubes. Caffeine, on the other hand, had no effect on miRNA levels. These results suggest the presence of a leucine-specific miRNA profile. Finally, we observed that miR-494-3p and miR-696 overexpression negatively regulate PGC-1α mRNA, an effect that is partially restored by leucine treatment. The present study constitutes the first report of a nutrient, especially leucine, regulating mitochondrial biogenesis through exercise-responsive miRNAs.

VII. Summary

In the present study, we examined the effect of leucine treatment on the expression of 3 miRNAs: miR-494-3p, miR-696, and miR-761, known as exercise-responsive miRNAs. As a common target of these miRNAs is PGC-1α, thus we further investigated their impact on mitochondrial biogenesis. We initially hypothesized that leucine promotes mitochondrial biogenesis partially through the regulation of PGC-1α mediated by these miRNAs. The main purpose of the study was to elucidate the relationship between leucine treatment, the exercise-responsive miRNAs, and mitochondrial biogenesis in C2C12 myotubes. The findings of each experiment are summarized as follows.

1. Effect of leucine treatment on miRNAs (miR-494-3p, miR-696, and miR-761) expression and mitochondrial biogenesis in C2C12 myotubes (Experiment I).

This experiment corroborated the effect of leucine treatment on protein synthesis while it induced a mild increase in mitochondrial biogenesis (mtDNA content). Moreover, we observed the downregulation of miR-494-3p and/or miR-696 after treatment with leucine. These results suggest a leucine-specific mechanism to regulate miRNAs whereas promoting mitochondrial biogenesis potentially via downregulation of miR-494-3p and/or miR-696 in skeletal muscle. A positive correlation between the expression of miR-494-3p and miR-696 was found, indicating possible coordination between these miRNAs' functions. Furthermore, the data presented constitute the first report of a nutrient, especially leucine, regulating the exercise-responsive miRNAs, miR-494-3p, and miR-696.

2. Relationship between leucine, miRNA levels, and mitochondrial biogenesis in C2C12 myotubes (Experiment II).

In this experiment, the double overexpression of miR-494-3p and miR-696 resulted in decreased PGC-1α and TFAM, both at mRNA and protein levels. Furthermore, the treatment

with leucine still upregulated PGC-1α gene expression, regardless the strong negative effect of miR-494-3p and miR-696 overexpression on its levels. Gain- and loss-of-function studies corroborated the upregulation of mtDNA copy numbers following leucine treatment. These results are in correspondence with our hypothesis and support the theory that leucine effects on mitochondrial biogenesis partially involve the regulation of PGC-1α-targeting miRNAs.

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Reference Paper

Effect of leucine on microRNAs and mitochondrial biogenesis in C2C12 myotubes

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Number of Tables: 1

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Running Head: Leucine affects miRNAs on mitochondria

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Abstract

The branched-chain amino acid leucine (Leu) has been known to induce mitochondrial biogenesis through the activation of PGC-1α. Some studies suggest that miR-494-3p, miR-696, and miR-761 play a role in this process by targeting PGC-1α, although it remains unclear whether nutrient stimulation affects their expression. Therefore, our study focused on the effect of Leu on these miRNA functions and how it affected mitochondrial biogenesis. Following 5 days of differentiation, C2C12 myotubes were treated with Leu (1 or 3 mM) in Leu-free DMEM without serum for 24-h. After treatment, the cells were harvested and DNA, RNA, and whole protein fraction were isolated for immunoblotting and qPCR analyses. Myotubes treated with 3 mM Leu increased mitochondrial DNA copy number, although no changes in OXPHOS proteins were observed. In addition, Leu treatments downregulated both miR-494-3p and miR-696 levels. When miR-494-3p and miR-696 levels were overexpressed, PGC-1α and TFAM genes and protein expressions were downregulated, although Leu treatment could stimulate PGC-1α mRNA even in miRNAs overexpression conditions. Furthermore, a main effect of Leu treatment was found on mtDNA copy number, which might indicate a protective effect of this amino acid from the overexpression of miRNAs in C2C12 myotubes. These results suggest that Leu regulates mitochondrial biogenesis possibly through modulation of PGC-1α-targeting miRNAs in skeletal muscle cells.

Keywords: Leucine, miR-494, miR-696, PGC-1α, mitochondrial biogenesis

Introduction

The branched-chain amino acid leucine (Leu) has emerged as a favorite amongst athletes and recreational exercisers to promote protein synthesis and muscle growth (8). Moreover, Leu has been reported to upregulate the protein and/or gene expression of peroxisome proliferator-activated γ (PPARγ)-receptor coactivator 1 α (PGC-1α) through diverse mechanisms leading to mitochondrial biogenesis (9,22).

MicroRNAs (miRNAs) are small non-coding RNA of approximately 22 nucleotides in length that have been reported to regulate ~60% of the protein-coding genes, thereby controlling most cellular processes (3,6). They exert their biological function by binding to the 3'-untranslated region (3'-UTR) of targeted mRNA, either by inhibiting translation or promoting mRNA degradation (12). Here we focused on three miRNAs, miR-494-3p, miR-696, and miR-761 that play a part in regulating mitochondrial biogenesis of skeletal muscle, although the exact regulatory mechanism has not been elucidated (19,24,26). These miRNAs are downregulated following treadmill running and voluntary wheel exercise thus contributing to skeletal-muscle adaptations in mice (24,26). It has been proposed that miR-494-3p, miR-696, and miR-761 regulate mitochondrial biogenesis partially by direct binding to the 3'UTR of the PGC-1α gene (11,15,24). Mitochondrial transcription factor A (TFAM), Forkhead box J3 (FoxJ3), PGC-1α, mitochondrial fission factor (MFF), and members of the MAPK pathway are other validated targets of these miRNAs (2,24,26). Among these genes, PGC-1α is highly important as a key regulator of cell metabolism, mitochondrial biogenesis, antioxidant defense, and inflammatory response in skeletal muscle (13,21).

The accumulated knowledge confirms the miRNAs connection with a wide range of biological processes that demands a strict regulatory mechanism to maintain cellular homeostasis. In the research field of skeletal muscle, miRNAs are focused as biomarkers of athletic performance, physical fatigue, and the incidence risk of disease development (8).

Within the vast family of miRNAs, miR-494-3p, miR-696, and miR-761 are known as exercise-responsive miRNAs due to their typical response after physical activity. Although discovered separately, they respond to similar stimuli and share target genes, with PGC-1α as one of the most relevant, thus regulating major cellular processes including mitochondrial biogenesis, fatty acid oxidation, and apoptosis (2,16,26). This might suggest a cooperative mechanism of action among these miRNAs. However, it remains unclear whether miR-494-3p, miR-696, and miR-761 are regulated by nutrients stimulation. Therefore, the present study focused mostly on the effect of Leu treatment on these miRNAs' functions. We hypothesized that: (i) Leu regulates the expression of miR-494-3p, miR-696, and miR-761 in skeletal muscle cells and (ii) Leu promotes mitochondrial biogenesis partially through the regulation of PGC-1α mediated by these miRNAs.

Materials and Methods

Cell Culture and Leucine treatment

This study was approved by the Gene Recombination Experiment Safety Committee of Kanazawa University (approval ID: Kindai 6-2145). Mouse C2C12 myoblasts were obtained from American Type Culture Collection (ATCC, VA, USA) and cultured in Dulbecco's modified Eagle's medium (high-glucose DMEM; Gibco, MA, USA) containing 10% fetal bovine serum, 1% penicillin-streptomycin, at 37°C in 5% CO_2 air atmosphere. At 80% of confluence (defined as Day 0), the medium was changed to DMEM supplemented with 2% calf serum, 1% penicillin-streptomycin, and 1% non-essential amino acids to induce myotubes formation. After 5 days of differentiation, the myotubes were starved in Leu-free custom medium (IFP, Yamagata, Japan) without serum for 24-h before treatment with Leu (1 or 3 mM; Nacalai Tesque, Kyoto, Japan) for further 24-h (Fig. 1). The starvation condition before treatment has been widely accepted to determine the effects of nutrients and other compounds

in vitro (4,5,9). As a control, 2% H_2O was added to the culture medium. Following treatment, cells were harvested and DNA, RNA, and whole-protein fractions were isolated for immunoblotting and qPCR analyses. All experiments were performed using cells of up to 9 passages.

***** insert Figure 1 *****

Myotubes transient transfection

After 2 days of differentiation, myotubes were double transfected with 30 pmol of mmu-miR-494-3p (#AM17100, ID: PM12409) and mmu-miR-696 (#AM17100, ID: PM11434) precursor sequence using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA), according to the protocol provided by the manufacturer. For the control group, cells were transfected with the precursor control, Pre-miR Negative Control #1 (AM17110) from Ambion by Life Technologies (Carlsbad, CA, USA). Briefly, precursor miRNAs and Lipofectamine RNAiMAX reagent were diluted in Opti-MEM I Reduced Serum Medium (Gibco, MA, USA). Next, the diluted precursor miRNAs and Lipofectamine reagent were mixed (1:1 ratio) and incubated at room temperature for 10 min, before adding it to cells in differentiation medium without serum. The transfection mix was removed 24-h later and cells were grown in differentiation medium for a similar period. Forty-eight hours post-transfection, the cells were treated with or without Leu (3 mM) for 24-h and then harvested for downstream analyses.

Mitochondrial DNA copy number

Cells were collected with (D)-PBS, centrifuged at 600 *g* for 5 min and the cell pellets were immediately frozen in liquid nitrogen before being stored at -80°C. Genomic DNA was extracted using the spin-column based DNeasy Blood & Tissue Kit (QIAGEN, Mississauga, ON, Canada) and diluted in Tris-EDTA (pH 8.0) for long-term storage. Real-time PCR was

performed using ABI 7300 system with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). The following primers were used: 5´-GCCGACTAAATCAAGCAACA -3´ (forward) and 5´- CAATGGGCATAAAGCTATGG -3´ (reverse) specific for cytochrome c oxidase subunit II (COXII, mitochondrial), and 5´-GCGACCAGCCCATTGTAGA -3´ (forward) and 5´- GCGTTCTGGGTAC CATCCTAAC-3´ (reverse) specific for mitochondrial uncoupling protein 2 (UCP2, nuclear) genes (Eurofins Genomics, Tokyo, Japan). The PCR reaction was set up as follows: UDG incubation for 2 min at 50°C, initial denaturation step at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. The relative copy number of mitochondrial to nuclear DNA were determined by the comparative method $2^{-\Delta\Delta Ct}$ using UCP2 as an internal control (26).

miRNA expression

Cells were prepared using the same conditions as for DNA extraction. Total RNA was isolated using the ReliaPrepTM miRNA Cell and Tissue Miniprep System (Promega, WI, USA) according to the manufacturer's instructions and quantified with Denovix Spectrophotometer (DS-11; Wilmington, DE, USA). Then, 20-60 ng of total RNA was reverse-transcribed into cDNA with the TaqMan MiRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For miRNA expression detection, TaqMan miRNA assay kits were used (mmu-miR-494-3p: 002365, mmu-miR-696: 001628, mmu-miR-761: 002030 and snU6: 001973, Thermo Fisher, MA, USA); the real-time qPCR was performed according to the manufacturer's instructions using TaqMan Universal PCR Master Mix, no AmpErase^{TM} UNG, (Thermo Fisher). The PCR reaction was set up as follows: initial denaturation step at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C.

Quantification of mRNA Expression

Real-time PCR was performed using total RNA samples. Double-stranded cDNA was synthesized from 25 ng of total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The mRNA expression of PGC-1α and TFAM was quantified with ABI 7300 system and software (Applied Biosystems, USA), following the manufacturer's instructions using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). Forward and reverse primers for the target genes are listed in Supplementary Table 1 (Eurofins Genomics, Tokyo, Japan). Conditions for amplification were 60 s at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 60°C for primer annealing and elongation. All samples were run in duplicate and data were analyzed according to the $2^{-\Delta\Delta Ct}$ method using GAPDH as the control housekeeping gene. The purity of the amplified product was checked through analysis of the melting curve carried out at the end of amplification.

Sample preparation and immunoblotting

Cell lysates were homogenized in RIPA buffer (20 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) containing a protease- and phosphatase-inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) using a pestle homogenizer (Nippi, Tokyo, Japan). Homogenates were then sonicated for 20 s at power 5 with an Ultrasonic Disruptor (UR-21P; Tomy Seiko, Tokyo Japan) and incubated for 30 min at 4°C in a rotator. Finally, lysates were centrifuged at 10,000 *g* for 20 min at 4 °C and the supernatant was used for immunoblotting (21,27). The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Samples of the whole protein fraction were solubilized in equal volume of 2x SDS sample buffer (125 mM Tris-HCl pH 6.8, 20% Glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.002% bromophenol blue), adjusted to the final concentration of 1.5 µg protein /µl with 1x SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% Glycerol, 2% SDS,

5% 2-mercaptoethanol, 0.001% bromophenol blue), and then incubated at 95°C for 5 min. Normalized samples were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated with blocking buffer followed by incubation with primary antibodies specific for oxidative phosphorylation complexes antibody cocktail (OXPHOS, 1:100; ab110413, Abcam, Cambridge, UK), PGC-1α (1:1,000; ab54481, Abcam), TFAM (1:1,000; 22586-1-AP, Proteintech, Rosemont, IL, USA), GAPDH (1:1,000; ab8245, Abcam), and α-tubulin (1:2,000; 3873S, Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. After exposure to a secondary antibody, reaction products were visualized using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). Band densities were measured with ImageJ software (version 1.52p; NIH, MD, USA).

Statistical Analysis

Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test for differences between Leu treatment and control group. The Pearson product-moment correlation coefficient was computed to assess the relationship between variables. A two-way ANOVA (transfection and treatment) was performed to assess main vs. interaction effects of miRNAs overexpression and Leu treatment. All statistical analyses were performed using IBM SPSS Statistics 25 (IBM Japan, Tokyo, Japan). The results are expressed as mean ± SEM with a significance level of $p < 0.05$, while a trend was defined as $p < 0.1$.

Results

Treatment of fully differentiated C2C12 myotubes with 3 mM Leu tended to increase the expression of mtDNA copy number determined by the COXII/UCP2 ratio ($p = 0.058$, Fig. 2A). On the contrary, no significant changes in the expression of the oxidative phosphorylation (OXPHOS) Complexes I-V was observed (Fig. 2B and 2C). Regarding the miRNA's

expression profile, miR-494-3p levels tended to be lower in both 1- and 3-mM Leu-treated groups ($p = 0.096$ and 0.084, respectively; Fig. 3A). A similar trend was also observed in the miR-696 level, but the miR-696 expression significantly decreased in the 3 mM Leu group ($p < 0.05$). These results might suggest a nutrient-mediated expression profile. Overall, there was a strong, positive linear correlation between miR-494-3p and miR-696 levels in C2C12 myotubes ($r = 0.503$, $p < 0.05$, $n = 24$; Fig. 3B). The results summarized in Figure 3B might indicate a cooperative effect between these two miRNAs, independent of the treatment applied. On the contrary, miR-761 levels were not affected by Leu treatment.

***** insert Figure 2 and 3 *****

Two-way ANOVA indicated a significant main effect of transfection in miR-494-3p and miR-696 expressions ($p < 0.05$, Fig. 4A). The expression level of both miR-494-3p and miR-696 was significantly higher in these miRNAs-transfected cells (abbreviated as miRs OE) than in non-transfected cells ($p < 0.05$). On the contrary, there was a significant main effect of treatment on mtDNA copy number ($p < 0.05$, Fig. 4B). Compared to the (-) Leu cells, mtDNA copy number was significantly greater in Leu-treated cells ($p < 0.05$). Furthermore, two-way ANOVA revealed significant main effects of transfection and treatment in PGC-1α mRNA expression ($p < 0.05$, Fig. 4C). The mRNA expression of PGC-1α was significantly lower in miRs OE than in control cells, whereas the value in Leu-treated cells was significantly higher than in (-) Leu cells. A similar trend, except for the main effect of treatment, was observed in PGC-1α protein expression and TFAM mRNA and protein expressions (Fig. 4D-F). These results indicate that miR-494-3p and miR-696 target PGC-1α while presenting a relationship between Leu treatment, miR-494-3p and miR-696, and PGC-1α in skeletal muscle.

***** insert Figure 4 *****

Discussion

Nutrients constitute an important factor in regulating skeletal muscle functions. In the present study, we aimed to determine the effect of leucine treatment on exercise-responsive miRNAs. Before testing our hypothesis about nutrient regulation of miR-494-3p, miR-696, and miR-761 expression, we verified the effect of Leu on mitochondrial content in C2C12 myotubes. We observed that Leu increased mtDNA copy number at 3 mM dose (Fig. 2A). In correspondence with our result, a study reported that 0.5 mM Leu was sufficient to increase mitochondrial content and mitochondrial biogenesis-related gene expression of sirtuin 3 (SIRT3), PGC-1α, COXII, and COXV in C2C12 myotubes after 48-h treatment (17). Furthermore, high-fat-diet-induced mitochondrial damage was partially restored by Leu supplementation and was accompanied by increases in the mtDNA content, TFAM expression, and ATP production, confirming the protective role of Leu in skeletal muscle (9,18). In contrast, Leu treatment did not affect the expression of both nuclear- and mitochondrial-encoded proteins detected by OXPHOS (Fig. 2B and 2C). This discrepancy may be partially explained by the interactions of miRNAs with mitochondrial-biogenesis genes. Another possibility might be the Leu-treatment period. Liang *et al.* (17) reported only a 5% increase in mtDNA after 24-h treatment with 0.5 mM Leu (in normal DMEM, final concentration 1.3 mM), with this effect being 2-fold higher at 48-h. Therefore, 24-h Leu treatment may be too short to induce large changes in mitochondrial biogenesis and the level of mitochondrial-related proteins.

Recently, Leu and essential amino acids have been shown to regulate miRNA expression in cultured skeletal muscle cells (7). Since the skeletal muscle is highly sensitive to nutrient availability, the miRNA profile in this tissue might exhibit a nutrient-specific response as well. In the present study, consistent with our hypothesis, miR-494-3p and miR-696 tended to be or were downregulated by Leu treatment (Fig. 3A).

We found a positive correlation between miR-494-3p and miR-696 expression across all the study groups, independent of the treatment applied (Fig. 3B). This could suggest coordinated functions of these miRNAs in skeletal muscle, independently of the nutritional status. Therefore, we double transfected myotubes with miR-494-3p and miR-696 precursors, followed by treatment with 3 mM Leu (24-h). Contrary to our previous observation, Leu treatment did not affect these miRNA levels in miRs OE cells (Fig. 4A), which might indicate that higher concentrations of Leu are necessary to counteract the overexpression levels achieved here. In this preliminary study, we corroborated the positive effect of Leu treatment on mtDNA copy number (Fig. 4B), suggesting a protective effect of this amino acid on mitochondrial biogenesis in myotubes. This effect was accompanied by an upregulation of the PGC-1α gene after Leu treatment, regardless of the strong downregulation of this factor by miRs OE condition at both mRNA and protein level (Fig. 4C and 4E). Similar to PGC-1α expression, the miRs OE was found to decrease TFAM mRNA and protein expression, while no protective effect of Leu was observed (Fig. 4D and 4F). These facts indicate that in our experimental conditions miR-494-3p and miR-696 overexpression regulate PGC-1α and TFAM expression, both validated targets of these miRNAs (11,26).

All the evidence seems to point towards the stimulation of PGC-1α and consequently, mitochondrial biogenesis, by Leu treatment with miR-494-3p and miR-696 as possible intermediaries. Therefore, Leu might increase mitochondrial content partially through the regulation of PGC-1α-targeting miRNAs, although we can only speculate about the exact mechanism. Regarding the control of miRNA expression, diverse regulatory levels have been proposed, such as feedback loops between miRNAs and their targets, miRNA sponges, epigenetic regulation, nutrients sensing pathways, and others (27,28). However, the exact relationship between nutrients and miRNAs biogenesis in skeletal muscle has not been elucidated.

There are several limitations of this study that should be addressed in future research. First, the study focused solely on the effect of Leu treatment of the miRNAs of interest and hypothesized about the underlying regulatory mechanism. Second, the difficulties with determining the significance of miRNA changes need to be taken into consideration since each miRNA has several targets and the resulting changes are often contradictory. Finally, no interactions were found between Leu treatment and miRs OE, possibly due to the small sample size used.

In summary, 24-h of Leu supplementation (3 mM) increased mitochondrial content in C2C12 myotubes, potentially via downregulation of miR-494-3p and/or miR-696. A positive correlation between the expression of miR-494-3p and miR-696 was found, indicating possible coordination between these miRNAs. Furthermore, the data presented constitute the first report of a nutrient, especially Leu, regulating the exercise-responsive miRNAs, miR-494-3p, and miR-696. Further experiments should be performed to clarify the relationship between these miRNAs' functions and different nutritional conditions.

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Conflict of interest

The author(s) declare that there is no conflict of interest regarding the publication of this article.

Author contributions

CPL and K. Masuda conceived the research; CPL, TS, K. Morino, and K. Masuda designed the project; CPL, TS, and RK performed the experiments; CPL and RK analyzed the data; CPL, TS, K. Morino, and K. Masuda interpreted the results of the experiments; CPL prepared the figures; CPL drafted the manuscript; All authors discussed the results and commented on the manuscript; CPL, TS, K. Morino, and K. Masuda edited and revised the manuscript; All authors approved the final version of the manuscript.

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Figure legends

Figure 1. Experimental design, treatment, and sample collection.

When the cells reached 80% confluence, the medium was exchanged to induce myotube differentiation for 5 days. Next, cells were starved with serum and Leu-free medium for 24-h followed by the addition of Leu (1 or 3 mM) to the medium and incubation for another 24-h. At the end of the treatment period, cells were harvested for downstream analyses.

Figure 2. Effect of Leu supplementation on mitochondrial biogenesis.

(A) Mitochondrial DNA copy number (mtDNA) after 24-h of treatment with Leu (1 or 3 mM). The relative copy number of mitochondrial to nuclear DNA were determined by the comparative method $2^{-\Delta\Delta Ct}$ using UCP2 as an internal control. (B) Immunoblot images of oxidative phosphorylation (OXPHOS) proteins and the loading control GAPDH. (C) Expression levels of OXPHOS complexes I-V after 24-h of treatment with Leu. Data of each group are expressed as the ratio relative to the average value of the control group (1.0). Values are presented as mean ± SEM (n = 5-8/group). #: $p < 0.1$, tendency as compared with the control condition by one-way ANOVA with Dunnett's post-hoc test.

Figure 3. Fold change in miRNA expression levels after treatment with Leu.

(A) Relative expression levels of miR-494-3p, miR-696, and miR-761 after treatment with Leu. Expression levels of these miRNAs were determined by the comparative method $2^{-\Delta\Delta Ct}$ using snU6 as an internal control and expressed as the ratio against control levels. (B) Correlation between miR-494-3p and miR-696 expression levels in Control (○), 1 mM Leu (□), 3 mM Leu (■) (n = 8/group, total n = 24). Pearson's correlation coefficient was obtained across all the data, independent of the treatment applied ($r = 0.503$ and $p < 0.05$). Data of each group are expressed as the ratio relative to the average value of the control group (1.0). Values in (A) are expressed as mean ± SEM. *: $p < 0.05$, different from the control group, #: $p < 0.1$, tendency as compared with the control condition by one-way ANOVA with Dunnett's post-hoc test.

Figure 4. Effects of miR-494-3p and miR-696 overexpression in C2C12 myotubes.

(A) Expression levels of 494-3p and miR-696 were determined by the comparative method $2^{-\Delta\Delta Ct}$ using snU6 as an internal control and expressed as the ratio against control levels. (B) Mitochondrial DNA copy number (mtDNA) expressed as the ratio of mitochondrial to nuclear DNA, was determined in cells double transfected with miRNA precursors, followed by Leu treatment. (C) mRNA, and (E) protein expression levels of PGC-1α in cells double transfected with miRNA precursors, followed by Leu treatment. The arrowhead (➤) indicates the quantified band (105 kDa). (D) mRNA, and (F) protein expression levels of TFAM in cells double transfected with miRNA precursors, followed by Leu treatment. Values are expressed as mean ± SEM (n = 3/group). The main effects of miRNA overexpression (miR OE) and Leu treatment were determined by two-way ANOVA test.

Supplementary Table 1. Primer sequences for real-time PCR.

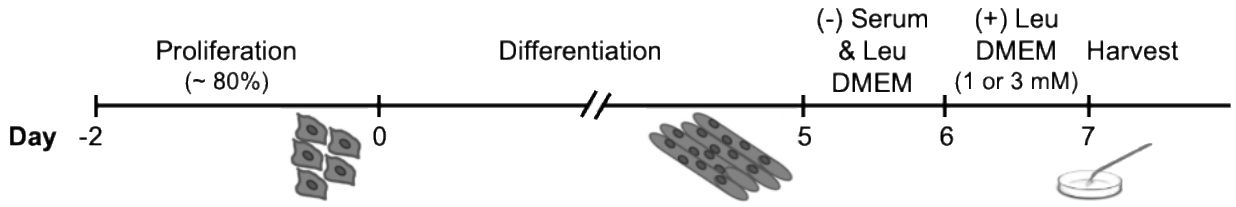

Figure 1.

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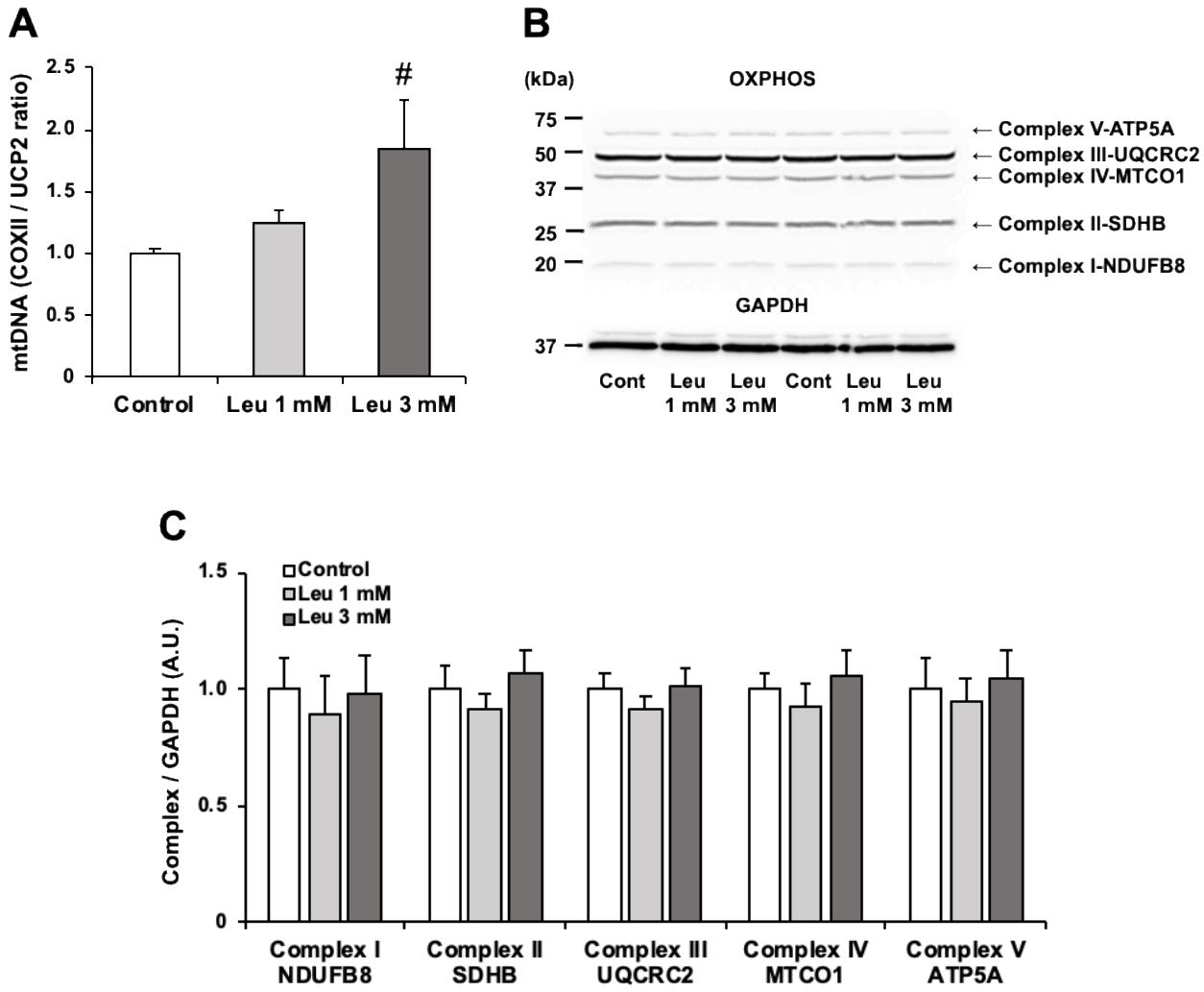

Figure 2.

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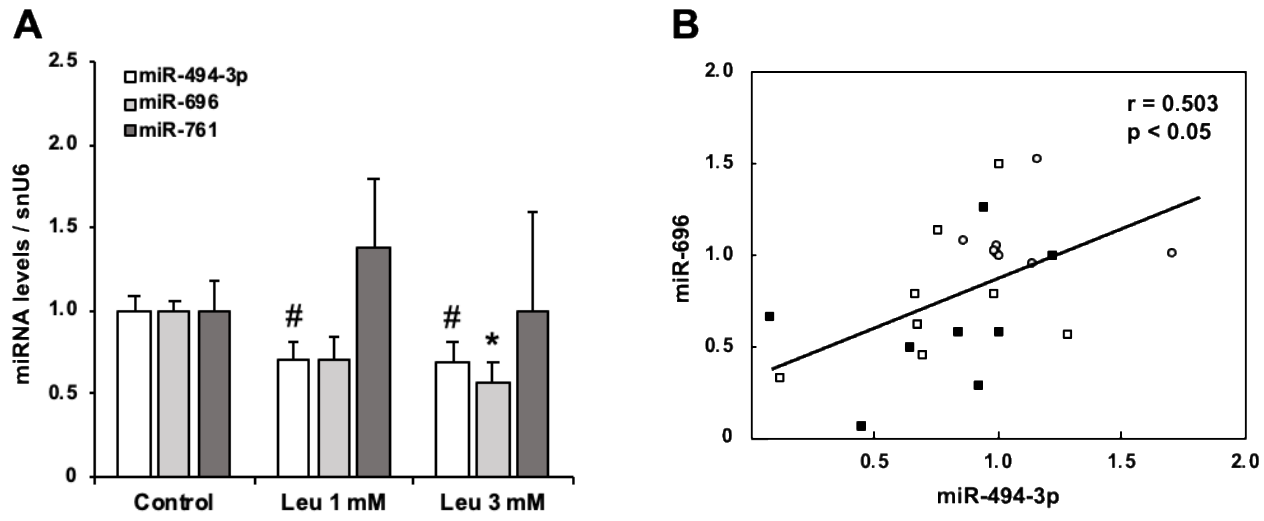

Figure 3.

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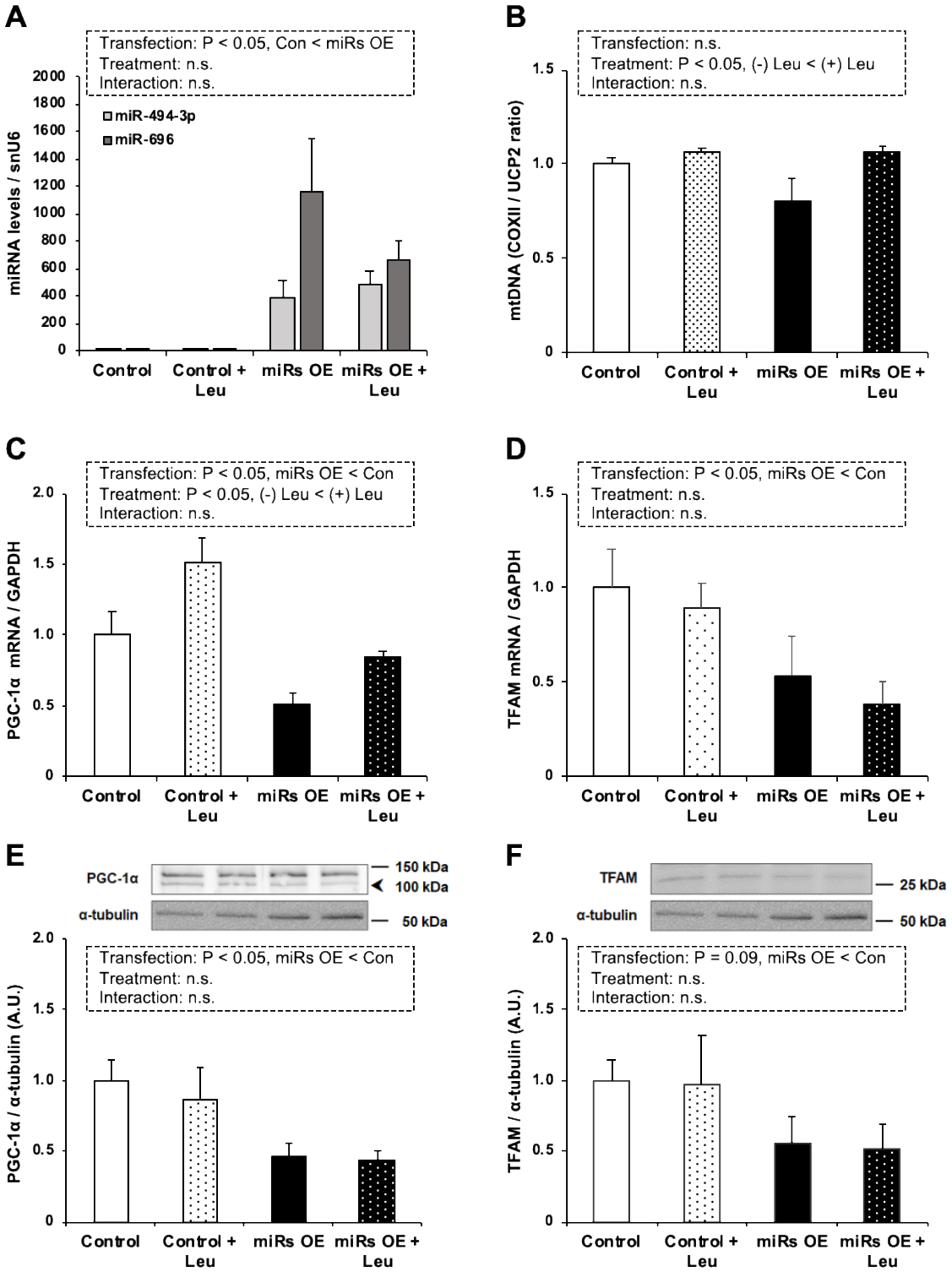

Figure 4.

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Supplementary Table 1. Primer sequences for real-time PCR.

PGC-1α	forward	AAG TGT GGA ACT CTC TGG AAC TG
	reverse	GGG TTA TCT TGG TTG GCT TTA TG
TFAM	forward	CAT TTA TGT ATC TGA AAG CTT CC
	reverse	CTC TTC CCA AGA CTT CAT TTC
GAPDH	forward	TGT GTC CGT CGT GGA TCT GA
	reverse	TTG CTG TTG AAG TCG CAG GAG

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