THE ROLE OF PGC-1α OVEREXPRESSION IN SKELETAL MUSCLE EXOSOME BIOGENESIS AND SECRETION

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CVD- Cardiovascular disease

ATP- Adenosine triphosphate

OXPHOS- Oxidative phosphorylation

EV- Extracellular vesicle

MFN1- Mitofusin 1

MFN2- Mitofusin 2

Drp1- Dynamin-related protein 1

cAMP- Cyclic adenosine monophosphate

PKA- Cyclic adenosine monophosphate-dependent protein kinase

mtDNA- Mitochondrial DNA

PGC-1α- Peroxisome-proliferator activated receptor gamma coactivator 1-alpha

NRF-1- Nuclear respiratory factor-1

NRF-2- Nuclear respiratory factor-2

ERRα- Estrogen-related receptor alpha

TFAM- Mitochondrial transcription factor A

AMPK- AMP-activated protein kinase

p38 MAPK- P38 mitogen activated protein kinase

ATF-2- Activating transcription factor-2

MEF2- Myocyte enhancer factor-2

ESCRT- Endosomal sorting complex required for transport

ILV- Intraluminal vesicle

MVB- Multivesicular body

OE- overexpression

CaP- calcium phosphate

ABSTRACT

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Skeletal muscle functions as an endocrine organ. Exosomes, small vesicles containing mRNAs, miRNAs, and proteins, are secreted from muscle cells and facilitate cell-to-cell communication. Our recent work found greater exosome release from oxidative compared to glycolytic muscle. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a key driver of mitochondrial biogenesis, a characteristic of oxidative muscle. It was hypothesized that PGC-1α regulates exosome biogenesis and secretion in skeletal muscle. The purpose of this study is to determine if PGC-1α regulates skeletal muscle exosome biogenesis and secretion. On day 4 of differentiation, human primary myotubes from vastus lateralis biopsies from lean donors (BMI < 25.0 kg/m2) were exposed to adenovirus encoding human PGC-1α or GFP control. On day 6 of differentiation, culture media was replaced with exosome-free media. On day 8, cells were collected for mRNA and protein analysis, and culture media was collected for exosome isolation. Overexpression of PGC-1α increases regulators of exosome biogenesis in the endosomal sorting complexes required for transport (ESCRT) pathway: Alix (CON: 1.0 ± 0.2 vs. PGC-1α: 7.6 ± 3.8), TSG-101 (CON: 1.0 ± 0.1 vs. PGC-1α: 7.3 ± 2.1), CD63 (CON: 1.0 ± 0.17 vs. PGC-1α: 3.7 ± 0.4), Clathrin (CON: 1.0 ± 0.2 vs. PGC-1α: 11.6 ± 2.5), and the secretion pathway: Rab27b (CON: 1.0 ± 0.3 vs. PGC-1α: 3.2 ± 0.3), STAM (CON: 1.0 ± 0.3 vs. PGC-1α: 7.3 ± 0.6), and VTA1 (CON: 1.0 ± 0.1 vs. PGC-1α: 7.3 ± 2.4). Exosome count and total extracellular vesicle count were not significantly different from control. Overexpression of PGC-1α increases gene

expression of regulators of exosome biogenesis and secretion in human primary myotubes. In the future, *in vitro* studies assessing exosomal content from PGC-1α OE cells as well as *in vivo* effects of PGC-1α OE on exosome production and release should be investigated to further understand the role PGC-1α plays in exosome secretion.

CHAPTER 1: INTRODUCTION

The systemic benefits of exercise have been extensively studied in humans. Exercise can increase cardiovascular fitness, lean muscle mass, and insulin sensitivity, as well as decrease the risk of all-cause mortality, obesity, diabetes, and cardiovascular disease. It is well established that exercise reduces the risk of cardiovascular disease (Whelton et al., 2002; Fagard & Cornelissen, 2007; Myers et al., 2002; Piepoli et al., 2004; van Tol et al., 2006). Cardiovascular disease (CVD) is a broad term that includes a variety of conditions such as coronary artery disease, hypertension, hyperlipidemia, congestive heart failure, valvular dysfunction, and arrythmias. Systematic reviews by Taylor and Heran established that a cardiac rehabilitation exercise program reduced all-cause and cardiovascular mortality and reduced total cholesterol levels, triglycerides, and systolic blood pressure in patients with previous myocardial infarction, coronary artery bypass graft, or percutaneous transluminal coronary angioplasty (Heran et al., 2011; R. S. Taylor et al., 2004). Regular physical training leads to greater reductions in body weight and BMI compared to diet alone, without loss of muscle mass from dieting (Shaw et al., 2006). Consistent exercise protects subjects who previously lost weight from gaining said weight back as well. A meta-analysis of six studies interested in the effects of exercise and diet on weight loss maintenance found that the groups that exercised more frequently had significantly greater weight loss maintenance than those who exercised less frequently (Anderson et al., 2001). Kraus et al. observed a significant reduction in low-density lipoprotein and intermediate-density lipoprotein and an increase in high-density lipoprotein following 8 months of high volume, high intensity exercise training in physically inactive, overweight men (Kraus et al., 2002). The importance of exercise in reducing the risk of developing chronic diseases like cardiovascular disease, obesity, and diabetes and maintaining overall health cannot be overstated.

Skeletal muscle accounts for ~40% of total body mass in humans and plays many roles in human physiology (Janssen et al., 2000). Certain organs, such as the brain, liver, and kidneys, remain relatively stable in their metabolic rates independent of environmental stressors throughout the day. Skeletal muscle metabolism, however, has the ability to fluctuate greatly depending on external stimuli. Zurlo et al. demonstrated that resting forearm oxygen uptake, a measurement of muscle metabolism, is related to resting metabolic rate and sleeping metabolic rate in healthy, young men (Zurlo et al., 1990). While the resting metabolic rate of skeletal muscle is relatively low compared to other organs, ~20-30% of total metabolism at rest is due to the sheer volume of skeletal muscle. During exercise, muscle metabolism can increase tremendously. At maximal intensities, muscle oxygen consumption may reach up to 90% of total body oxygen intake (Zurlo et al., 1990). During prolonged, submaximal bouts of exercise, skeletal muscle primarily uses oxidative phosphorylation for adenosine triphosphate (ATP) generation (Hargreaves, 2000). Oxidative phosphorylation (OXPHOS) uses stored glycogen, free fatty acids, and blood glucose as energy substrates. Energy metabolism varies greatly depending on energy demands during rest or exercise, and skeletal muscle is crucial for substrate metabolism and metabolic rate.

Recently, attention has been brought to a variety of extracellular vesicles and their role in cell-to-cell communication. Extracellular vesicles (EVs) are defined as any extracellular structures with a lipid bilayer. A variety of cell types, including adipocytes, skeletal muscle fibers, and cancer cells, as well as plant cells and bacteria, can release extracellular vesicles. Typically, EVs are between 100-1000 nm, while a subset of vesicles 30-150 nm in size are termed exosomes. For the purpose of this proposal only exosomes will be discussed. The exosome response to exercise, as well as their specific cargo and its impact once received by a

cell, are key questions regarding their importance in exercise physiology. Rigamonti et al. measured total exosome count from plasma following moderate-intensity exercise (60% VO_2 max) in normal and obese men and women. The study sought to identify if differences in exosome count were present in a tissue-, sex-, and BMI-related manner post-exercise. Total exosome count was significantly lower in women versus men, while significant increases in exosome count were seen post-exercise in all groups (Rigamonti et al., 2019). Exosomes have the potential to be a novel therapeutic approach to disease treatment. Chronic kidney disease reduces the expression of miR-29, a microRNA important in prevention of skeletal muscle atrophy and kidney fibrosis. When exosomes containing miR-29 were injected intramuscularly, significant decreases in muscle atrophy and kidney fibrosis were observed via Ying Yang 1 and transforming growth factor beta, respectively (H. Wang et al., 2019). Exosomes have the ability to directly influence cellular processes and have the potential to deliver therapeutic interventions in the future.

1.1-Oxidative Skeletal Muscle

Skeletal muscle is highly plastic, capable of adapting to various mechanical and neurological stimuli. Adaptations in stimuli can lead to remodeling of myofibrillar components, rebuilding intracellular signaling pathways (Hargreaves, 2000), mitochondrial biogenesis (D. Hood, 2009); Z. P. Chen et al., 2000; Dolmetsch, Lewis, Goodnow, & Healy, 1997), protein synthesis (Biolo et al., 1995), myofiber nuclei (Lee et al., 2018), and myofiber-capillary density (Cocks & Wagenmakers, 2016). Human skeletal muscle is composed of a heterogeneous mix of fiber types which are classified by the predominant isoform of myosin heavy-chain present (Herbison et al., 1982). Type I fibers, also known as slow-twitch fibers, are highly oxidative in nature, high in mitochondrial content, and resistant to fatigue. Type II fibers, or fast-twitch, are

primarily glycolytic. Type II fibers display a greater capacity for strength and power, while also fatiguing quicker than their Type I counterparts. Two subcategories of Type II fibers exist in humans, Types IIa and IIx. IIx fibers produce more peak tension and peak power than IIa and I fibers (Widrick et al., 1996). A portion of fibers contain characteristics of both Type I and II fibers and are categorized as "hybrid" fibers. The fiber type composition of specific muscles is dependent on many variables including training, health, obesity, and genetics.

Skeletal muscle is highly plastic in nature. Fiber type shifts occur with specific exercise training regimens (Staron, Hikida, Hagerman, Dudley, & Murray, 1984; Tesch & Karlsson, 1985). One study demonstrated the percentage of slow-twitch fibers in untrained males and females to be 52.6% and 51.0%, respectively (Costill et al., 1976). Costill observed fiber type composition in a variety of different athlete populations as well. Male and female sprinters had the lowest slow-twitch fiber percentage (24.0 and 27.4%), while distance runners had the highest percentage (69.4% in males). Esbjörnsson, et al. demonstrated that 6 weeks of high intensity sprint cycling shifted fiber type composition from 45 to 38% Type I and 40 to 52% Type II fibers in trained men (Esbjörnsson et al., 1993). Sixteen weeks of resistance training in obese men resulted in a significant increase in Type IIx fibers (23 to 42%) (Stuart et al., 2017). Fiber type shifting can occur in the elderly as well. Following maximal strength training, a significant increase in Type II fibers was observed along with hypertrophy of those same fibers (E. Wang et al., 2017). These data demonstrate the ability of skeletal muscle to adapt to the demands placed on it during training.

1.2-Skeletal Muscle Mitochondria

During exercise, skeletal muscle is one of the most metabolically active tissues in the body. Mitochondria serve as the primary producers of ATP necessary for skeletal muscle

metabolism and function. Two different mitochondrial populations exist in skeletal muscle: intermyofibrillar mitochondria and subsarcolemmal mitochondria (Palmer et al., 1977). Subsarcolemmal mitochondria appear to be involved in cell signaling, while intermyofibrillar mitochondria are energy generators for muscle contraction capable of 1.4-1.7 times the oxidative capacity of SS mitochondria (Palmer et al., 1977). Skeletal muscle mitochondrial function and preservation is critically important during normal aging and certain disease states. (D. Hood, 2009). Considerable evidence supports the idea that mitochondrial content and function decrease following disuse, which can occur during bedrest (Buso et al., 2019; Ringholm et al., 2011), denervation (Wicks & Hood, 1991), limb immobilization (Min et al., 2011; Abadi et al., 2009), or even space flight (Ohira et al., 1996). Loss of mitochondrial content and function can occur in pulmonary disease, heart failure, cancer, and Type 2 diabetes (Gururaja Rao, 2017; Zhou & Tian, 2018; Lowell & Shulman, 2005; Prakash, Pabelick, & Sieck, 2017). Substantial evidence supports the idea that mitochondrial loss and dysfunction leads to skeletal muscle mass atrophy (Brown et al., 2017; Liu et al., 2016). Loss of metabolic regulation can lead to detriments in muscle function and mass along with many other negative health outcomes.

Mitochondrial maintenance is a product of the balance between muscle protein synthesis and protein degradation. Regular maintenance of mitochondria involves an autophagic process termed “mitophagy” to remove dysfunctional organelles. Mitophagy is the product of mitochondrial fusion and fission. The fusion and fission of mitochondria regulates mitochondrial content in the cell. Fusion, mitochondrial joining and elongation, is primarily regulated by the proteins mitofusin 1 and 2 (MFN1 and MFN2) and optic atrophy 1 (B. S. Gordon et al., 2013; Russell, Foletta, Snow, & Wadley, 2014). Conversely, fission leads to the disruption of mitochondrial membranes and degradation of dysfunctional mitochondria via dynamin related-

protein 1 (Drp1) (Imoto et al., 1998). If mitochondrial protein degradation exceeds mitochondrial protein synthesis, a net loss in mitochondrial protein content occurs.

Cellular mitochondrial content can be manipulated *in vitro* including: electrical pulse stimulation (EPS), intracellular calcium increase, and activation of exercise-associated pathways (Di Benedetto et al., 2014; Sparks et al., 2011). Sparks et al. used pulsed forskolin and ionomycin (PFI) to increase mitochondrial biogenesis and fusion in human primary myotubes. Forskolin activates cyclic adenosine monophosphate (cAMP) and cyclic adenosine monophosphate-dependent protein kinase (PKA) (Alasbahi & Melzig, 2012). PKA regulates mitochondrial fission through the phosphorylation of Drp1 at Ser 656 (Cribbs & Strack, 2007). Knockdown of ChChd3, a PKA substrate, resulted in fragmentation of mitochondria and reduced function in HeLa cells (Darshi et al., 2011). Along with cAMP and PKA, changes in intracellular calcium concentration can induce mitochondrial changes as well. While calcium concentration remains relatively stable at rest, usually 50-100 nM in cytoplasm, during contraction concentrations can rise beyond 100 nM (Rizzuto & Pozzan, 2006; Endo, 2009). Ionomycin, a calcium ionophore, increases calcium efflux in skeletal muscle cells *in vitro*. Increases in calcium flux can activate phosphatases such as calcineurin, leading to downstream changes in gene transcription. Increasing cytosolic calcium in human primary myotubes led to increases in the expression of PGC-1α, NRF-1, NRF-2, and TFAM, all crucial proteins for mitochondrial biogenesis (Ojuka et al., 2003a). A-23187, another calcium ionophore, increased cytochrome c transcription in a concentration and time-dependent manor (Freyssenet, Di Carlo, et al., 1999). By increasing cAMP/PKA signaling and intracellular calcium, skeletal muscle mitochondrial biogenesis can be increased *in vitro* (Sparks et al., 2011).

1.3-PGC-1α in Skeletal Muscle

Regulation of gene expression and protein synthesis of nuclear and mitochondrial genes is another key component to retaining mitochondrial content in aging, disease, and disuse. Mitochondria are unique in that there is a coordinated relationship between nuclear and mitochondrial encoded mRNAs and proteins. Mitochondrial DNA (mtDNA), while miniscule compared to nuclear DNA, contains 16,659 nucleotides and codes for 13 mRNAs, 22 tRNAs, and 2 rRNAs. (D. A. Hood, 2001). All 13 proteins encoded in the mtDNA are a part of the electron transport chain necessary for oxidative metabolism.

The primary driver of mitochondrial biogenesis is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). PGC-1α is a transcriptional coactivator of a number of mitochondrial and nuclear genes, and is known as a "mitochondrial master regulator". PGC-1α is highly expressed in metabolically active tissues such as the brain, heart, liver, and skeletal muscle (P. Puigserver et al., 1998). Specifically, PGC-1α content is greater in Type I fibers versus Type II and controls the formation of Type I fibers (Lin et al., 2002). PGC-1α targets genes crucial for mitochondrial biogenesis such as nuclear respiratory factor-1 and -2 (NRF-1 and NRF-2) and estrogen-related receptor alpha (ERRα) for transcription. The NRFs are key mitochondrial transcription factors that target mitochondrial genes such as mitochondrial transcription factor A (TFAM), a transcription factor that translocates to the mitochondria to promote replication and transcription of mitochondrial DNA, and cytochrome c oxidase subunit IV, a key enzyme in the electron transport chain (Prakash, Pabelick, & Sieck, 2017; Pere Puigserver & Spiegelman, 2003). ERRα is a transcriptional coactivator capable of translocating to the mitochondria to increase transcription of mtDNA as well as nuclear encoded genes

(Ranhotra, 2015). Inhibition of PGC-1α leads to a reduction in activation of genes controlling production of mitochondrial proteins and transcription of mitochondrial DNA (mtDNA).

Chronic endurance exercise training leads to alterations in a number of signaling pathways, most notably those regulated by calcineurin, AMP-activated protein kinase (AMPK), and p38 mitogen activated protein kinase (p38 MAPK) (Dolmetsch, Lewis, Goodnow, & Healy, 1997; Fujii et al., 2000). Calcineurin is a calcium-activated protein involved in the upregulation of several Type I fiber-associated genes like myoglobin and troponin-1 slow (Bassel-Duby & Olson, 2006). Calcineurin Aα and Aβ knockout mice had reductions in Type I fiber content of the soleus muscle (Parsons et al., 2003). Calcineurin also modulates PGC-1α activity via myocyte enhancer factor 2 (MEF2) (Czubryt et al., 2003). AMP-activated protein kinase (AMPK), a protein kinase involved in the maintenance of cellular metabolism and energy homeostasis, becomes activated during exercise (Chen et al., 2000; Fujii et al., 2000). AMPK subunit activation following exercise is fiber type-dependent (Kristensen et al., 2015) and PGC-1α is directly phosphorylated by AMPK (Kristensen et al., 2015; Jäger, Handschin, St-Pierre, & Spiegelman, 2007). Furthermore, administration of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside for 14 days significantly increased PGC-1α protein in rat soleus and EDL (Suwa et al., 2003). PGC-1α translocation to the subsarcolemmal mitochondria is an AMPK-dependent process as well (Smith et al., 2013). p38 MAP kinase directly phosphorylates activating transcription factor-2 (ATF-2) and myocyte enhancer factor-2 (MEF2). ATF-2 targets PGC-1α for phosphorylation, leading to an increase in PGC-1α activity (Wright et al., 2007) and MEF2 was found to be a substrate for p38 MAPK (Zhou & Tian, 2018). Overexpression of a negative form of p38γ MAPK in mice reduced PGC-1α mRNA expression (Pogozelski et al., 2009).

Thus, exercise-regulated protein kinases calcineurin, AMPK, and p38 MAPK are crucial for the transcription and activation of PGC-1α in skeletal muscle.

PGC-1α increases following both acute and chronic bouts of exercise. Both PGC-1α mRNA and protein significantly increase post exercise in animals and humans (Taylor et al., 2005; Baar et al., 2002). Endurance and resistance exercise, treadmill walking at 80% HR max, and 10x10 RM leg press respectively, upregulated PGC-1α in physically active young men (Silvennoinen et al., 2015). Transgenic mice overexpressing PGC-1α displayed elevated mitochondrial content and Type I fibers (Lin et al., 2002). Oppositely, reduced endurance capacity, Type I fibers, and mitochondrial gene expression were observed in PGC-1α muscle-specific knockout mice (Handschin et al., 2007). In skeletal muscle, many of the mitochondrial adaptations observed during exercise training are dependent on PGC-1α.

1.4-Exosomes

The first record of what are now referred to as exosomes was published in 1983 by Harding, et al. In the study, rat reticulocytes were treated *in vitro* with radioactive-labeled ^{125}I-transferrin in the cell culture medium (Harding et al., 1983). After a 35-minute incubation period, culture medium was removed and replaced with medium containing unlabeled transferrin. It was observed that radioactivity increased in the medium following this incubation period and that ^{125}I-transferrin remained biochemically viable. Harding et al. concluded that there was some process for packaging and expelling specific molecules in the cell. Pan, et al. used electron microscopy to image the entire endo/exocytosis phenomena in sheep reticulocytes and found the reticulocytes secrete gold labelled exosomes after the multivesicular body fuses with the plasma membrane (Pan et al., 1985).

Since Harding's breakthrough study, exosomes have been discovered in a growing number of cell types, each with various functions in cell-to-cell communication (Shyong, Chang, & Lin, 2018; Dautova et al., 2018; Savina, Furlán, Vidal, & Colombo, 2003). Exosomes are small, nanosized particles (50-150nm), secreted from cells that contain proteins, mRNAs, and miRNAs (Pap et al., 2009). Exosomes have the potential to be taken up by tissues and have a direct influence on recipient cell physiology (Andre et al., 2002). Three methods exist for the incorporation of exosomes into a cell: 1) endocytosis of exosomes into the cytosol, 2) fusion to the plasma membrane of recipient cell, and 3) internalization of exosomes or stimulation of internal signaling pathways via ligands (Chang & Wang, 2019). When isolated from a mouse mast cell line and used to treat human mast cells, exosomes led to the production of proteins not found previously in HMC-1 cells (Valadi et al., 2007). Isolated exosomes from human mast cells were shown to contain approximately 1,300 unique mRNAs (Valadi et al., 2007).

1.5-Exosome Biogenesis and Secretion

The precise mechanisms of exosome secretion remain unknown; however, recent work has begun to elucidate some of the machinery controlling exosome release. Once plasma membrane components, ligands, or other molecules make it past the plasma membrane they are transported to early endosomes (Stuffers et al., 2009a). Endosomes sort through the contents received to either be recycled back to the plasma membrane or degraded via lysosomes. The sorting of these contents was observed to be regulated via the endosomal sorting complex required for transport, also known as the endosomal sorting complex required for transport (ESCRT) protein group (Stuffers et al., 2009a). This machinery is comprised of four protein

complexes: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, along with VPS4, VTA1, and Alix (Colombo et al., 2013).

Interest in the ESCRT pathway has increased greatly following its discovery in 2001 (McDonald & Martin-Serrano, 2009). The ESCRT pathway plays a key role in intraluminal vesicle (ILV) and multivesicular body (MVB) biogenesis. When biomolecules reach the endosome to be sorted, the endosome invaginates and becomes an internal compartment for its contents (Hurley & Emr, 2006). Packaged contents sorted into the MVB are referred to as intralumenal vesicles (ILVs); although once the MVB fuses with the plasma membrane and releases its contents, the ILVs are called exosomes. There are many components of the ESCRT pathway involved in exosome secretion. The ESCRT-0 protein complex is responsible for identification and sorting of ubiquitinated proteins into the endosome. When a plasma membrane protein reaches the MVB surface to be sorted, ESCRT-0, along with clathrin and ubiquitin ligases deliver it into the MVB. ESCRT-0 in humans is comprised of HRS, STAM1, and STAM2. Downstream of ESCRT-0, ESCRT-1 and ESCRT-2 form a supercomplex comprised of TSG-101, VSPS28, VSP37 (A, B, C, and D), and MVB12a in ESCRT-1, while ESCRT-II is made up by VPS22, VPS25, and VPS36. Once sorted, ESCRT-I and ESCRT-II complexes facilitate budding of the limiting membrane of the MVB into the lumen along with the ubiquitinated cargo. Exosomes form as a result of the budding of the internal vesicles in multivesicular bodies (Colombo et al., 2013). Silencing genes in the ESCRT-0 complex (HRS, STAM1) and ESCRT-1 complex (TSG-101) significantly altered exosome secretion in HeLa-CIITA cells (Colombo et al., 2013). ESCRT-III then severs the inward bud of the membrane following ESCRT-I and II. Finally, VPS4-ATPase cleaves different components, primarily ESCRT-III, from the membrane resulting in ILVs (Bittel & Jaiswal, 2019). Additionally, Alix,

an ESCRT accessory protein, serves multiple functions in exosome biogenesis. Namely, Alix assists in the recruitment of ESCRT-III for cleaving of the MVB membrane (Hurley & Hanson, 2010). Alix also functions to bring in deubiquitinating enzymes, targeting proteins on course for degradation and removing their ubiquitin tags, leading to secretion instead. Using this multi-step process, the fusion of multivesicular bodies to the cell plasma membrane leads to exocytosis of contents, namely exosomes (Denzer et al., 2000).

1.6-Skeletal Muscle Exosomes

Previous studies have shown that skeletal muscle, like a variety of other cell types, has the ability to package and secrete exosomes (Guescini et al., 2010; Nie et al., 2019). Skeletal muscle exosomes were first isolated from murine skeletal muscle cells (Guescini et al., 2010a). C2C12 myoblasts grown in culture secrete exosomes into the cell culture media. Following the ultracentrifugation of the conditioned media, exosomes could be pelleted and isolated for proteomic and genomic analysis. The presence of known exosome markers TSG-101 and Alix provided evidence that these isolated vesicles were exosomes. Along with myoblasts, which are immature muscle stem cells, differentiated human myotubes have also been shown to secrete exosomes *in vitro* (Romancino et al., 2013a). Exosomes are vesicles that facilitate intercellular crosstalk. Packaging of specific biologically-active molecules has been demonstrated in exosomes from C2C12 myotubes. miR-133b, miR-1, and miR-206 expression are highest in C2C12 cells and exosomes during differentiation, indicating that cells may be exporting unwanted proteins during terminal differentiation to reduce their intracellular concentrations (Forterre et al., 2014a). Additionally, exosomes isolated from C2C12 myotubes contained miRNAs capable of silencing Sirt1, an inducer of cell differentiation, indicating these exosomes might play a role in inducing differentiation in nearby immature myoblasts. (Forterre et al.,

2014a). Isolated exosomes from muscle derived fibroblasts increased fibrotic mRNAs in skeletal muscle (Zanotti et al., 2018a). Additionally, myotubes treated with the inflammatory cytokines TNF-α and INF-γ released exosomes that increased muscle atrophy-related mRNAs in myoblasts (Kim et al., 2018a).

A number of treatments can increase exosome biogenesis and secretion. Under various circumstances endoplasmic reticulum (ER) homeostasis can be challenged, resulting in an accumulation of abnormal proteins. Increasing ER stress significantly increased MVB count and exosome release via downregulation of the ER stress transducers inositol required enzyme 1 and PKR-like ER kinase (Kanemoto et al., 2016). Adiponectin, a protein synthesized by adipocytes only, significantly increases exosome biogenesis and release as well. Adiponectin is capable of accumulating in cells with adiponectin/T-cadherin systems such as cardiac muscle, vascular endothelium, and skeletal muscle. Treatment using adiponectin significantly increased exosome production and release *in vitro* and *in vivo* using mouse models (Obata et al., 2018). Intracellular calcium concentration increases exosome release in a variety of cell types including RAW264.7 cells, HeLa cells, human vascular endothelium cells, and skeletal muscle (Shyong et al., 2018; Dautova et al., 2018; Matsuzaka et al., 2016; Savina et al., 2003). In RAW264.7 cells, treatment with calcium phosphate significantly increased exosome secretion by over two-fold (Shyong et al., 2018). Treatment using monensin, a calcium ionophore, significantly increased the size of MVBs and quantity of exosomes released into the conditioned medium in murine skeletal muscle cells (Matsuzaka et al., 2016). Monensin, along with caffeine, a ryanodine receptor activator, ameliorated the effects of GW4869, an inhibitor of exosome secretion. Recently, our lab has found that oxidative muscle releases more exosomes than glycolytic muscle. Exosomes isolated from the soleus and red portions of the gastrocnemius and tibialis anterior exhibited greater

acetylcholinesterase activity, an assay used for exosome quantification, compared to exosomes from extensor digitorum longus and white portions of the gastrocnemius and tibialis anterior (Nie et al., 2019a). The mechanisms behind these differences in exosome secretion from oxidative and glycolytic muscle have yet to be discovered.

1.7-Summary

Skeletal muscle is a highly plastic and actively metabolic tissue that comprises ~40% of body mass. Oxidative muscle fibers are characterized by an elevated mitochondrial content, oxidative metabolism, and endurance capacity. Exercise increases the activity of a number of kinases involved in energy metabolism as well as intracellular calcium flux. Exercise results in a robust increase in PGC-1α expression. PGC-1α is a prominent transcriptional coactivator and is known as a "master regulator of mitochondria". Overexpressing PGC-1α has been shown to increase mitochondrial content and oxidative capacity *in vivo* and *in vitro* and shift skeletal muscle to an oxidative phenotype. Skeletal muscle functions as an endocrine organ by secreting exosomes which are small vesicles capable of influencing cellular physiology through mRNAs, miRNAs, and proteins. Recently our laboratory demonstrated that oxidative muscle secretes more exosomes than glycolytic muscle. The mechanisms behind these findings have yet to be elucidated and require further investigation.

1.8-Aims

To determine if: (1) skeletal muscle mitochondria regulate exosome biogenesis and release; and (2) if skeletal muscle exosome biogenesis and release are PGC-1α dependent.

1.9-Hypothesis

Overexpression of PGC-1α increases exosome biogenesis and number of secreted exosomes in human primary myotubes.

CHAPTER 2: METHODS

2.1-Skeletal Muscle Cell Culture

Human skeletal muscle satellite cells were obtained previously from muscle biopsies of lean subjects. Muscle biopsies of the vastus lateralis were collected using the Bergstrom technique. Following the procedure, collected muscle tissue was transported in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, MO) to a sterile cell culture hood. Muscle tissue was minced into small pieces and resuspended using a dispase and collagenase mixture. Cell media was then centrifuged, and the cell pellet resuspended in Skeletal Muscle Growth Medium (SKGM) (Cell Applications Inc., San Diego, CA). Cells were grown on 10 cm culture dishes until reaching 80% confluence. Cells were detached, pelleted, and resuspended in a mixture of 90% Fetal Bovine Serum (FBS) (Atlanta Biologics, Flowery Branch, GA) and 10% DMSO (Thermo Fisher Scientific), and aliquoted in cryotubes for long term storage. The cryotubes were placed in an isopropanol containing freezing container (Thermo Fisher Scientific) and placed in a -80°C freezer for 24 hours before being transferred to a liquid nitrogen container for long term storage.

2.2-Adenovirus Amplification

Adenovirus tagged with either green fluorescent protein (GFP) or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) were used for the following cell culture experiments (Huang et al., 2017). Adenovirus amplification was conducted accordingly. Briefly, HEK 293A cells were grown to 80-90% confluence on a 15 cm dish. 2.5 µl crude virus was added to DMEM containing 5% FBS and a 1% mixture of penicillin and streptomycin. Cells were incubated at 37°C and 5% CO_2 to allow the adenovirus to amplify.

Light microscopy was used to confirm viral transfection. Transfected cells display cytopathic effect (CPE) which presents as cells balling up and detaching from the plate. Upon reaching 50% of cells with CPE, cells were pelleted at 170 × *g* for 5 minutes and washed with phosphate buffered saline (PBS), spun and washed again, and then resuspended in 1 ml of PBS. Cells were then frozen completely in a dry ice and ethanol bath, thawed completely in 37°C water bath, and vortexed for 30 seconds. This freeze-thaw cycle was repeated 3 more times. Finally, cells were spun at 3,500 × *g* for 10 minutes at room temperature to pellet cell debris. The supernatant following centrifugation was the crude adenovirus preparation.

The crude adenovirus preparation was used to transfect 25 – 15 cm dishes of HEK 293A cells once the dishes reach 80-90% confluence. Briefly, 800 µl crude virus was added to 500 ml DMEM with 50 ml FBS and 5 ml penicillin/streptomycin mixture. 22 ml of the medium-virus mixture was added to each 15 cm dish. After 2-3 days, the freeze-thaw protocol described above was repeated. Following the first centrifugation, cells were resuspended in 5 ml PBS. Volumes from three 50 ml tubes were mixed (four 50 ml tubes total), centrifuged and the supernatant aspirated off. The cell pellet was resuspended in 8 ml PBS, transferred to the next tube, next pellet resuspended, etc. Approximately 9 ml of cell suspension in a 50 ml tube remained. This suspension was transferred to a 15 ml tube and four repeat freeze-thaw cycles were performed as described previously. Finally, the 15 ml tube was centrifuged at 3,500 × *g* for 15 minutes. The supernatant contained amplified crude adenovirus containing either GFP or PGC-1α. The supernatant was transferred to a new 15 ml tube for long term storage at -80°C.

To determine viral titer, an end-point dilution assay was performed. Briefly, HEK 293A cells were grown to 70% confluence on two 96-well plates, one for GFP and one for PGC-1α. Serial dilutions of crude adenovirus were mixed by transferring 120 µl diluted virus to 1080 µl

medium. Dilutions ranging from $1:10^{-6}$ to $1:10^{-13}$ were used. Following preparation of dilutions, 100 µl diluted virus was added to each well in columns 1-10 along with 100 µl virus-free medium. 200 µl virus-free medium was added to columns 11-12. These wells served as controls. Transfected plates were incubated for 10 days at 37°C and 5% CO_2. CPE was assessed using light microscopy. The wells in each dilution row should be checked for CPE, and the number of positive CPE wells expressed as a fraction out of 10. Viral titer was calculated using the following equation:

Titer (pfu/ml) = $10^{1+Z\,(x\,-\,0.5)}$, where

Z = Log(10) of the dilution

X = the sum of the fractions of CPE-positive wells.

The viral titer must meet three criteria in order to be considered reliable: 1) control wells show free of CPE, 2) wells with lowest viral dilution are all CPE-positive, and 3) wells with highest viral dilution are all CPE-negative. The viral titer used for the following experiments was 1.0 x 10^7 pfu/ml for both GFP and PGC-1α based on preliminary data confirming adequate transfection.

2.3-Skeletal Muscle Adenovirus Treatment

Human primary myoblasts from six subjects were grown in SKGM. Cells from passages 3 to 5 were used, and cells were passaged at 80-90% confluence. Upon reaching 80-90% confluence, cell medium was switched to differentiation medium comprised of DMEM with 2% FBS and 1% penicillin and streptomycin. On day 4 of differentiation, cell medium was switched to either control medium (virus free), GFP-Adenovirus (GFP-Ad) differentiation medium, or PGC-1α-Adenovirus (PGC-1α-Ad) differentiation medium. Cells were incubated for two days

following transfection. On day 6 of differentiation, cell medium was aspirated off, washed twice with sterile PBS, and switched to exosome-free differentiation medium of DMEM containing 2% exosome-free Horse Serum (HS). On day 8 of differentiation, conditioned medium was collected from wells, and exosomes were isolated using ExoQuick-TC^{TM} Exosome Precipitation Solution (ExoQuick) (System Biosciences, Palo Alto, CA). Cell lysates were collected for mRNA and protein analysis.

2.4-Exosome Isolation

Exosomes from cultured human myotubes on day 8 of differentiation were isolated using ExoQuick solution. Briefly, conditioned medium was collected in 15 ml tubes. Conditioned medium was spun at 3000 × *g* for 15 minutes to remove cell debris. Following centrifugation, ExoQuick solution was added to conditioned medium at a 5:1 dilution of culture medium to ExoQuick. Solution was pipetted up and down thoroughly to mix into medium. The conditioned medium was refrigerated at 4°C overnight to precipitate exosomes. The following day 15 ml tubes were spun at 1,500 × *g* for 30 minutes to pellet exosomes. Conditioned medium was aspirated off, leaving the exosome pellet. The exosome pellet was spun a second time at 1,500 × *g* for 5 minutes. The remaining conditioned medium should be aspirated off, and the exosome pellet resuspended in 1 ml PBS for long term storage at -80°C.

2.5-Exosome Quantification

Skeletal muscle exosome quantification was conducted using a Malvern Panalytical Nanosight LM10 (Malvern Panalytical, Almelo, Netherlands). Briefly, isolated exosomes were resuspended in 1 ml PBS. Samples were drawn up into a syringe and slowly injected into the

Nanosight apparatus. Nanosight software and laser were turned on. A 20x magnification microscope was used to visualize particles. The microscope was focused onto the particles in the sample, using the blue laser as a reference point. The Nanosight camera was then autofocused using the Nanosight software. Once focused, four 30 seconds videos of the sample were captured. The Nanosight software uses laser light scattering and Brownian motion to quantify nanoparticle size and quantity. The Stokes-Einstein equation was used to calculate particle diameter. Size ranges and quantities for exosomes were measured.

2.6-RNA Isolation, Reverse Transcription, and real-time PCR

Total RNA was extracted using Trizol according to the manufacturer's instructions (Thermo Fisher Scientific). Briefly, 1 ml Trizol was added to each well and pipetted up and down several times to lyse cells thoroughly. Cells homogenates were collected in 1.5 ml tubes. 200 ul chloroform was then added to the Trizol solution and vigorously shaken for 30 seconds. Samples were centrifuged and the supernatant layer removed. Isopropanol was then added to the supernatant and centrifuged to pellet total RNA. The RNA pellet was washed twice with 100% ethanol to remove impurities and resuspended in 30 ul RNAase-free UltraPure distilled water (Invitrogen, Carlsbad, CA). Total RNA concentration was measured via Nanodrop 2000c (Thermo Fisher Scientific). mRNA reverse transcription was conducted using random hexamer primers with M-MLV Reverse Transcriptase (Thermo Fisher Scientific). The resultant single-stranded cDNA was used for real-time PCR (rt-PCR). rt-PCR was completed using the SYBR green technique on a CFX Connect Real-Time PCR detection System (BioRad, Herculeus, CA). Relative gene expression was calculated using the ΔΔCt relative quantification method previously described by Livak and Schmittgen (A. M. Gordon et al., 2000). A housekeeping

gene was used to determine relative gene expression for each sample. The list of primer sequences used for gene analysis can be found in Table 2.

Table 2. Primer sequences for mRNA expression

Gene name	**Gene ID**	**Forward (5'-3')**	**Reverse (5'-3')**
Beta-actin	60	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
PGC-1α	10891	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTCACATCTAGTTCA
ERRα	2101	AGGGTTCCTCGGAGACAGAG	AGGGTTCCTCGGAGACAGAG
TFAM	7019	ATGGCGTTTCTCCGAAGCAT	TCCGCCCTATAAGCATCTTGA
Alix	10015	ATGGCGACATTCATCTCGGTG	CGCTTGGGTAAGTCTGCTGG
CD63	967	CAGTGGTCATCATCGCAGTG	CAGTGGTCATCATCGCAGTG
Clathrin	1213	CAGTGGTCATCATCGCAGTG	CAGTGGTCATCATCGCAGTG
STAM	8027	AATCCCTTCGATCAGGATGTTGA	AATCCCTTCGATCAGGATGTTGA
VTA1	51534	CTCCCCGCACAGTTCAAGAG	CTCCCCGCACAGTTCAAGAG
VPS4a	27183	CTCCCCGCACAGTTCAAGAG	CCGTGTTTCTCTTTGCTTCGTA
TSG-101	7251	CCGTGTTTCTCTTTGCTTCGTA	CCGTGTTTCTCTTTGCTTCGTA
Rab27b	5874	TAGACTTTCGGGAAAAACGTGTG	AGAAGCTCTGTTGACTGGTGA

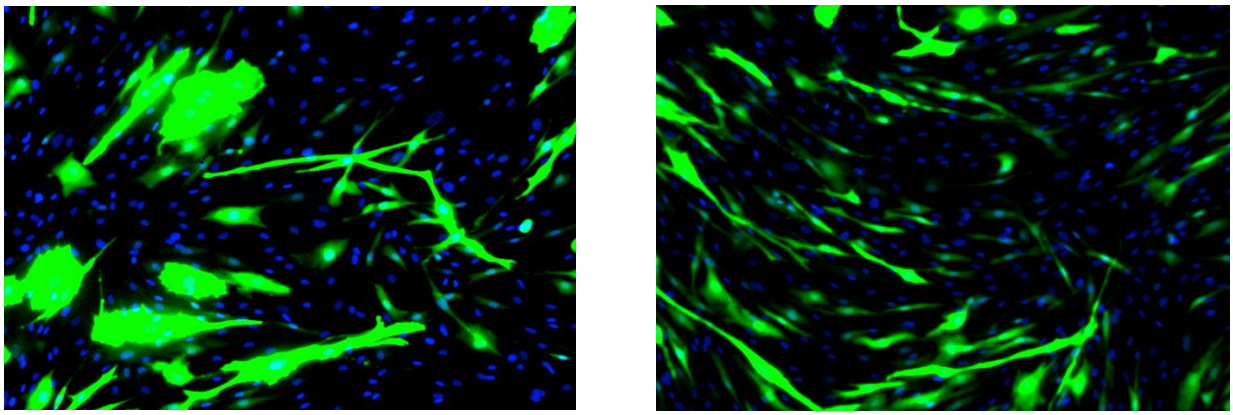
2.7-Statistical Analysis

Statistical analysis was conducted using RM ANOVA. Statistical significance was set at $P \leq 0.05$ for all variables. Data was reported as Mean ± SE.

CHAPTER 3: RESULTS

3.1-Adenovirus Transfection

Confirmation of successful adenovirus transfection is shown below in Figure 1. GFP-Ad and PGC-1α-Ad are tagged with green fluorescent protein (GFP), allowing images to be taken on a fluorescent microscope. Human primary myotubes will fluoresce green if transfection is successful. Preliminary experiments showed adequate transfection using a dosage of $1.0x10^7$ pfu/ml. Plaque forming units (PFU) is a measure of active virus in the sample.



GFP-Ad PGC-1α-Ad

Figure 1. Fluorescent microscopy images of day 8 human primary myotubes transfected with either GFP-Ad (left) or PGC-1α-Ad (right). Dosage used for the following experiments was $1.0x10^7$ pfu/ml.

3.2-Mitochondrial Biogenesis

To further confirm adenoviral transfection using PGC-1α-Ad, a number of genes involved in mitochondrial biogenesis were measured on day 8 of differentiation. Following 2 days of adenovirus treatment, PGC-1α gene expression increased by over 1,000-fold, although this increase was non-significant due to considerable variability (Range: 111.6-2874.2) (Figure 2). However, both TFAM and ERRα, which are downstream targets of PGC-1α, trended towards significant increases in expression. (Figure 2).

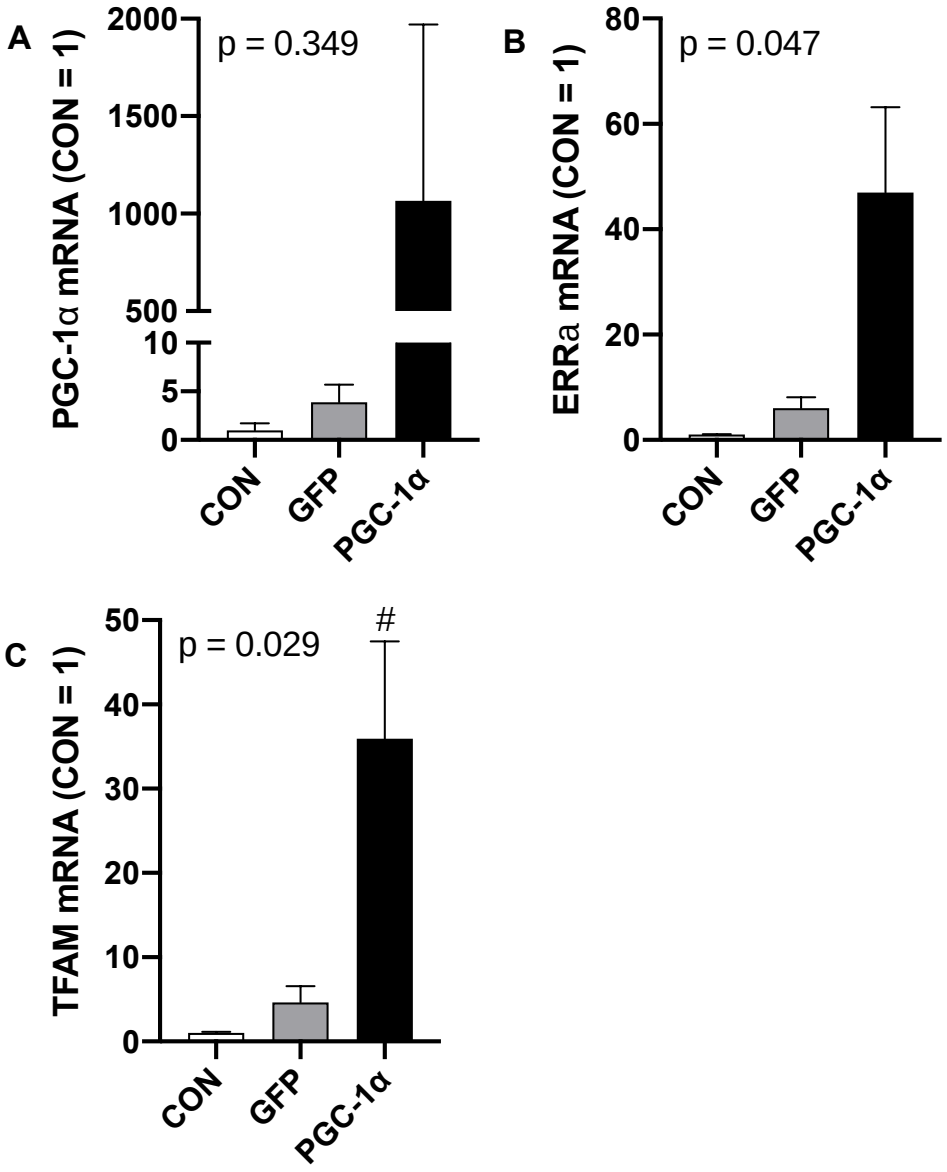


Figure 2. Trends toward significant increases in mitochondrial biogenesis markers PGC-1α (Graph A), ERRα (Graph B) and TFAM (Graph C) were found following 2 days of PGC-1α-Ad treatment in human primary myotubes. Gene expression was measured on day 8 of differentiation following 2 days of virus-free (control), GFP-Ad, or PGC-1α-Ad treatment. CON=1 for each gene. RM ANOVA. # = significantly different than CON. * = significantly different than all other groups. *n*=3 subjects/group.

3.3-Exosome Surface Markers

There are a number of proteins found on the surface of skeletal muscle exosomes. These proteins are indicative of exosome biogenesis. Following 2 days of PGC-1α-Ad treatment, significant increases in the exosome surface markers CD63, Alix and Clathrin were observed (Figure 3). There was a trend towards significant increases in TSG-101 gene expression (Figure 3).

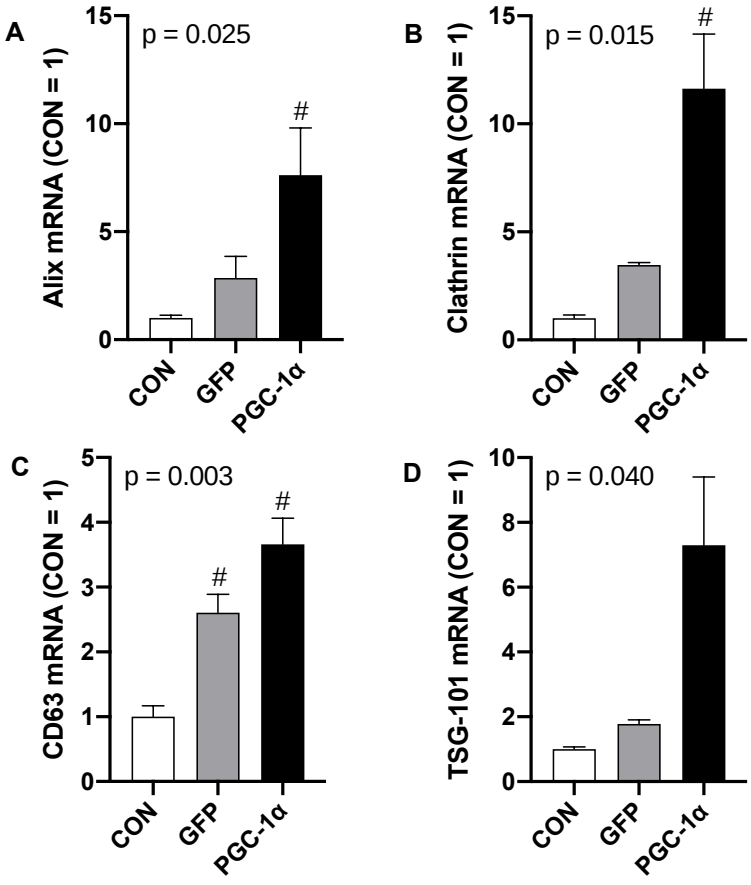


Figure 3. A trend toward a significant increase in the exosome surface marker TSG-101 (Graph D) was observed following 2 days of PGC-1α-Ad treatment in human primary myotubes. Significant increases in Alix (Graph A), Clathrin (Graph B) and CD63 (Graph C) gene expression were found. Gene expression was measured on day 8 of differentiation following 2 days of virus-free (control), GFP-Ad, or PGC-1α-Ad treatment. CON=1 for each gene. RM ANOVA. # = significantly different than CON. * = significantly different than all other groups. *n*=3 subjects/group.

3.4-Endosomal Sorting Complex Required for Transport (ESCRT) Pathway

The Endosomal Sorting Complex Required for Transport (ESCRT) pathway is crucial for exosome biogenesis and secretion. Genes in the ESCRT pathway were analyzed on day 8 of differentiation following 2 days of PGC-1α-Ad treatment. Significant increases in gene expression were seen for STAM, VTA1 and Rab27b compared to a virus-free control (Figure 4). A trend toward a significant increase was seen for VPS4a, a protein involved in cleaving ESCRT-III components from the membrane which results in ILVs (Figure 4).

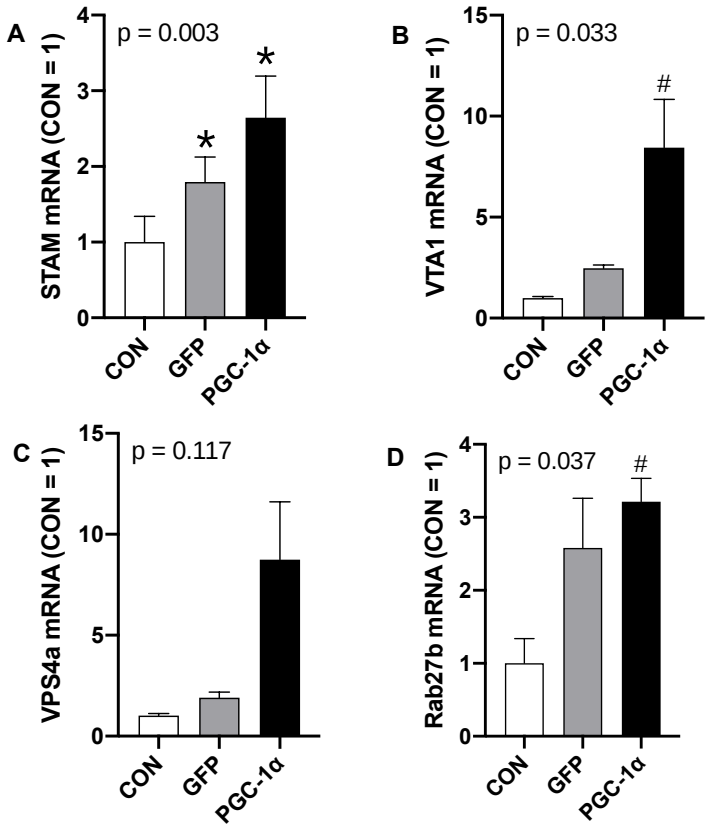


Figure 4. Trends toward significant increases were seen for VPS4a (Graph C). Significant increases were observed for STAM (Graph A), VTA1 (Graph B) and Rab27b (Graph D) gene expression. Gene expression was measured on day 8 of differentiation following 2 days of virus-free (control), GFP-Ad, or PGC-1α-Ad treatment. CON=1 for each gene. RM ANOVA. # = significantly different than CON. * = significantly different than all other groups. *n*=3 subjects/group.

3.5-Nanosight Exosome and Total EV Count

Skeletal muscle exosome quantification was conducted using a Malvern Panalytical Nanosight LM10 (Malvern Panalytical, Almelo, Netherlands). The Nanosight apparatus was used to calculate nanoparticle size and quantity distributions for each sample. No significant increase in particle count was found following PGC-1α-Ad treatment (Figure 5). Similarly, no significant increase in exosome count was found following GFP-Ad treatment (Figure 5).

Likewise, total extracellular vesicle count was measured a Malvern Panalytical Nanosight LM10 (Malvern Panalytical, Almelo, Netherlands). No significant increase in total extracellular vesicles was found following PGC-1α-Ad treatment (Figure 5). No significant increase was found following GFP-Ad treatment either (Figure 5).

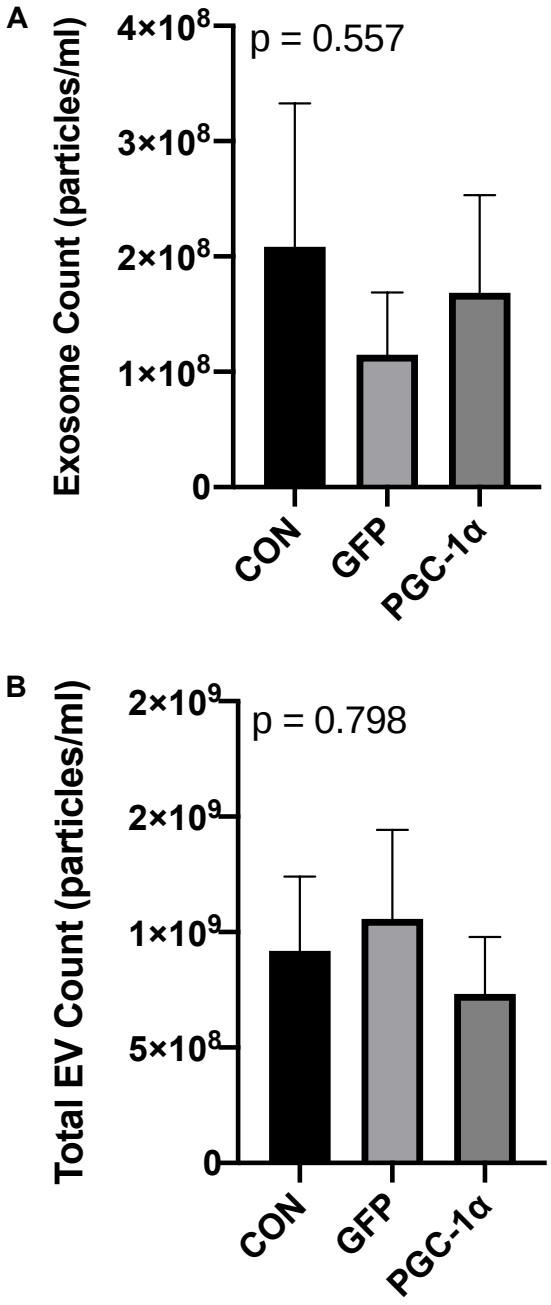


Figure 5. A. No significant increase in exosome count was found following PGC-1α-Ad treatment. Exosome count was measured on day 8 of differentiation following 2 days of virus-free (control), GFP-Ad, or PGC-1α-Ad treatment. B. Total extracellular vesicle count was measured on day 8 of differentiation. No significant increase in total extracellular vesicle count was found following GFP-Ad or PGC-1α-Ad treatment. CON=1 for each gene. RM ANOVA. # = significantly different than CON. * = significantly different than all other groups. *n*=3 subjects/group.

CHAPTER 4: DISCUSSION

4.1-Main Findings

The main finding of this study is that overexpression (OE) of PGC-1α in human primary myotubes increases the gene expression of Alix, Clathrin, CD63, STAM, VTA1, and Rab27b, each of which is involved in exosome biogenesis and/or exosome secretion. The gene expression of other exosome surface markers and the ESCRT-pathway trended towards significance or significantly increased following PGC-1α OE. However, exosome and extracellular vesicle count were not altered by PGC-1α OE.

4.2-Mitochondrial Biogenesis

A number of viable treatments exist to increase mitochondrial biogenesis in cell culture models. Some of these treatments include: electrical pulse stimulation, increases in calcium flux, and PGC-1α-Ad treatment. PGC-1α was first discovered as a novel transcription factor involved in adaptive thermogenesis of brown adipose tissue and skeletal muscle in mice (P. Puigserver et al., 1998), and is known as a "mitochondrial master regulator". PGC-1α modulates the transcription and activation of a number of nuclear encoded genes including ERRα, NRF-1 and NRF-2, as well as mitochondrial genes such as TFAM (Gleyzer et al., 2005; Schreiber et al., 2003). NRF-1 and NRF-2 target mitochondrial transcription factors like TFAM, which translate to the mitochondria and increase mitochondrial DNA replication and transcription, while ERRα translocates to the mitochondria directly for similar outcomes in mtDNA. Transfection in both cardiac and skeletal myocytes with PGC-1α-tagged adenovirus was shown to be successful in increasing mitochondrial biogenesis (Huang et al., 2017; Lehman et al., 2000). Pilot data confirmed successful transfection using GFP-Ad and PGC-1α-Ad by fluorescent microscopy

where GFP will emit a green light upon transfection (Figure 1). Following two days of PGC-1α-Ad treatment, we analyzed gene expression of key mitochondrial genes to ensure PGC-1α-Ad worked as intended. Our data are consistent with others in human primary myotubes where overexpression of PGC-1α increased regulators of mitochondrial biogenesis (Huang et al., 2017).

4.3-Skeletal Muscle Exosome Markers

Skeletal muscle cells, like all other cells types, have the ability to create and secrete exosomes which can directly influence recipient cellular physiology (Guescini et al., 2010; Nie et al., 2019). Myoblasts and myocytes can release exosomes into the cell culture media when grown *in vitro* (Guescini et al., 2010; Romancino et al., 2013). Previous studies have demonstrated that exosomes carry biologically active molecules such as proteins, mRNAs, and miRNAs (Forterre et al., 2014; Zanotti et al., 2018). Exosomes have the ability to transfer phenotypes from one cell to another. When exposed to inflammatory cytokines, C2C12 myotubes released exosomes that induced inflammation and atrophic signaling in treated myoblasts (Kim et al., 2018). Exosomes can improve cell function as well. Exosomes isolated from C2C12 myotubes improved HUVEC proliferation, migration, and tube formation (Nie et al., 2019). In the study from Nie et al., our laboratory demonstrated that slow-twitch, oxidative muscle (high in mitochondrial content) released more exosomes compared to fast-twitch, glycolytic muscle (low in mitochondrial content). To our knowledge, this is currently the only study showing a potential relationship between muscle mitochondria and exosome release. After two days of PGC-1α-Ad treatment, day 8 human primary myotubes showed trends or significant increases in known exosome surface markers. Our current findings suggest that increasing PGC-

1α and mitochondria in human primary myotubes increases exosome production and secretion in skeletal muscle.

Regular exercise is a potent inducer of PGC-1α via a muscle contraction-mediated process. Chronic adaptations to exercise are the result of repeated acute bouts of exercise and associated signaling. During muscle contraction, an efflux of sarcoplasmic reticulum Ca^{2+} floods the cytosol. This increase in sarcoplasmic Ca^{2+} not only increases protein content of transcriptional coactivators such as PGC-1α, but also transcription of key mitochondrial genes NRF-1, NRF-2, cytochrome c, and TFAM (Freyssenet, Connor, et al., 1999; Ojuka et al., 2003). Increasing PGC-1α protein results in an increase in both oxygen consumption and mitochondrial uncoupling in C2C12 myotubes (Wu et al., 1999). The effect of exercise on exosome release in response to both acute and chronic training has not yet been elucidated. Following one 30 minute bout of submaximal cycling at 60% of VO_2 max, circulating EVs significantly increased in all tested groups (men, women, lean, obese) (Rigamonti et al., 2019). Similarly, markers of EVs isolated from serum following an incremental cycling test significantly increased immediately post exercise (Frühbeis et al., 2015). Interest in the potential benefits of exercise-associated exosomes in relation to cardiovascular protection, glucose tolerance, and obesity has recently grown. When injected intravenously in mice, GFP-labeled exosomes travel to various organs including skeletal muscle (Aswad et al., 2014). In addition, when treated with high levels of palmitate, both C2C12 myotubes and mice increased the release of exosomes higher in lipid content and suggesting transferability of muscle cell exposure to exosomes and exosomal targets. Injection of exosomes isolated from mouse embryonic stem cells following myocardial infarction in mice significantly increased ejection fraction, resident cardiac progenitor cells, and myocardial capillarization (Khan et al., 2015). The potential for skeletal muscle exosomes to

influence organ physiology in an endocrine and paracrine manner, and the influence of exercise in the process, remain key questions to be addressed.

4.4-Skeletal Muscle ESCRT Pathway

In skeletal muscle, as in other cell types, there exist ESCRT-dependent and ESCRT-independent pathways involved in exosome biogenesis and secretion. While MVB generation and budding on the intraluminal membrane into ILVs is ESCRT-dependent in yeast cells, eukaryotic cells from mammals have shown a retention of their ability to secrete exosomes following silencing of necessary ESCRT-pathway proteins (Odorizzi et al., 1998; Stuffers et al., 2009). However, reduction of ESCRT-components like TSG-101 and Hrs leads to significant changes in MVB and ILV production. Early endosomes produced from cells lacking specific ESCRT machinery have been shown to become enlarged and house few ILVs or are improperly folded (Doyotte et al., 2005; Razi & Futter, 2006). Thus, the ESCRT-pathway remains the most important pathway involved in exosome biogenesis and secretion.

Various treatments can increase exosome biogenesis and secretion. Increasing intracellular calcium using calcium phosphate (CaP) or calcium ionophores has been shown to increase exosome secretion in many cell types including skeletal muscle (Dautova et al., 2018; Savina et al., 2003; Shyong et al., 2018). Seventy-two-hour treatment of both THP-1 and RAW264.7 cells with CaP particles significantly increased the number of secreted exosomes (Shyong et al., 2018). Similarly, monensin, a calcium ionophore, significantly increased acetylcholinesterase activity, a measurement of exosome content, in K562 cells (Savina et al., 2003). Causing endoplasmic reticulum stress or treating cells with adiponectin have both been shown to increase exosome release as well (Kanemoto et al., 2016; Obata et al., 2018). We

observed that following two days of adenovirus treatment, cells with an increase in PGC-1α and mitochondrial biogenesis exhibited significant or trended towards significant increases in gene expression of proteins in the ESCRT-pathway. To our knowledge, this is the first time linking PGC-1α and mitochondria to the endosomal sorting complex required for transport (ESCRT) pathway. The potential for PGC-1α overexpression as a method for stimulating exosome biogenesis and secretion remains promising and could provide a deeper understanding of exosome regulation in the future.

4.5-Future Studies

Further understanding of the precise mechanisms between PGC-1α and exosome biogenesis and release need to be examined in future studies. Future *in vitro* studies should use additional methods of exosome quantification such as electron microscopy and vesicle flow cytometry to assess whether exosome secretion increases following PGC-1α overexpression. PGC-1α OE and exosome secretion should be observed in cell types other than skeletal muscle to investigate if similar increases in exosome machinery occur. Preferential packaging of biomolecules such as proteins and RNAs into exosomes is still not well understood. Examining the contents of PGC-1α OE exosomes could give insight into the potential benefits of skeletal muscle exosomes following aerobic training. Subsequent *in vitro* studies should look into whether exosomes harvested from PGC-1α OE cells can restore function in dysfunctional cell models. Likewise, *in vivo* rodent and human research may look into the effects of aerobic exercise training as a potential method for stimulating skeletal muscle exosome release.

4.6-Limitations

There are a number of limitations in regards to the current study. Due to the COVID-19 quarantine, a number of laboratory experiments could not be completed in time for the submission of this thesis. Western blotting to assess cell protein content for the aforementioned mitochondrial proteins, exosome markers and ESCRT-pathway components was not finished. Additional methods of exosome quantification would be beneficial in confirming that exosome secretion is unchanged in response to PGC-1α OE. In addition, a secondary piece of our project involved increasing mitochondrial biogenesis via a PGC-1α-independent pathway using forskolin, an activator of cAMP, and ionomycin, a calcium ionophore, to test whether exosome biogenesis and secretion relies on PGC-1α content. Supplementary methods of mitochondrial quantification are needed. Measurement of mitochondrial DNA (mtDNA) or quantification using MitoTracker would further confirm that PGC-1α-Ad treatment was successful. While the cell culture portion of the project was completed, the necessary benchwork remains incomplete. Western blotting, real-time PCR, and exosome quantification must be run for these samples. While *in vitro* studies are helpful in identifying mechanisms of cellular physiology, future *in vivo* studies are needed to further establish what has been observed in this study. *In vivo* studies using adenoviral transfection in rodents or exercise training in humans would further confirm if PGC-1α plays a major role in exosome production and release.

4.7-Conclusion

In conclusion, the current study demonstrates that overexpression of peroxisome-proliferator activated receptor gamma coactivator 1-alpha (PGC-1α) in human primary myotubes increases gene expression of skeletal muscle exosome markers and the endosomal sorting

complex required for transport (ESCRT) pathway. To our knowledge, this is the first study showing a relationship between PGC-1α overexpression and exosome biogenesis and secretion. In the future, *in vitro* studies assessing exosomal content from PGC-1α OE cells as well as *in vivo* effects of PGC-1α OE on exosome production and release should be investigated to further understand the role PGC-1α plays in exosome secretion.

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