8-2018

Acute Effects of Contractile Activity on Skeletal Muscle Exosomes

Ron T. Garner
Purdue University

ACUTE EFFECTS OF CONTRACTILE ACTIVITY ON SKELETAL MUSCLE EXOSOMES

by

Ron T. Garner

A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Health and Kinesiology

West Lafayette, Indiana

August 2018

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Timothy P. Gavin, Chair
Department of Health and Kinesiology
Dr. Bruno Tesini-Roseguini
Department of Health and Kinesiology
Dr. Shawn S. Donkin
Department of Animal Sciences
Dr. Shihuan Kuang
Department of Animal Sciences

Approved by:

Dr. David B. Klenosky
Head of the Graduate Program

To my beautiful wife, Maggie, for her countless years of support and sustaining love.

To my unconquerable children, Hayden, Grace, Rose, and Nolan, for their constant efforts to encourage me to complete this journey.

ACKNOWLEDGMENTS

I recognize a lot of effort and encouragement from many over the years to complete this endeavor. I have dedicated this work and my life to Maggie, Hayden, Grace, Rose, and Nolan.

I acknowledge Dr. Tim Gavin for his years of support, respect and care for my family, and for giving me the room to expand my knowledge through perseverance, trial and error.

I want to thank my committee members, Dr. Roseguini, Dr. Kuang, and Dr. Donkin, who have each uniquely shaped my thinking, preparation, and approach to science.

I also want to acknowledge Dr. Yaohui Nie, without whom I never would have learned the skills necessary to complete this dissertation.

I acknowledge my friends and family, who were encouraging and complimentary of my efforts, to remind me that the journey was not for the faint of heart. To my Mom and Dad for their support in my efforts, and to help care for my family when I needed them most. For the friends I met in the process of time who shared their faith and optimism with me when I had little of my own. To my fellow graduate students who helped me to endure the final challenges of the Ph.D. journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A+REx – concurrent exercise

AEx – aerobic exercise

Alix – apoptotic linked gene-2 interacting protein X

AMPK – adenosine monophosphate protein kinase

BMI – body mass index

CaMKII – calcium-calmodulin protein kinase II

CVD – cardiovascular disease

EC – endothelial cell

EPS – electrical pulse stimulation

ESCRT – endosomal sorting complex required for transport

FBS – fetal bovine serum

HGS – hepatocyte growth factor-regulated tyrosine kinase substrate

HOMA-IR – homeostatic model assessment – insulin resistance

IL-6 – interleukin-6

LN – lean

MAPK – mitogen activated protein kinase P38

MARCKS – maristoylated alanine-rich C-kinase substrate

MHC – myosin heavy chain II

miR – micro ribonucleic acid

mRNA – messenger ribonucleic acid

MVB – multivesicular body

MyoG – myogenin

NF-κβ – nuclear factor light-chain-enhancer of activated B cells

OB - obese

PBS – phosphate buffer solution

REx – resistance exercise

SkM – skeletal muscle

SkMCC – skeletal muscle cell culture

SNARE – soluble NSF attachment protein receptor complexes

STAM – signal transducing adaptor molecule

T2D – type II diabetes mellitus

TNF-α – tumor necrosis factor alpha

TSG-101 – tumor susceptibility gene – 101

TSP-1 – thrombospondin-1

$\dot{V}E$ – minute ventilation

VEGF – vascular endothelial growth factor

$\dot{V}O_{2MAX}$ – maximal oxygen consumption

VPS4a – vacuolar protein sorting mutant

VTA1 – vesicle trafficking 1

ABSTRACT

Author: Garner, Ron, T. PhD
Institution: Purdue University
Degree Received: August 2018
Title: Acute Effects of Contractile Activity on Skeletal Muscle Exosomes
Major Professor: Timothy P. Gavin

Introduction: Skeletal muscle functions as an endocrine organ, and a key way to accomplish that is through exosomes. The internal budding of a multivesicular body (MVB) forms exosomes and contains mRNA, miRNA, and proteins. Through a multifaceted approach, the following three experiments were designed to better understand the effect of contractile activity on skeletal muscle MVB/exosome biogenesis and release.

Study 1: Exosome-related pathway response to acute exercise. Sedentary, lean, healthy male subjects (n=12) performed 45 min cycling (AEx), followed by single leg knee extensor exercise (A+REx). A biopsy was obtained prior to exercise and 1 hr post-exercise from each leg. Stimulation of MVB biogenesis occurred in the A+REx group, with miRNA biogenesis activated by both AEx and A+REx. Overall, there appeared to be no additive effect of REx for angiogenesis or mitochondrial improvements following acute exercise. Our data displays no total protein changes for known exosome surface markers. Increased MVB biogenesis and release indicate exciting possibilities for exercise stimulated exosome release.

Study 2: Effects of obesity and acute resistance exercise on skeletal muscle intercellular communication pathways. To more fully determine the effect of resistance exercise (REx) alone on MVB/exosome signaling and obesity on skeletal muscle angiogenesis, the following study was performed: Lean (LN) and obese (OB) (n=8 each group) sedentary human subjects performed single leg knee extension exercise at 80% 1-RM, 3 sets, 8-12 reps. Muscle biopsies were obtained at rest, 15 min, and 3 hr post-exercise. With OB, baseline anti-angiogenic miRNA and diminished MVB biogenesis were displayed, along with an increased anti-angiogenic factor thrombospondin-1 in response to acute REx, indicating plausible pathways to explain diminished capillary

density of Type II muscle fibers. No total changes were observed in VEGF between LN and OB, though a trend for reduced VEGF existed in the OB group compared to LN.

Study 3: Role of CaMKII in skeletal muscle exosome release. To determine a candidate pathway linked with calcium signaling, muscle contraction, and exosome release, specific attention was given to the role of calcium-calmodulin kinase II (CaMKII). Skeletal muscle biopsies were obtained from lean, healthy, male college-aged subjects (n=8) and satellite cells isolated for use in cell culture (SkMCC). To ensure proper differentiation, SkMCC had markers Pax7, Myogenin (MyoG), and myosin heavy chain (MHC) measured via Western blot on days 0, 2, 4, 6, and 8. SkMCC were stimulated for 30 min with electrical pulse stimulation (EPS), an exercise mimetic, to validate activation of *in vivo* exercise pathways adenosine monophosphate kinase (AMPK), mitogen activated protein kinase (MAPK) and CaMKII. SkMCC underwent 24 h of EPS with and without the addition of KN-93, a chemical inhibitor of CaMKII. Quantification of isolated exosomes following EPS was accomplished by use of a NanoSight, RT-PCR, and Western blot analysis. The main finding, despite an identifiable change in MHC and formation of myotubes, minimized contractile activity *in vitro* leading to inadequate activation of metabolic pathways and MVB release. Strong trends for the phosphorylation of AMPK, interaction of CaMKII with clathrin gene expression, reduced gene expression of PDK4, and reduced total nanoparticles in response to EPS indicate that changes occurred, though not to the anticipated levels based on preliminary data.

CHAPTER 1. REVIEW OF THE LITERATURE

Introduction (Review of Literature)

Physical inactivity is a pervasive health problem. It is well documented that regular physical activity reduces the risk for several diseases including cardiovascular disease (CVD) (60), obesity, type 2 diabetes (T2D), and some forms of cancer, yet only 1 in 5 Americans meet the 2008 Physical Activity Guidelines (9). Inadequate physical activity is associated with an estimated $131 billion in health care expenditures (9). This review will address several issues on health including aerobic and resistance exercise, skeletal muscle cell signaling, and the impact of obesity.

Obesity

Obesity rates in the United States continue to be a prevalent issue. Despite concentrated efforts to increase awareness of physical activity, diet, health, and wellness, the obesity rate continues to increase. From 2000 to 2016, the rate of obesity has increased from 30.5% to 39.6% of adults in the U.S. (21). Obesity is associated with increased risk for a host of factors related to the metabolic syndrome, such as dyslipidemia, impaired fasting glucose, hypertension, and insulin resistance (13). A common dysfunction in these conditions is impaired extracellular communication, which may lead to an increased risk of T2D (19, 24). Exercise is a well-known intervention to reduce the rates of obesity (64). However, impaired intercellular communication observed with obesity could limit the effects of exercise.

Aerobic Exercise

Aerobic exercise reduces the risk of CVD (60) and increases total aerobic capacity. A long-term clinical study showed that every 1-metabolic equivalent (3.5 ml O2 x kg-1 x min-1) increase in aerobic capacity came a 12% improvement in survival (31). The protective benefits of physical activity and exercise are well displayed in this study, even in subjects with known CVD. Aerobic exercise also reduces abdominal fat, even without total weight loss, a risk factor for CVD associated with obesity (49).

Skeletal muscle represents 40% of body weight and approximately the same metabolic energy use (70). Activation of skeletal muscle in response to exercise causes several cell-signaling cascades to occur, including adenosine monophosphate kinase (AMPK), which activates fatty acid oxidation and glucose transport to maintain energy demands (23). AMPK activation also stimulates angiogenesis, control of food intake and whole-body energy expenditure, and mitochondrial biogenesis (45). Activation of mitogen activated protein kinases (MAPK) also stimulates glucose and fatty acid oxidation. The isoforms of MAPK have been shown to be differentially activated based on the intensity and duration of exercise (27). These are important factors to understand differing responses of MAPK to various exercise interventions. Exercise-stimulated pathways shown *in vivo* related to calcium signaling include CaMKII (1, 47, 48), AMPK (45), and MAPK (27).

Using omics (proteomics, genomics, etc.) analysis of individual exercise bouts helps to reveal large pieces of information which were previously poorly understood. Single muscle fiber analysis of contraction-mediated skeletal muscle analysis has identified over 48 individual myokines released following exercise (40). The release of myokines will

also vary based on fitness level (55). These insights aid future experimental designs for subject recruitment, outcomes, and the intensity of an exercise intervention.

Resistance Exercise

The primary results from performing resistance exercise training is increased muscle mass, strength, and power through hypertrophy signaling. Increased muscle mass increases basal metabolic rate (42). However, with age, muscle mass begins to decline (12) and adiposity increase (66). Maintaining muscle mass over the lifetime is an important principle to reduce obesity and maintain activities of daily living, enjoying a higher quality of life (44).

Skeletal muscle hypertrophy signaling is well established through the IGF-1/AKT/mTOR pathway (54). The phosphorylation of P70S6 kinase, downstream of the mTOR signaling cascade, is correlated with the phenotypical changes of muscle hypertrophy (4). As with aerobic exercise, increased technology and precision of measuring the outcomes of resistance training focuses on individual proteins altered, based on intensity. Some of these outcomes include muscle remodeling, immune system modulation, and regeneration of the extracellular matrix of skeletal muscle (40).

Skeletal Muscle as an Endocrine Organ

Myokines, which are cytokines or other peptides released by skeletal muscle, can act in a paracrine or endocrine-like fashion to surrounding tissue (38). Aerobic exercise is a known stimulus to release myokines through contraction (38, 39). Myokines, including the interleukin family and myostatin, are released to enhance metabolism, immune system health, and are pro-inflammatory in their effect (38).

Exosomes

Another method for cell to cell communication is via exosomes, small vesicles (30-100nm) released by tissues such as mast and epithelial cells (28). These vesicles contain proteins, mRNA and miRNA which can regulate target tissues. For example, skeletal muscle myotube derived exosomes decrease myoblast proliferation by downregulating the cell cycle protein cyclin D and increase myoblast differentiation by upregulating the muscle development and repair protein myogenin (16, 17).

Exosome Biogenesis

Exosomes form from the internal budding of a multivesicular body (MVB), housed within the cell membrane. The formation of MVB's entails the endosomal sorting complex required for transport (ESCRT) proteins which have four complexes: ESCRT-0, I, II, and III. ESCRT-0 is responsible for recognition and sequestering of ubiquitinylated proteins and associates with several well-known accessory proteins, including signal transducing adaptor molecule (STAM), hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) and tumor susceptibility gene 101 (TSG-101) (11, 58).

As part of the ESCRT-I complex, TSG-101 is most commonly known as a marker of exosomes and recruits either the apoptotic linked gene-2 interacting protein X (Alix) protein or the ESCRT-II complex (11, 26, 57). Alix or ESCRT-II will then recruit ESCRT-III, which is primarily responsible for scission of the MVB within the cell membrane (65). Once fused with the plasma membrane, exosomes are released (7, 26). MVB's not released from the cell are degraded by a trafficking protein, vacuolar protein sorting mutant (Vps4a),

from the AAA ATPase family (5). Increased Vps4a would indicate increased degradation of MVB's, reducing the potential for exosome formation.

Exosome Release

Exosome production and release is a coordinated and well-documented process (3, 20, 46). The Rab family has been identified as a key player in the release of the vesicles from tissue, including MVB's (37). Specific family members include Rab11, 27a, 27b, 35, and Syntaxin 1A. MVB fusion with the plasma membrane (and subsequent exosome release) is increased in response to altered pH, hypoxia, and oxidative stress, all of which are altered in response to exercise (46). Increased expression of the Rab family indicates increased activity at the endosome, potentially leading to more exosome release.

Exosome Content

The content of individual exosomes will determine the effect in target tissues. Exosomes may be "antigen containing," effecting immune system response (28, 59) or "RNA containing," effecting functions based on the RNA encased (22, 28, 62). The previous references indicate a range of exosome content which include whole proteins, messenger RNA (mRNA) or exosomal shuttle RNA (esRNA, micro RNA (miR), small interfering RNA, and interfering RNA. Each play critical roles in cell-to-cell communication. An esRNA is a mRNA which is "shuttled" from one tissue to another and retains the ability to transcribe protein in the new tissue (62). miRNA, which are short (~20 nucleotides) non-protein coding RNA, can effectively block protein transcription by binding to or cleaving mRNA. This blocks mRNA in post-transcriptional modification or an "epigenetic" fashion (14). Additionally, the selection of miRNA is not random, but

certain miR will be given preferential packaging using methods that are still not entirely known (20, 67).

microRNA

Specific miR known to impact muscle function continue to be understood. Those of paramount importance to this review include miR related to angiogenesis, muscle function, or known exercise responses. Angiogenesis is decreased with miR-206 and miR-503 by impacting VEGF and NFκB expression (25, 56) and miR-503 directly downregulates FGF2 and VEGFα gene expression (69). Increased miR-503 expression contributes to impaired angiogenesis observed in T2DM (8). An angiogenic promoter, miR-130a, blocks the anti-angiogenic GAX and HOXA5 homeobox genes in vascular endothelial cells (10). Endothelial cells express large levels of miR-126, which is also pro-angiogenic in response to ischemia-induced angiogenesis (63).

For regular muscle function, miR-181a is expressed during skeletal muscle differentiation as a negative feedback system, which downregulates Hox11A and represses the differentiation process (32). Mature skeletal muscle cells deficient of miR-133a are associated with decreased mitochondrial function and impaired exercise tolerance (33). This helps to identify potential targets and benefits of miR in response to exercise. Several reports have identified skeletal muscle miRNA altered in response to acute exercise at various time points (34, 50, 51, 61).

Exosome Cross-Talk

Documentation of skeletal muscle exosome release in humans has been limited. Prior research has shown MVB's released into the circulation in young, healthy, physically

active men in a dose-response manner following maximal cycling and running exercise (18). However, the samples collected were serum MVB's, which limit our ability to determine any specific release from skeletal muscle. In a recent review of exosomes and MVB's, it was shown that immediately after aerobic exercise, there is lower expression of Alix in exercised compared to sedentary mice (52). Alix has been identified as a calcium-dependent pathway with the ESCRT-III pathway (30). With a large amount of calcium released from the sarcoplasmic reticulum in skeletal muscle, evidence would suggest a potential calcium pathway related to muscle contraction and exosome release.

It is well known that obese individuals have high macrophage activity and chronic inflammation (41). Physical activity can reduce this inflammation state associated with obesity (6). One source where chronic inflammation can occur is from exosomes released by adipocytes, which led to increased macrophage activity (68). Macrophages increase common inflammatory markers observed with obesity; tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kβ), all of which reduce insulin sensitivity (29). Mice fed a high fat diet released skeletal muscle exosomes which transferred the deleterious effects of insulin resistance and were incorporated in metabolic tissues, the liver and pancreas (2). In humans, exosomes contained 55 differentially expressed miRNA in obese compared to lean, focusing primarily on the TGF-β and Wnt/β-Catenin pathways (15). These pathways are involved in increased inflammation and altered cell signaling. Altogether, these articles display that exosomes in obesity may alter insulin signaling, inflammation, and negatively affect paracrine-like signaling to adjacent tissues *in vitro* and *in vivo*.

Electrical Pulse Stimulation

Primary human muscle cultures are powerful tools to understand cellular phenotypes. There are advantages and drawbacks to each type of experimental approach, including comparisons of *in vitro* to *in vivo* responses to similar stimuli (36). An advantage to using *in vitro* modeling systems with skeletal muscle is the knowledge that what is released from muscle can be captured and accurately identified.

Using an *in vitro* model for exercise through electrical pulse stimulation (EPS) is well established (35, 53). Recent work in mouse primary skeletal muscle cells as well as C2C12 cells, an immortalized murine skeletal muscle cell line, has shown that exosomes isolated from myotubes will suppress myoblast expression (17). The ability to isolate exosomes from skeletal muscle to use as treatment allows powerful information on intrinsic and extrinsic control or "cross-talk" between cells (16).

Hypothesis

It was hypothesized that acute exercise stimulates the biogenesis of MVB's. Additionally, it was hypothesized that reduced exosome production occurs in response to acute exercise in obesity compared to normal weight subjects. To test our hypothesis, the following three studies were performed:

1. Skeletal muscle exosome response to acute aerobic and concurrent exercise.
2. Skeletal muscle exosome and angiogenic miRNA response to acute resistance exercise in lean and obese men and women.
3. *In vitro* mechanistic analysis of skeletal muscle exosome release in primary human skeletal muscle cells via electrical pulse stimulation (EPS).

CHAPTER 2. EXOSOME AND EXOSOME-RELATED PATHWAY RESPONSES TO ACUTE EXERCISE

Ron T Garner[1], Jessica S Solfest[1], Yaohui Nie[1], Shihuan Kuang[2], Julianne Stout[3], Timothy P Gavin[1]

[1]Department of Health and Kinesiology and Max E. Wastl Human Performance Laboratory, Purdue University, West Lafayette, IN
[2]Department of Animal Science, Purdue University, West Lafayette, IN
[3]Indiana University School of Medicine–West Lafayette, West Lafayette, IN

Corresponding Author: Timothy P Gavin, Purdue University, Health and Kinesiology Department, 800 West Stadium Avenue, West Lafayette, IN, 47906.
Email: gavin1@purdue.edu

Abstract

Introduction**:** Exercise training promotes beneficial adaptations and recommendations for activity include aerobic (AEx) and resistance (REx) exercise on most days of the week. A potential method of communication in response to exercise includes microvesicles, particularly exosomes, which contain mRNA, miRNA, and proteins. Exosomes are known to regulate transcription in target tissues and would be a viable signaling mechanism following exercise. However, little is known about exosome biosynthesis and release following varied types of exercise. Our hypothesis is that 1) skeletal muscle exosomes are released in response to AEx and concurrent (A+REx) exercise and 2) contain muscle-specific miRNA used to enhance angiogenesis.

Methods: Twelve sedentary, healthy male subjects (21.6 ± 1.8 years) exercised on a cycle ergometer for 45 minutes (AEx) followed by single leg knee extensor exercise (A+REx). Muscle biopsies were obtained from the vastus lateralis in a single leg at rest and one-hour post-exercise from each leg. Western blot and rt-PCR analysis of known multivesicular body (MVB) biogenesis, release, and cell surface markers as well as miRNA machinery and mitochondrial and angiogenic factors were measured. miRNA related to angiogenesis (miRNA's 126, 130a, 133a, 206, and 503) were also measured. Data were compared using one-way repeated measures ANOVA and Pearson Correlations.

Results: MVB biogenesis was increased for clathrin and alix, with trends in other MVB complexes and CD-63 in response to A+REx. No change in the release pathway in response to AEx or A+REx, but trends for increased expression are observed. Whole muscle protein analysis showed no changes in alix, TSG-101, or CD-63. Gene expression increased for VEGF and PGC-1a, indicating an angiogenic and mitochondrial response to AEx and A+REx. miRNA processing pathway exportin-5 and dicer increased following each exercise condition.

Conclusion: Our data displays increased gene expression for select MVB biogenesis markers, trends for increased gene expression of MVB release, yet no total protein changes for known exosome surface markers. Although total protein of exosome surface markers were unchanged, increased gene expression of MVB biogenesis indicates an exciting possibility for an exercise-induced pathway to potential exosome release.

Keywords: Exosomes, miRNA, Concurrent, angiogenesis

Introduction

Skeletal muscle represents 30-40% of body mass and metabolic energy use (49) and participates in intercellular communication in part via myokines, muscle released cytokines (32, 33). There is mounting evidence that many cell types including skeletal muscle also communicate via the release of exosomes, 30-100 nm microvesicles containing proteins, mRNA and miRNA that regulate other cells function (26). Skeletal muscle myotube derived exosomes decrease myoblast proliferation by downregulating the cell cycle protein, cyclin D, and increase myoblast differentiation by upregulating the muscle development and repair protein, myogenin (11, 12). Injected C2C12 myotube-derived exosomes can be incorporated into metabolic tissues such as the liver and pancreas in mice (1). These reports establish skeletal muscle exosomes as a potential source for paracrine and endocrine signaling, however data on the regulation of exosome biogenesis in humans is limited (38).

Exosome biogenesis occurs from the internal budding of multivesicular bodies (MVB) via four protein complexes known as the endosomal sorting complex required for transport (ESCRT) 0, I, II, and III (Figure 1) (8). ESCRT-0 recognizes and sequesters ubiquitinylated proteins and associates with the accessory proteins signal transducing adaptor molecule (STAM), hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), and tumor susceptibility gene 101 (TSG-101) (8, 43). As part of the ESCRT-I complex, TSG-101 recruits either the apoptotic linked gene-2 interacting protein X (Alix) or the ESCRT-II complex (8, 23, 42). Alix or ESCRT-II then recruits ESCRT-III, which is primarily responsible for scission of the MVB within the cell membrane (48) resulting in MVB fusion with the plasma membrane and exosome release (4, 23). MVBs not fused

with the cell are degraded by a trafficking protein, vacuolar protein sorting mutant (Vps4a, Vps4B) in complex with vesicle trafficking 1 (Vta1) (3, 21).

Exosome release from the cell is a coordinated and well-documented process (2, 20, 34) and occurs through MVB fusion to the plasma membrane via soluble NSF attachment protein receptor complexes (SNARE) (2). Rab proteins are known to regulate SNARE protein function through direct or indirect contact (5, 22). Specific members of the Rab family identified with MVB/exosome regulation include Rab11, 27a, 27b, 35, and the syntaxin family gene Syntaxin 1A (2, 31). Increased expression of Rab and syntaxin families indicates increased activity at the endosome, the location of MVB biogenesis (23, 31). As MVB biogenesis and release increases, miRNA content may also increase. To produce mature miRNA, primary and pre miRNA are formed by drosha and pasha in the nucleus, pre-miRNA are then transported to the cytoplasm by exportin-5, and pre-miRNA are cleaved by dicer to form mature miRNA (10).

Regular physical activity reduces the risk of several chronic diseases (45). Previous work from our laboratory on the angiogenic myokine vascular endothelial growth factor (VEGF) found rapid increases in VEGF mRNA in response to both acute AEx (17) and REx (15) as well as the secretion of VEGF during acute AEx (18). VEGF is important for muscle basal capillarization and angiogenesis in response to exercise (29, 30). Interestingly, cardiac muscle progenitor cells are known to secrete angiogenic exosomes in response to hypoxia (19). Recent work suggests acute AEx increases circulating MVB's (47), however little information exists on the response of the muscle microvesicle pathways in response to acute exercise.

In the current report, we investigated if acute exercise increases expression of MVB biogenesis and release pathways. With miRNA being important constituents of exosomes, the expression of the miRNA processing pathway and selected miRNA in skeletal muscle were investigated. Finally, given current interests in concurrent exercise, responses to acute aerobic (AEx) and aerobic+resistance (A+REx) were analyzed. It was hypothesized that skeletal muscle MVB biogenesis increases in response to acute aerobic and concurrent exercise. Additionally, it was hypothesized that acute exercise increases miRNA processing proteins as well as specific miRNA related to angiogenesis and muscle function in response to acute aerobic and concurrent exercise.

Methods

Subjects. Twelve sedentary, young (18-30 y of age) men volunteered to participate after an explanation of the study and informed consent approved by the University Institutional Review Board. All subjects were healthy non-smokers, with no history of cardiopulmonary disease. Sedentary subjects were defined as participating in less than 1 hr of strenuous physical activity per week. Subject characteristics are listed in Table 1.

Determination of $\dot{V}O_{2MAX}$ and 1-RM. Maximal oxygen consumption ($\dot{V}O_{2MAX}$) was measured on an electronically braked cycle ergometer (Lode, Excaliber Sport, Groningen, Netherlands) as previously described (24). Minute ventilation ($\dot{V}E$), oxygen uptake ($\dot{V}O_2$), and carbon dioxide production ($\dot{V}CO_2$) were continuously monitored via open circuit spirometry (True Max 2400, Parvo Medics, Salt Lake City, UT). Heart rate (model T31, Polar Electro Inc., Woodbury, NY) and rating of perceived exertion (RPE) were measured continuously. The test began with a 5-min warm-up at 50W, following which workload increased 25W every min until volitional fatigue. Subjects were verbally encouraged to

continue for as long as possible. The criterion used to assess $\dot{V}O_{2MAX}$ included: 1) a heart rate in excess of 90% of age predicted max (220 - age); 2) a respiratory exchange ratio greater than or equal to 1.10; and 3) identification of a plateau (< 150 ml/min increase) in $\dot{V}O_2$ despite a further increase in workload. In all tests, at least two of three criteria were met.

After a 15-min rest period following the $\dot{V}O_{2MAX}$ test, single leg knee extensor (KE) one repetition maximum (1-RM) was determined for the dominant leg. The initial weight of 13.6 kg was progressively increased by 4.5 kg until they were unable to continue. Subjects were given 1 min rest between each attempt. The highest successfully lifted weight was designated as 1-RM.

Experimental design. At least two weeks after the determination of $\dot{V}O_{2MAX}$ and 1-RM, subjects reported to the Max E. Wastl Human Performance Laboratory between 0600 and 0700 after a 12 hr fast. Subjects performed 45 min of two-legged cycle ergometry at 55% of $\dot{V}O_{2MAX}$. Directly following AEx, subjects completed single leg KE exercise, three sets of 8-12 repetitions at 55% of the 1-RM workload, with a 2-min rest interval between sets (A+REx). Given VEGF mRNA is increased 2-fold and 4-fold at 0 and 2 hr after aerobic exercise (17) and increases in circulating exosomes occur at 1 hr after aerobic exercise (47), we investigated responses from vastus lateralis biopsies prior to and 1 hr after the acute AEx and A+REx bouts. The initial, resting biopsy was alternated between AEx and A+REx legs across subjects. Biopsies for AEx and A+REx were alternated between being the 2nd and 3rd biopsy across subjects to minimize potential time bias post-exercise. Biopsies (rest and acute exercise) when taken from the same leg were separated

by at least 3 cm as previously described (15, 17). Samples were stored at –80ºC until analysis.

Quantitative real-time PCR. Total RNA from skeletal muscle was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). For mRNA reverse transcription, first-strand cDNA was generated by random hexamer primers with MMLV Reverse Transcriptase (Invitrogen). For mature miRNA reverse transcription, multiple adenosine nucleotides were first added to the 3' end of the total RNA with *Escherichia Coli* DNA polymerase (New England BioLabs, Ipswich, MA) and cDNA was then synthesized with a Poly T primer including an adaptor sequence using MMLV Reverse Transcriptase. To determine mature miRNA, the specific miRNA sequence was used as the forward primer and the adapter sequence from the Poly T primer was used as the universal reverse primer for all miRNAs. Real-time PCR reactions were performed with a SYBR green PCR kit in a Roche LightCycler® 480 System (Roche, Indianapolis, IN). Gene expression was determined with the $2^{-\Delta\Delta Ct}$ relative quantification method and was normalized to 18s for mRNA or RNU24 for miRNA (25). Primer sequences are listed in Table 2. Due to inadequate muscle sampling, one subject was excluded from all gene expression analysis (n=11)

Muscle protein isolation and analysis. Twenty mg of frozen muscle was homogenized on ice in RIPA buffer (50mm Tris HCL 7.4 pH, 150mm NaCl, 2mm EDTA, 0.1% SDS, 0.1% Triton x-100, 0.5% NaDeoxycholate) using a handheld variable speed homogenizer (LabGEN7, Cole-Palmer, Vernon Hills, IL). Phosphatase inhibitors (0.2 mM Na_3VO_4, 50 mM NaF) and a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO) were added to prevent modification of proteins post-homogenization. Total sample protein

concentration was determined by the bicinchoninic acid method (BCA protein assay kit, Bio-Rad Laboratories, Hercules, CA).

From muscle homogenates, 30-60 µg of total protein was fractionated for Western blot analysis on SDS-polyacrylamide gels, transferred to reduced-fluorescence PVDF membrane, and incubated with one of the following primary antibodies: Alix (#2171s, Cell Signaling Technology), TSG-101 (#sc-7964, Santa Cruz Biotechnology), and CD-63 (#sc-5275, Santa Cruz). Either florescence-conjugated (#925-32210, LI-COR) or horseradish peroxide–conjugated (#7074 Anti-Rabbit, #7076 Anti-Mouse, Cell Signaling) secondary antibodies were incubated with the membrane and detected using florescence (Odyssey, LI-COR, Lincoln, NE) or chemiluminescence (PerkinElmer, Waltham, MA). Densitometric analysis was performed manually using Image J (SciJava) or Image Lab software (Bio-Rad). Results were normalized to alpha tubulin (#2144, Cell Signaling).

Statistical Treatment. One-way repeated measures analysis of variance (ANOVA) was used. Following a significant F ratio, a Fisher's LSD was used. Pearson correlations were performed to investigate relationships between variables. Significance was established at $p \leq 0.05$ for all statistical sets and data reported are Mean ± SE.

Results

MVB biogenesis and release pathways. To assess the MVB biogenesis pathway in response to exercise, key components in the formation of MVB's (HGS, clathrin, TSG-101, Alix, STAM, VTA1, VSP4a) were analyzed using rt-PCR analysis. Clathrin and alix mRNA were increased in response to exercise (Figure 2). Post-hoc analysis identified increases in clathrin and alix mRNA following A+REx. There was a trend toward increased gene expression for HGS where A+REx increased ~25%. There was no

difference in mRNA for CD-63, TSG-101, STAM, VTA1, or VPS4a in response to exercise.

To investigate an increase in MVB release following acute exercise, gene expression for Rab family (Rab11A, 27A, 27B, 35) and Syntaxin1A proteins were analyzed (Figure 3). A trend for increased gene expression for Rab27A and Rab35 was observed, where A+REx increased ~60% and ~40% respectively. There was no change in Rab11A, Rab27B, or Syntaxin1A in response to acute exercise.

Exosome surface marker protein expression. To determine MVB/exosome release in response to acute exercise, exosome surface marker and MVB biogenesis protein levels (alix, CD-63, TSG-101) were measured before and after acute exercise via Western blot analysis. At 1 hr post-exercise, there were no differences identified for protein expression in AEx or A+REx conditions for alix, TSG-101, or CD-63 (Figure 4).

miRNA biogenesis processing. Essential parts of the miRNA biogenesis pathway include drosha, exportin-5, and dicer. The mRNA for dicer and exportin-5 were increased, with no change in drosha (Figure 5). Post-hoc analysis identified increased dicer and exportin-5 with AEx and A+REx. These data suggest a coordinated response of the transport of pre-miRNA from the nucleus and the processing of mature miRNA in skeletal muscle. Pearson correlation analysis revealed relationships between exosome surface markers and miRNA processing in both AEx and A+REx conditions, consistent with potential coordination between miRNA and MVB expression (Table 3). No relationships were identified between MVB biogenesis/release pathways and the miRNA processing pathway.

Angiogenic and mitochondrial mRNA and miRNA. To explore the acute response of angiogenesis and mitochondria 1 hr post-exercise, the gene expression of VEGF and PGC-1α was examined. Both VEGF and PGC-1α were increased ~5-fold by AEx and A+REx, suggesting no additive effect with REx. There were no changes in response to exercise in angiogenic (miR-126, -130a, -206, and -503) or mitochondrial (miR-133a) miRNA (Figure 6). A moderate negative correlation was observed between $\dot{V}O_{2Max}$ and miR-130a AEx (r=-0.63; p=0.04) (data not shown).

Discussion

In the current report, we investigated if acute, moderate intensity cycling exercise with or without the addition of REx increases components of the exosome pathway: MVB formation, MVB fusion/exosome release, exosome surface markers, and miRNA. We identified increases in the gene expression of components of MVB biogenesis (alix and clathrin) and miRNA processing (exportin-5 and dicer), but no changes in MVB release.

MVB biogenesis and release. There are over 23 proteins associated with the ESCRT exosome biogenesis pathway (8). In the current report, key components in each of the ESCRT pathway complexes were analyzed to identify potential changes in MVB biogenesis in response to acute exercise. Increased gene expression was observed for clathrin and alix, an early phase of MVB biogenesis and accessory protein, which may indicate a response of MVB biogenesis following acute exercise in humans.

Microvesicles increased in circulation immediately following maximal cycling exercise in humans (13), and visual evidence demonstrated reduced skeletal muscle alix protein immediately following submaximal exercise in mice (38). Despite this evidence, the present investigation observed no change in alix total protein 1 hr post-exercise.

However, increased gene expression of alix and clathrin and trends for increased HGS (~25%) and STAM (~25%) following A+REx identify stimulation of the MVB biogenesis pathway. Although alix total protein did not change, increased gene expression for alix may indicate any changes which occurred returned to baseline 1 hr post-exercise. Compared to the former studies, subjects completed submaximal cycling efforts (55% $\dot{V}O_{2MAX}$) and samples were not obtained immediately post-exercise. Based on this finding, future studies should increase exercise intensity and collect samples immediately post-exercise to fully identify alterations of MVB biogenesis in human skeletal muscle.

In K562 and T-cells, calcium is important in exosome release (40, 41). Rab11 is associated with MVB release and calcium dependent pathways (39). As calcium release and sequestration are required for skeletal muscle contraction, a potential signaling pathway in human skeletal muscle exosome release is identified through Rab and syntaxin family proteins. However, gene expression of Rab and Syntaxin family proteins was unchanged in the present study. A trend for increased gene expression of Rab27A (~55%) and Rab35 (~40%) were displayed with A+REx. As there are no known time course studies related to the release of skeletal muscle exosomes, future studies should include specific acute exercise effects on the timing of MVB biogenesis and release (AEx, REx, A+REx, etc.).

Exosome surface marker identification. Exosome surface markers may be more important than we have understood to this point to identify function or size of particles. Several reports discuss varying surface markers for microparticles (8, 38, 44). Alix and CD-63 are released in different volumes from C2C12 myoblasts/myotubes during different days of differentiation (35). One report noted that when alix was knocked down *in vitro*,

released microparticles contained more major histocompatibility complex II components, which alters the size of the vesicle (8). Taken together, these data indicate exosome markers may be associated with a specific function or released in response to a specific stimulus (i.e. AEx vs. A+REx). The understanding gained from the current investigation for gene or protein expression in response to AEx and A+REx could be altered based on the exosome marker selected. To avoid this, we analyzed several commonly known markers of exosomes in whole muscle homogenates. A differential regulation of exosome sub-type based on size or surface marker could show exciting possibilities of altered exosome release based on the type of activity performed or content contained therein.

miRNA biogenesis. Following 60 min of acute cycling exercise, drosha, exportin-5, and dicer gene expression were increased ~30-35% 3-hours post-exercise (36). Consistent with this data, gene expression for exportin-5 and dicer were increased 1 hr post-exercise in the current report. In conjunction with increased miRNA biogenesis, there was a coordinated gene expression response to AEx and A+REx (Table 3) with exosome surface markers/MVB biogenesis. Although increases in exosome marker gene expression does not signify an increase of exosomes in skeletal muscle, it gives evidence that both pathways (miRNA and MVB's) may be activated by an acute exercise stimulus.

Exercise, muscle, angiogenesis, and mitochondria. It is well known that exercise leads to increased expression of VEGF and PGC-1α (14, 16, 27). Prior research has identified increased VEGF in response to AEx or REx alone and known responses occur 2 hr post-exercise (15, 16). The data herein demonstrates that VEGF gene expression is increased at 1 hr post-exercise and that no additive response occurs with the addition of REx to AEx. PGC-1α gene expression was also increased 1 hr post-exercise in both conditions. Due to

this known response, any changes with miRNA measured in the current investigation should have occurred. This gives confidence that acute AEx and A+REx at the intensity and time selected does not stimulate known angiogenic (miR-126, -130a, 206, -503) or mitogenic (miR-133a) miRNA.

Several reports have identified skeletal muscle miRNA altered in response to acute exercise at various time points (28, 36, 37, 46). In one case, evidence shows altered miRNA response to resistance training separated by low and high responders to lean body mass gained during training (9). For the present investigation, a moderate negative correlation was observed between $\dot{V}O_{2MAX}$ and miR-130a AEx (r=-0.63; p=0.04). This gives insight into angiogenic potential in subjects with the lowest $\dot{V}O_{2MAX}$ values. miR-130a is an angiogenic promoter which blocks the anti-angiogenic GAX and HOXA5 homeobox genes in vascular endothelial cells (7). Subjects with the lowest $\dot{V}O_{2MAX}$ displayed greater miR130a expression in response to AEx, increasing the angiogenic response compared to those with normal $\dot{V}O_{2MAX}$ values. No correlations were observed with $\dot{V}O_{2MAX}$ and VEGF, yet the greater miR-130a identifies a unique potential therapeutic target to enhance angiogenesis in subjects with diminished $\dot{V}O_{2MAX}$ values. Greater miR-503 previously exhibited diminished endothelial cell function (6), however, no relationships were identified for miR-503 and $\dot{V}O_{2MAX}$ in the current investigation. A moderate positive correlation was observed with miR-503 Pre and PGC-1α A+REx response (r=0.57, p=0.01). This indicates that the addition of REx to AEx may help to stimulate PGC-1α to combat the negative endothelial function at baseline.

In summary, our data display increased gene expression of MVB biogenesis and known exosome surface markers. In addition, gene expression of enhanced miRNA processing,

VEGF, and PGC-1α response 1 hr post-exercise identify novel adaptations to acute exercise. These data indicate exciting potential for exercise stimulated MVB/exosome biogenesis. The new possibilities to understand skeletal muscle exosome response to varied exercise type, time, and intensity gives rise to future research.

References

1. Aswad H, Forterre A, Wiklander OP et al. Exosomes participate in the alteration of muscle homeostasis during lipid-induced insulin resistance in mice. *Diabetologia*. 2014;57(10):2155-64.
2. Azmi AS, Bao B, Sarkar FH. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer and Metastasis Reviews*. 2013;32(3-4):623-42.
3. Babst M, Sato TK, Banta LM, Emr SD. Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *The EMBO journal*. 1997;16(8):1820-31.
4. Booth AM, Fang Y, Fallon JK, Yang J-M, Hildreth JE, Gould SJ. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. *J Cell biol*. 2006;172(6):923-35.
5. Cai H, Reinisch K, Ferro-Novick S. Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. *Developmental cell*. 2007;12(5):671-82.
6. Caporali A, Meloni M, Völlenkle C et al. Deregulation of microRNA-503 contributes to diabetes mellitus–induced impairment of endothelial function and reparative angiogenesis after limb ischemia. *Circulation*. 2011:CIRCULATIONAHA. 110.952325.
7. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood*. 2008;111(3):1217-26.
8. Colombo M, Moita C, van Niel G et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci*. 2013;126(24):5553-65.
9. Davidsen PK, Gallagher IJ, Hartman JW et al. High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression. *Journal of applied physiology*. 2010;110(2):309-17.
10. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. *Nature Reviews Cancer*. 2006;6(4):259-69.
11. Forterre A, Jalabert A, Berger E et al. Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk? *PLoS One*. 2014;9(1):e84153.

12. Forterre A, Jalabert A, Chikh K et al. Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. *Cell cycle*. 2014;13(1):78-89.
13. Fruhbeis C, Helmig S, Tug S, Simon P, Kramer-Albers EM. Physical exercise induces rapid release of small extracellular vesicles into the circulation. *J Extracell Vesicles*. 2015;4:28239.
14. Gavin TP. Basal and exercise-induced regulation of skeletal muscle capillarization. *Exercise and sport sciences reviews*. 2009;37(2).
15. Gavin TP, Drew JL, Kubik CJ, Pofahl WE, Hickner RC. Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta Physiol*. 2007;191:139-46.
16. Gavin TP, Robinson CB, Yeager RC, England JA, Nifong LW, Hickner RC. Angiogenic growth factor response to acute systemic exercise in human skeletal muscle. *Journal of applied physiology*. 2004;96(1):19-24.
17. Gavin TP, Robinson CB, Yeager RC, England JA, Nifong LW, Hickner RC. Angiogenic growth factor response to acute systemic exercise in human skeletal muscle. *J Appl Physiol*. 2004;96(1):19-24.
18. Gavin TP, Ruster RS, Carrithers JA et al. No difference in the skeletal muscle angiogenic response to aerobic exercise training between young and aged men. *J Physiol*. 2007;585(Pt 1):231-9.
19. Gray WD, French KM, Ghosh-Choudhary S et al. Identification of therapeutic covariant microRNA clusters in hypoxia-treated cardiac progenitor cell exosomes using systems biology. *Circ Res*. 2015;116(2):255-63.
20. Guay C, Regazzi R. Exosomes as new players in metabolic organ cross-talk. *Diabetes, Obesity and Metabolism*. 2017;19(S1):137-46.
21. Henne WM, Buchkovich NJ, Emr SD. The ESCRT pathway. *Developmental cell*. 2011;21(1):77-91.
22. Hutagalung AH, Novick PJ. Role of Rab GTPases in membrane traffic and cell physiology. *Physiological reviews*. 2011;91(1):119-49.
23. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. *Immunology letters*. 2006;107(2):102-8.
24. Kraus RM, Stallings HW, 3rd, Yeager RC, Gavin TP. Circulating plasma VEGF response to exercise in sedentary and endurance-trained men. *J Appl Physiol (1985)*. 2004;96(4):1445-50.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods*. 2001;25(4):402-8.
26. Lotvall J, Valadi H. Cell to cell signalling via exosomes through esRNA. *Cell adhesion & migration*. 2007;1(3):156-8.
27. Mathai AS, Bonen A, Benton CR, Robinson DL, Graham TE. Rapid exercise-induced changes in PGC-1α mRNA and protein in human skeletal muscle. *Journal of applied physiology*. 2008;105(4):1098-105.
28. Nielsen S, Scheele C, Yfanti C et al. Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle. *The Journal of physiology*. 2010;588(20):4029-37.
29. Olfert IM, Howlett RA, Tang K et al. Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. *J Physiol*. 2009;587(Pt 8):1755-67.

30. Olfert IM, Howlett RA, Wagner PD, Breen EC. Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. *Am J Physiol Regul Integr Comp Physiol*. 2010;299(4):R1059-67.
31. Ostrowski M, Carmo NB, Krumeich S et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature cell biology*. 2010;12(1):19-30.
32. Pedersen BK, Åkerström TC, Nielsen AR, Fischer CP. Role of myokines in exercise and metabolism. *Journal of applied physiology*. 2007;103(3):1093-8.
33. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nature Reviews Endocrinology*. 2012;8:457.
34. Roma-Rodrigues C, Fernandes AR, Baptista PV. Exosome in tumour microenvironment: overview of the crosstalk between normal and cancer cells. *BioMed research international*. 2014;2014.
35. Romancino DP, Paterniti G, Campos Y et al. Identification and characterization of the nano-sized vesicles released by muscle cells. *FEBS letters*. 2013;587(9):1379-84.
36. Russell AP, Lamon S, Boon H et al. Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. *The Journal of physiology*. 2013;591(18):4637-53.
37. Safdar A, Abadi A, Akhtar M, Hettinga BP, Tarnopolsky MA. miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice. *PloS one*. 2009;4(5):e5610.
38. Safdar A, Saleem A, Tarnopolsky MA. The potential of endurance exercise-derived exosomes to treat metabolic diseases. *Nat Rev Endocrinol*. 2016;12(9):504-17.
39. Savina A, Furlán M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *Journal of Biological Chemistry*. 2003;278(22):20083-90.
40. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. *Journal of cell science*. 2002;115(12):2505-15.
41. Soo CY, Song Y, Zheng Y et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology*. 2012;136(2):192-7.
42. Sundquist WI, Schubert HL, Kelly BN, Hill GC, Holton JM, Hill CP. Ubiquitin recognition by the human TSG101 protein. *Molecular cell*. 2004;13(6):783-9.
43. Tamai K, Tanaka N, Nakano T et al. Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. *Biochemical and biophysical research communications*. 2010;399(3):384-90.
44. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nature Reviews Immunology*. 2009;9(8):581-93.
45. Thompson PD, Buchner D, Piña IL et al. Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease. *Circulation*. 2003;107(24):3109-16.
46. Uhlemann M, Möbius-Winkler S, Fikenzer S et al. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. *European journal of preventive cardiology*. 2014;21(4):484-91.

47. Whitham M, Parker BL, Friedrichsen M et al. Extracellular Vesicles Provide a Means for Tissue Crosstalk during Exercise. *Cell Metab*. 2018;27(1):237-51 e4.
48. Wollert T, Wunder C, Lippincott-Schwartz J, Hurley JH. Membrane scission by the ESCRT-III complex. *Nature*. 2009;458(7235):172-7.
49. Zurlo F, Larson K, Bogardus C, Ravussin E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *Journal of Clinical Investigation*. 1990;86(5):1423.

Table 1. Subject Characteristics. BMI, body mass index; $\dot{V}o_{2MAX}$, maximal oxygen consumption; KE, maximal knee extensor strength. Mean ± SE. $N = 12$.

	Mean ± SE	Range (Min-Max)
Age, y	22.3 ± 1.7	18-30
Height, m	1.80 ± 0.02	1.73-1.85
Mass, kg	75.5 ± 4.4	57.7-90.9
BMI, kg/m^2	23.3 ± 1.4	17.3-29.7
$\dot{V}O_{2MAX}$, l×min^{-1}	2.93 ± 0.20	1.88-3.68
$\dot{V}O_{2MAX}$, ml×kg^{-1}×min^{-1}	39.0 ± 4.0	24.6-54.8
KE, kg	35.6 ± 3.1	27.3-50.0
KE, kg×kg^{-1}	0.47 ± 0.04	0.36-0.68

Table 2. Gene sequences of mRNA and miRNA, obtained from the Harvard Primer Bank. Apoptotic linked gene-2 interacting protein X (Alix); Tumor Susceptibility Gene-101 (TSG-101); hepatocyte growth factor-regulated tyrosine kinase substrate (HGS); signal transducing adaptor molecule (STAM); vesicle trafficking 1 (Vta1); vacuolar protein sorting mutant (Vps4a).

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
Alix	ATGGCGACATTCATCTCGGTG	CGCTTGGGTAAGTCTGCTGG
Clathrin	ATTCTGCCAATTCGTTTTCAGGA	GCTTTCAGTGCAATTACTTTGCT
TSG-101	GAGAGCCAGCTCAAGAAAATGG	TGAGGTTCATTAGTTCCCTGGA
CD63	CAGTGGTCATCATCGCAGTG	ATCGAAGCAGTGTGGTTGTTT
Dicer	GAGCTGTCCTATCAGATCAGGG	ACTTGTTGAGCAACCTGGTTT
Exportin-5	ATCCTGGAACACGTTGTCAAG	CACTACAATTCGAGACAGAGCAT
Drosha	TGTCACAGAATGTCGTTCCAC	GGGCCTAAAGGATGGTGCT
HGS	CTCCTGTTGGAGACAGATTGGG	GTGTGGGTTCTTGTCGTTGAC
STAM	AATCCCTTCGATCAGGATGTTGA	CGAGACTGACCAACTTTATCACA
VTA1	CTCCCCGCACAGTTCAAGAG	AACGACAGTAATAAGCCACCAC
Vps4a	CCACGCTATCAAGTATGAGGC	CCGTGTTTCTCTTTGCTTCGTA
Rab11A	CAACAAGAAGCATCCAGGTTGA	GCACCTACAGCTCCACGATAAT
Rab27A	GCTTTGGGAGACTCTGGTGTA	TCAATGCCCACTGTTGTGATAAA
Rab27B	TAGACTTTCGGGAAAAACGTGTG	AGAAGCTCTGTTGACTGGTGA
Rab35	TACTGTTGCGTTTTGCAGACA	CCCCGATAATACGTGGAGGTG
Syntaxin 1A	CGAGACCGCTTCATGGATGAG	ACTTGGAACGAACTTTGTTTGC
miR		
126	TCGTACCGTGAGTAATAATGCG	
130a	CAGTGCAATGTTAAAAGGGCAT	
133a	TTTGGTCCCCTTCAACCAGCTG	
206	TGGAATGTAAGGAAGTGTGTGG	
503	TAGCAGCGGGAACAGTTCTGCAG	

Table 3. Pearson correlation values (r) displaying interactions between miRNA processing and exosome surface markers/MVB biogenesis for AEx (A), and A+REx (B). *p≤0.05.

A.	Drosha mRNA	Exportin-5 mRNA	Dicer mRNA
Alix mRNA	0.42	0.82*	0.75*
TSG-101 mRNA	0.35	0.75*	0.71*
CD63 mRNA	0.73*	0.71*	0.34

B.	Drosha mRNA	Exportin-5 mRNA	Dicer mRNA
Alix mRNA	0.77*	0.75*	0.81*
TSG-101 mRNA	0.94*	0.77*	0.82*
CD63 mRNA	0.79*	0.28	0.85*

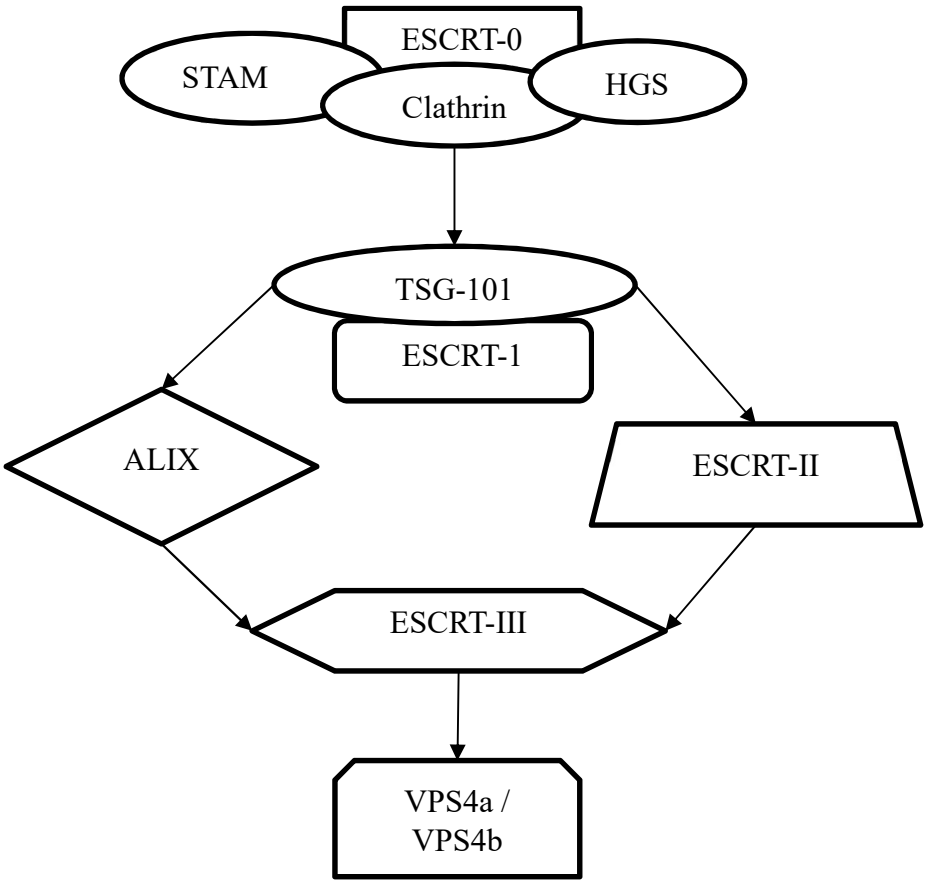

Figure 1. Overview of MVB biogenesis pathway. Endosomal complex required for transport (ESCRT) 0, I, II, and III. Hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), clathrin, signal transducing adaptor molecule (STAM), tumor susceptibility gene-101 (TSG-101), apoptotic linked gene-2 interacting protein X (Alix), and vacuolar protein sorting mutant (Vps4a, Vps4b).

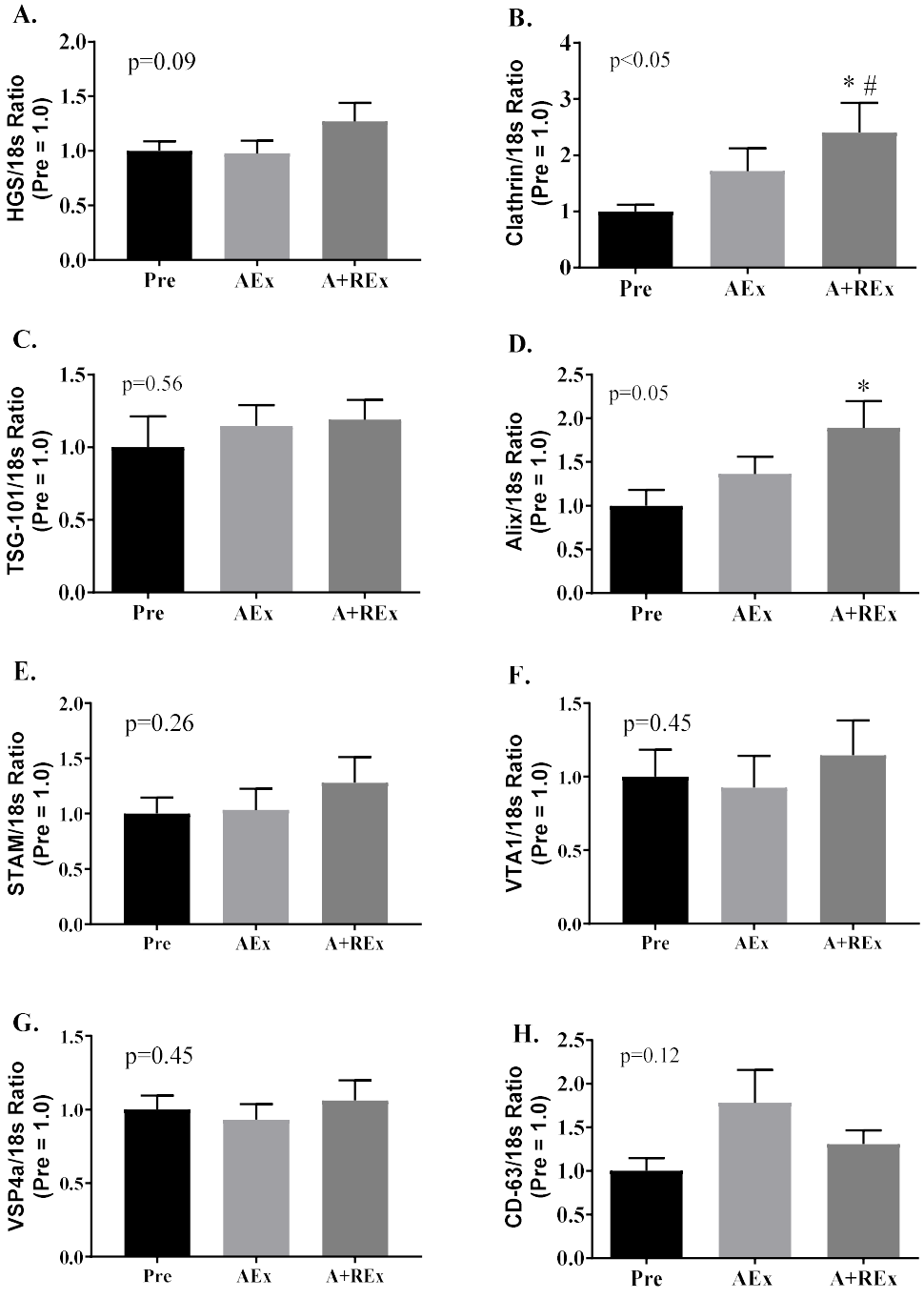

Figure 2. Gene expression of MVB biogenesis/ESCRT in response to acute aerobic (AEx) and concurrent (A+REx) exercise. ESCRT-0 proteins hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) (A) and clathrin (B), signal transducing adaptor molecule (STAM) (E), ESCRT-I proteins Tumor Susceptibility Gene-101 (TSG-101) (C), accessory protein Apoptotic linked gene-2 interacting protein X (Alix) (D), ESCRT-III proteins vesicle trafficking 1 (Vta1) (F), and vacuolar protein sorting mutant (Vps4a) (G), and tetraspanin CD-63 (H). *p≤0.05 vs Pre; #p≤0.05 vs AEx. Mean ± SE, n=11.

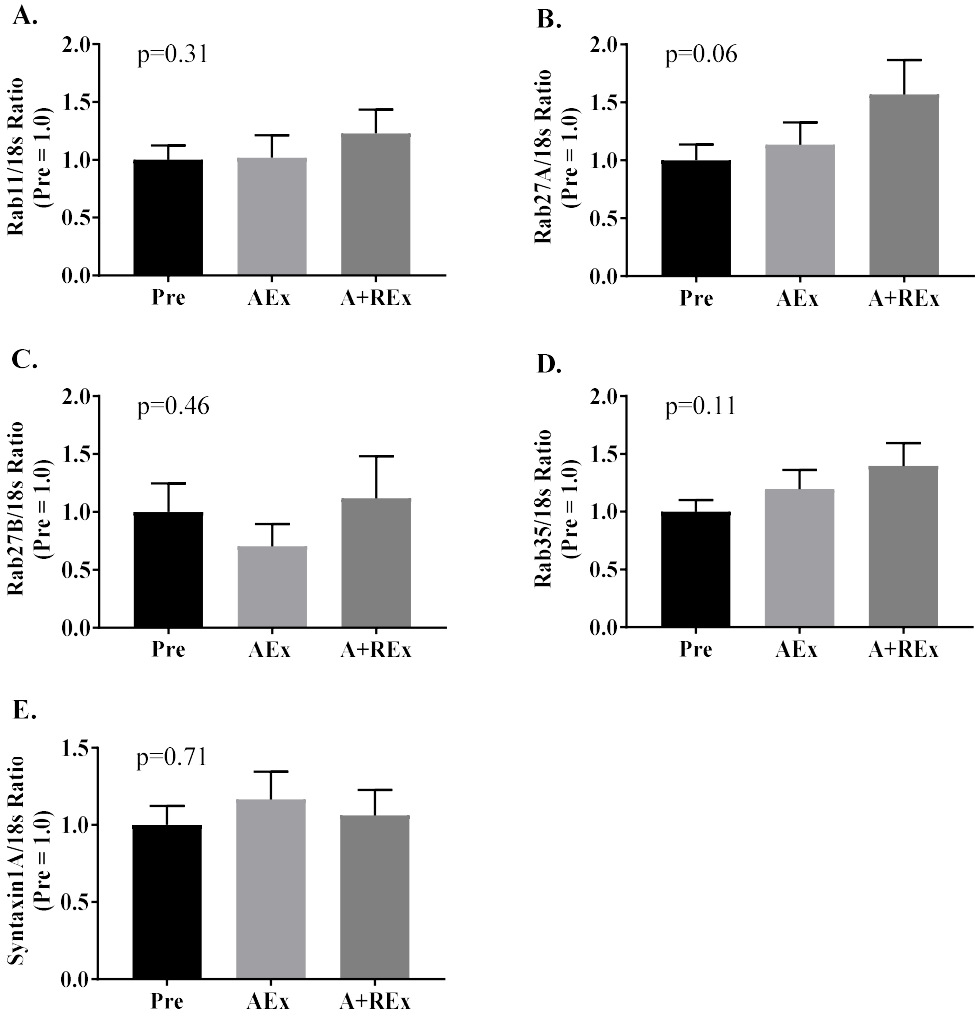

Figure 3. Gene expression of MVB/exosome release in response to acute aerobic (AEx) and concurrent (A+REx) exercise for Rab11 (A), Rab27A (B), Rab27B (C), Rab35 (D), and Syntaxin1A (E). Rab and syntaxin family proteins are associated with MVB fusion with the membrane, leading to exosome release. Mean ± SE, n=11.

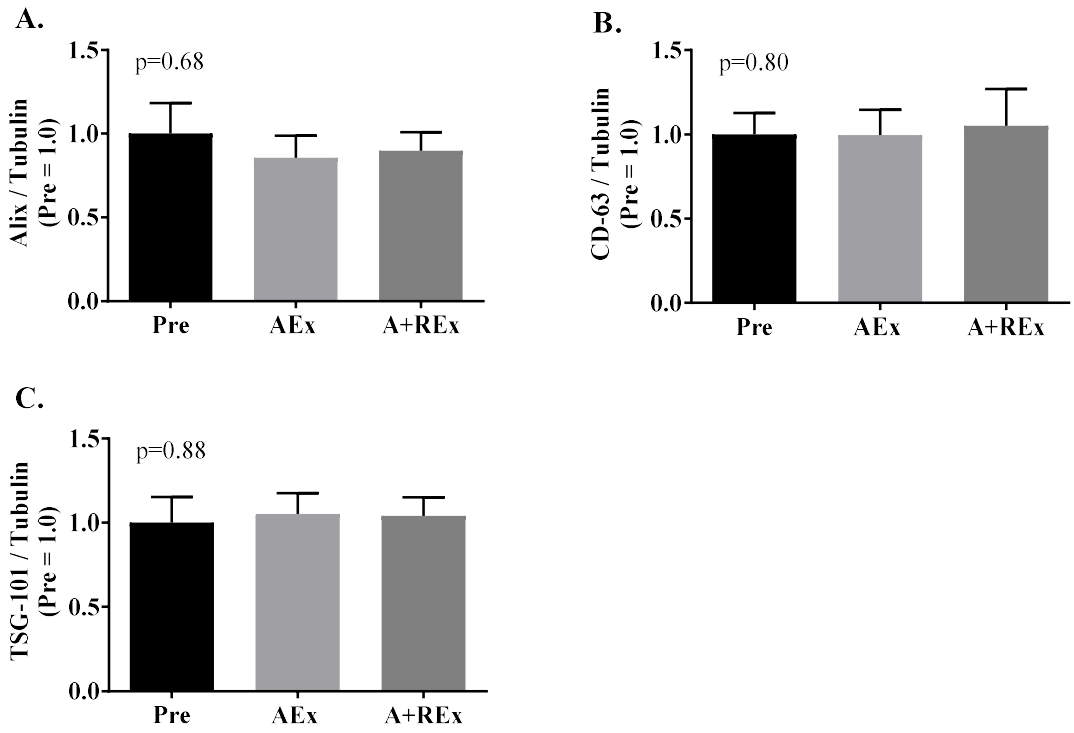

Figure 4. Protein analysis of exosome surface markers in response to acute aerobic (AEx) and concurrent (A+REx) exercise for alix (A), CD-63 (B), and TSG-101 (C). Mean ± SE, n=12.

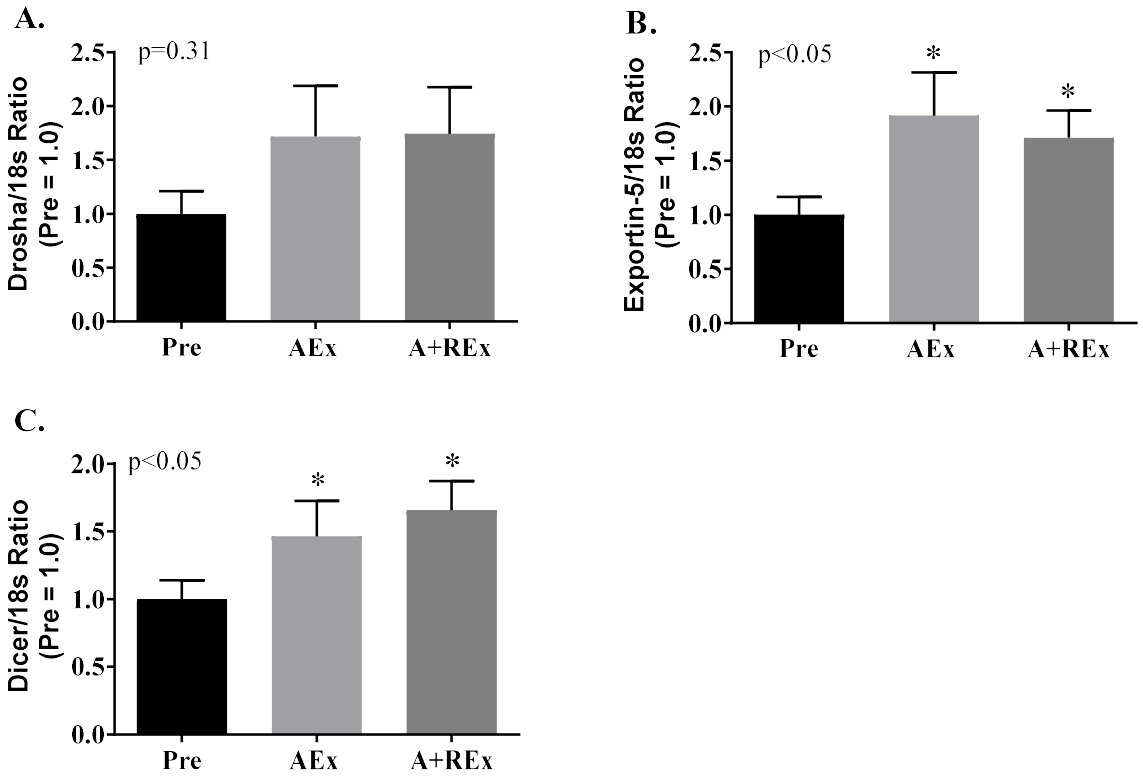

Figure 5. Gene expression of miRNA processing pathway drosha (A), exportin-5 (B), and dicer (C) in response to acute aerobic (AEx) and concurrent (A+REx) exercise. *p≤0.05 vs Pre. Mean ± SE, n=11.

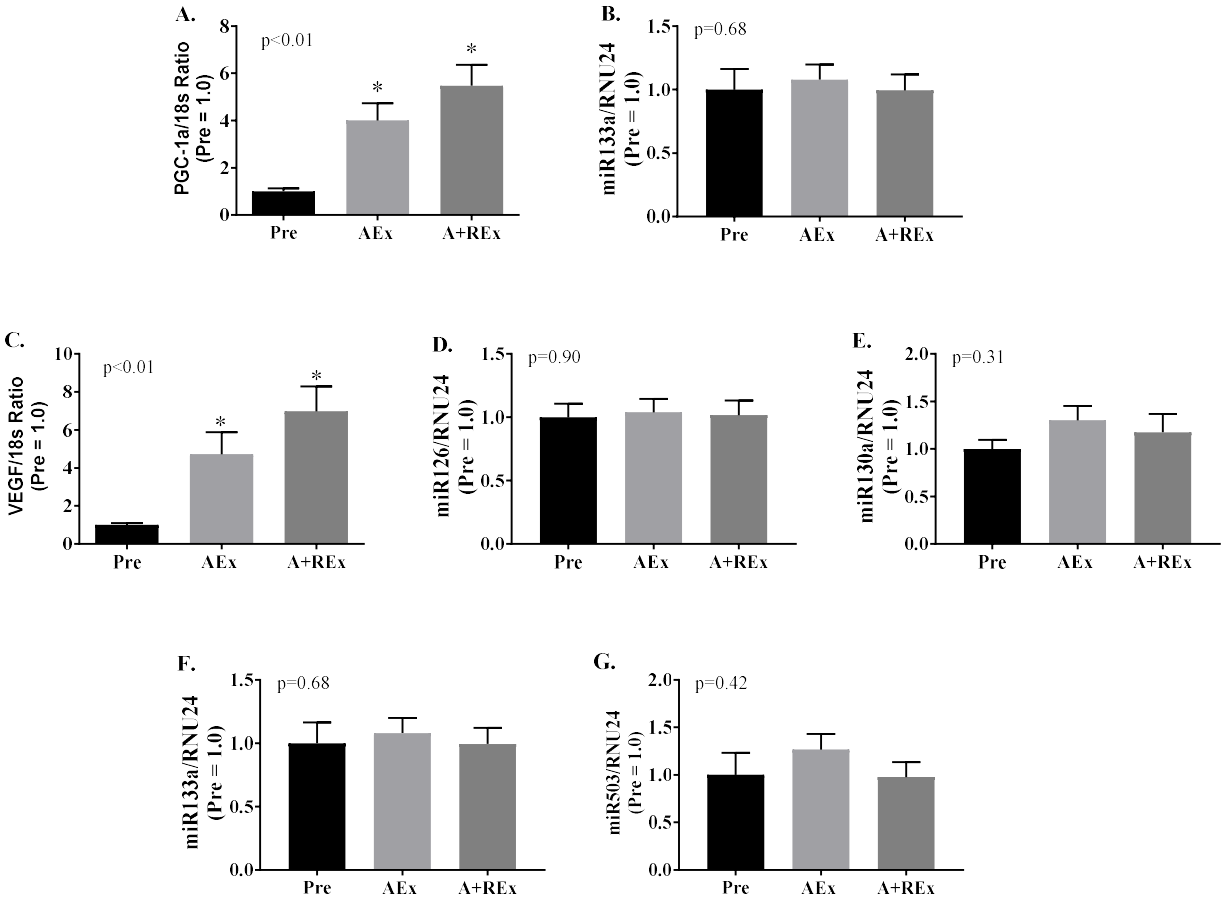

Figure 6. Analysis of mitochondrial and angiogenic gene expression and miRNA in response to acute aerobic (AEx) and concurrent (A+REx) exercise. For mitochondrial function, PGC-1α (A) and miR-133a (B) were measured. For angiogenesis, VEGF (C), and miR-126 (D), -130a (E), -206 (F) and -503 (G) were measured. *p≤0.05 vs pre. Mean

CHAPTER 3. EFFECTS OF OBESITY AND ACUTE RESISTANCE EXERCISE ON SKELETAL MUSCLE INTERCELLULAR COMMUNICATION PATHWAYS

Ron T. Garner *, Jessica A. Weiss *, Yaohui Nie, Shihuan Kuang, and Timothy P. Gavin
Purdue University, Max E. Wastl Human Performance Laboratory

*Contributed equally. JAW recruited subjects, analyzed data, and authored portions of the methods section. RTG prepared manuscript, assisted with muscle biopsies, processed all muscle samples, and analyzed data.

Corresponding Author: Timothy P. Gavin, Purdue University, Health and Kinesiology Department, 800 West Stadium Avenue, West Lafayette, IN, 47906.
Email: gavin1@purdue.edu

Abstract

Introduction: Obesity (OB) continues to be a prevalent cause for concern and disrupts cellular communication and skeletal muscle capillarization. Exosomes, small microvesicles, are known to pass genetic material (mRNA, miRNA) and whole proteins and are affected by aerobic exercise. Resistance exercise (REx) stimulates angiogenesis with unknown effects on exosome biogenesis. Additionally, little is known about the impact of a single bout of REx on angiogenesis. Our hypothesis was that OB impacts basal exosome biogenesis, and a single bout of REx will stimulate exosome biogenesis and angiogenesis in LN subjects, but that OB will diminish the REx effect.

Methods: LN (LN) and OB (n=8 each group) sedentary human subjects participated in the study. Subjects underwent $\dot{V}O_{2MAX}$ and 1-RM testing to establish their fitness level. On a separate day, subjects performed single leg knee extension exercise at 80% 1-RM, 3

sets, 8-12 reps. Muscle biopsies were obtained at rest, 15 min, and 3 hr post-exercise. Histological and chemical analysis of skeletal muscle was performed, including q-PCR, Western blots, and fiber type and capillary staining.

Results: Basal exosome biogenesis was reduced in OB compared to LN with no REx effect in either group. Exosome surface marker protein and gene expression was altered in response to REx in both groups. miRNA related to angiogenesis was altered in obesity at baseline, with no REx effect. Both groups have intact vascular endothelial growth factor (VEGF) response following REx, with increased anti-angiogenic signaling, thrombospondin-1 (TSP-1) in the OB group. Capillary density in OB subjects is impaired in Type II muscle fibers.

Discussion: With obesity, baseline anti-angiogenic miRNA, diminished MVB biogenesis, and increased TSP-1 in response to acute REx indicate plausible pathways to explain diminished capillary density of Type II muscle fibers. Variable changes to increase and decrease exosome surface markers were identified in response to REx, with no changes in MVB biogenesis in either group. Potential therapeutic targets to improve angiogenesis in OB should be identified for transport in exosomes, such as miR-206, -503, and 130a.

Key Words: Exosomes, MVB, Angiogenesis

Introduction

Obesity (OB) rates in the United States continue to be a prevalent issue and continue to rise with an increase from 30.5% to 39.6% of adults between 2000 and 2016 (19). OB is associated with reduced skeletal muscle oxidative capacity (42), increased insulin resistance (9), impaired intercellular communication (18, 20), and increased capillary cross-sectional area and diminished density (17, 32). This reduces the number of

capillaries per fiber of OB individuals, limiting the critical role capillaries play in delivering substrates, such as oxygen, glucose, and insulin to skeletal muscle (25).

Intercellular communication between skeletal muscle fibers and adjacent endothelial cells is important for capillarization and is regulated by several factors including nitric oxide (50), vascular endothelial growth factor (VEGF) (12, 45), and thrombospondin-1 (TSP-1) (24). Maintaining proper capillarization ensures adequate diffusion capacity to skeletal muscle (23) and intercellular communication plays an important role in this regulation. Skeletal muscle plays a critical role in cross-talk to adjacent tissues through myokines or "exerkines" released through exercise (37, 40).

In addition to paracrine growth factor regulation, exosomes, 30-100 nm microvesicles, might also regulate capillarization via cell-to-cell communication in muscle (26, 29). The effects of obesity with exosome release at baseline and during acute exercise is unknown. Exosomes are formed from the internal budding of multivesicular bodies (MVB) and contain mRNA, proteins, and miRNA that vary by cellular source (4). This process entails coordination of many pathways, including MVB formation with clathrin, TSG-101, and alix (4), and miRNA processing, drosha, exportin-5, and dicer (10). Angiogenic miRNA could play a role in basal changes of muscle capillarization by effecting VEGF, such as miR-130a, -206, and -503 (3, 43, 49).

Previously, our laboratory reported no difference in VEGF at rest between OB and LN subjects as well as no differences in the VEGF response to acute aerobic exercise (17). This is consistent with findings that muscle capillarization is increased in response to aerobic training in OB women (31). Resistance exercise (REx) training is well known to promote muscle fiber hypertrophy and angiogenesis. Given muscle fiber hypertrophy and

lower capillary density in obesity (17), responses to acute REx might provide insight into the impaired angiogenic response to chronic muscle overload and hypertrophy in obesity. It is known that angiogenic growth factors between LN and OB are no different at rest, however, the effect on exosome biogenesis or miRNA in OB human muscle at rest or in response to acute REx is unknown. Accordingly, the present study was designed to investigate the hypothesis that obesity alters resting and resistance exercise-induced expression of angiogenic growth factors, MVB biogenesis, or miRNA in human muscle.

Methods

Subjects. Sedentary, non-smoking, LN (n=8) and OB (n=8) college-aged (18-35) males (4 LN, 4 OB) and females (4 LN, 4 OB) volunteered to participate after an explanation of the study and informed consent approved by the University Institutional Review Board. Sedentary was defined as participating in physical activity fewer than 20 min/d, 3 d/wk. Subject characteristics are listed in Table 4.

Measurement of maximal oxygen ($\dot{V}O_{2Max}$) consumption. Maximal oxygen consumption ($\dot{V}O_{2Max}$) was measured on an electronically braked cycle ergometer (Lode, Excaliber Sport, Groningen, Netherlands) as previously described (30). Minute ventilation ($\dot{V}E$) oxygen uptake ($\dot{V}O_2$), and carbon dioxide production ($\dot{V}CO_2$) were continuously monitored via open circuit spirometry (True Max 2400, Parvo Medics, Salt Lake City, UT). Heart rate (model T31, Polar Electro Inc., Woodbury, NY) and rating of perceived exertion (RPE) were recorded at the end of each stage. The test began with a 5-min warm-up at 50W. After the warm-up, workload increased 25W every min until volitional fatigue. Participants were verbally encouraged to continue for as long as possible. The criterion used to assess $\dot{V}O_{2MAX}$ included: 1) a heart rate above 90% of age predicted max (220 -

age); 2) a respiratory exchange ratio greater than or equal to 1.10; and 3) identification of a plateau (≤ 150 ml/min increase) in $\dot{V}O_2$ despite a further increase in workload (21). In all tests, at least two of three criteria were met.

1-repetition maximum testing. After a 15-min rest period following the $\dot{V}O_{2MAX}$ test, one leg knee extensor (KE), one repetition maximum (1-RM) was determined in dominant and non-dominant randomly assigned legs. An initial weight of 13.6 kg was progressively increased by 4.5 kg until participants were unable to continue. One minute rest between each attempt was given and the highest successfully lifted weight was designated as 1-RM.

Experimental design. At least one week following the initial visit, participants completed three sets of single leg REx of 8-12 repetitions at 80% of 1-RM in either the dominant or non-dominant leg (randomly assigned). A baseline biopsy was obtained from the non-exercise leg immediately prior to exercise, followed by biopsies in REx leg at 15 min and 3 hr post-exercise. Known VEGF responses have been observed at 2 hr and 4 hr post-exercise for REx (15), and exosome release immediately following exercise in mice has been identified (40), therefore, the time points of 15 min and 3 hr post-exercise were selected. Biopsies taken from the same leg were separated by at least 3 cm (5). Samples were stored at -80°C until analysis.

Quantitative real-time PCR. Total RNA from skeletal muscle was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). For mRNA reverse transcription, first-strand cDNA was generated by random hexamer primers with MMLV Reverse Transcriptase (Invitrogen). For mature miRNA reverse transcription, multiple adenosine nucleotides were first added to the 3' end of the total RNA with *Escherichia Coli* DNA polymerase (New England BioLabs, Ipswich, MA) and cDNA was then synthesized with a Poly T

primer including an adaptor sequence using MMLV Reverse Transcriptase. To determine mature miRNA, the specific miRNA sequence was used as the forward primer and the adapter sequence from the Poly T primer was used as the universal reverse primer for all miRNAs. Real-time PCR reactions were performed with a SYBR green PCR kit in a BioRad CFX96 Real-Time System (Bio-Rad, Hercules, CA). Gene expression was determined with the 2-ΔΔCt relative quantification method and was normalized to 18s for mRNA or RNU24 for miRNA (33). Primer sequences for miRNA and mRNA are listed in Table 5.

Muscle protein isolation and analysis. Twenty mg of frozen muscle was homogenized in RIPA buffer (50mm Tris HCL 7.4 pH, 150mm NaCl, 2mm EDTA, 0.1% SDS, 0.1% Triton x-100, 0.5% NaDeoxycholate) using a Bead Ruptor 12 bead homogenizer (Omni, Kennesaw, GA). Phosphatase inhibitors (0.2 mM Na_3VO_4, 50 mM NaF, and a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO)) were added to prevent modification of proteins post-homogenization. Total sample protein concentration was determined by the bicinchoninic acid method (BCA protein assay kit, Bio-Rad Laboratories, Hercules, CA).

From muscle homogenates, 30-60 µg of total protein was fractionated for Western blot on SDS-polyacrylamide gels, transferred to a reduced-fluorescence PVDF membrane, and incubated with one of the following antibodies: alix (#2171s, Cell Signaling Technology), TSG-101 (#sc-7964, Santa Cruz Biotechnology), CD-63 (#sc-5275, Santa Cruz), and thrombospondin-1 (TSP-1) (#14778, Cell Signaling). Following primary incubation horseradish peroxide–conjugated (#7074 Anti-Rabbit, #7076 Anti-Mouse, Cell Signaling) secondary antibodies were incubated with the membrane and detected using florescence

(Bio-Rad, Hercules, CA). Densitometric analysis was performed using Image Lab software (Bio-Rad). Results were normalized to alpha tubulin (#2144, Cell Signaling), or ponceau S stain (P3504, Sigma Aldrich).

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed for human VEGF (R&D, DVE00, Minneapolis, MN). Protocol from the manufacturer's instructions were followed and muscle homogenates were prepared at 0.25 µg/µl for a total volume of 100 µl as previously described (16). Briefly, 100 µl of each sample was analyzed and optical density was determined using a microplate reader (Multiskan, Fisher Scientific, Waltham, MA) at 450 nm and corrected by readings at 550 nm.

Muscle cross-section. Morphological analysis was completed as described previously (44). Twenty mg skeletal muscle tissue was embedded in an OCT / tragacanth mixture (~40mg tragacanth/1ml OCT) and frozen in liquid nitrogen cooled isopentane. Frozen muscle was then cut into 10µm cross sections by using a Leica CM1850 cryostat (Leica, Wetzlar, Germany). Frozen sections were fixed in acetone for 30 s, permeabilized and blocked in PBS containing 5% goat serum, 3% bovine serum albumin (BSA), 0.2% Triton X-100, and 0.1% sodium azide for 1 hr. For capillaries, mouse anti-human CD31 primary antibody (1:1000, BD Biosciences, Franklin Lakes, NJ). Cell Membranes were identified by staining with dystrophin (1:500; Abcam, Cambridge, MA, USA). For fiber type, samples were directly permeabilized and blocked with blocking buffer and MyHC1 (type 1) antibody from mAB clones (American Type Culture Collection, Manassas, VA, USA).

Following primary antibody, sections were incubated with fluorescent labeled anti-IgG secondary antibodies, goat anti-rabbit 647 for dystrophin (1:1000 Thermo Fisher Scientific, Waltham, MA, USA), goat anti-mouse 568 for CD31(1:1000 Thermo Fisher Scientific) for

1 hr. Fluorescent images were captured with a CoolSnap HQ charge coupled camera (Photometrics, Tucson, AZ, USA) by using a Leica DM6000 microscope. Capillary number was quantified using the manual count tool in Photoshop (Adobe, San Jose, CA, USA). Fiber types were traced and recorded by immunostaining images (red for type 1, non-stained for type 2). Fiber size was determined by the magic wand tool in Photoshop; measurement was performed after setting the measurement scale (1 pixel= 0.57 μm; 100x magnification) (Adobe, San Jose, CA, USA). Three subjects (1 LN, 2 OB) were excluded from morphological analysis due to improper mounting of tissue samples at the Pre biopsy which prevented proper staining and subsequent analysis.

The following measurements of capillarization were made: 1) the number of capillaries around a muscle fiber, 2) capillary-to-fiber ratio, 3) capillary-to-fiber perimeter exchange index (CFPE) and 4) capillary density.

Statistical analysis. Unpaired Student's t-tests were used to analyze differences in subject characteristics between LN and OB. Otherwise, a two-way mixed-plot factorial ANOVA (group × condition) was used. Following a significant F ratio, a Fisher's LSD was used. Linear regression was performed to investigate relationships between variables. Significance was established at $P \leq 0.05$ for all statistical sets and data reported as Mean ± SE. All data were analyzed using Prism Graphpad Software Version 7 (La Jolla, CA, USA) or Microsoft Excel 2016 (Version 1708, Redmond, WA, USA).

Results

Subject characteristics. By design, body mass index (BMI) was greater in the OB group compared to LN. OB subjects also had lower relative $\dot{V}O_{2Max}$ values, and increased fasting insulin, HOMA-IR, and triglycerides (Table 4).

MVB biogenesis and release pathway. To determine the effect of obesity and REx on MVB biogenesis, gene expression of ESCRT proteins was determined. A main effect for BMI was observed for HGS and VPS4a mRNA (Figure 7). No differences were observed for BMI with clathrin, TSG-101, alix, STAM, or VTA1. In response to REx, a trend was observed for clathrin, with increased gene expression 3 hr compared to Pre. No other changes were observed for ESCRT protein gene expression with REx. There was an increase in CD63 mRNA in response to exercise at 3 hr compared to Pre and 15 min independent of group (Figure 7).

To identify potential changes in MVB release, gene expression of Rab and syntaxin family proteins known to be involved in MVB release were determined. There were no changes in gene expression for Rab 11, 27A, 27B, 35 or Syntaxin1A between LN and OB subjects at rest or in response to REx (Figure 8).

miRNA processing biogenesis. To detect the effect of obesity and REx on potential miRNA formation, gene expression for drosha, exportin-5, and dicer were measured. No observed differences at rest in OB subjects or in response to REx were observed for miRNA processing (Figure 9).

Exosome surface marker protein expression. Common exosome surface markers include alix, CD63, and TSG-101, therefore, Western blot analysis was performed for these proteins to identify potential changes in muscle exosome content. There was no effect of BMI for alix, TSG-101, or CD63. Changes were identified for alix and TSG-101 total protein in response to REx, and a trend for CD-63 (Figure 10). Post-hoc analysis revealed decreased alix protein 15 min and 3 hr post-exercise vs. Pre and 3 hr vs 15 min post-

exercise. TSG-101 protein displayed increased protein content in response to REx at 3 hr. The trend in CD63 showed reduced protein content 15 min vs Pre.

Skeletal muscle angiogenesis. No main effect for BMI was observed in the gene expression of VEGF or FLK-1. VEGF gene expression was increased (~3 fold) at 3 hr post-exercise in response to REx, with no change in FLK-1. TSP-1, displayed an interaction effect, with no change in LN subject's response and increased gene expression Pre vs. 15 min and 15 min vs. 3 hr in the OB group (Figure 11). At rest, a main effect in the OB group is noted for miR-130a, -206, and -503 with no REx induced changes in miR-126, -130a, -206, or -503 (Figure 11).

Whole muscle VEGF protein was measured via ELISA (Figure 12). There was no change in VEGF in response to REx with a trending main effect of lower VEGF protein in OB compared to LN.

Skeletal muscle mitochondrial regulators. Gene expression of PGC-1α and miR133a were measured to determine mitochondrial biogenesis at rest and acute REx. There was no effect of BMI on PGC-1α at rest, but increased expression 3 hr post-REx (~2 fold) (Figure 13). There were no differences between groups or in response to REx for miR133a.

Muscle morphology. There was no BMI effect observed for capillary counts, capillary-to-fiber ratio, or CFPE for total, type I or type II muscle fibers (Figure 14). Capillary density was reduced in OB type II fibers compared to LN.

Discussion

There are many differences in skeletal muscle between LN and OB individuals. In the current report, differences between LN and OB were investigated in pathways associated with intercellular communication at rest and following acute REx. The principal findings

are: 1) REx increased TSP-1 mRNA only in OB; 2) lower expression of components of MVB biogenesis and competing anti- and pro-angiogenic miRNA are present in OB; and 3) REx-induced increases in PGC-1α and VEGF mRNA were similar between groups. Taken together, the findings provide novel insight into the effects of obesity and REx on skeletal muscle intercellular communication pathways, which are still poorly understood (40).

Angiogenesis and anti-angiogenic effect of REx. Global deletion of TSP-1 increases skeletal muscle capillarization and exercise capacity in mice (34) and increased TSP-1 gene expression is associated with increased obesity, inflammation, and insulin resistance (47). In contrast with these data, the current investigation displays an observably low TSP-1 gene expression in OB at rest compared to lean (~70%), and a large TSP-1 response in OB 3 hr post-REx. Additionally, a trend for lower VEGF protein at rest in OB compared to LN supports the diminished capillary density observed in the OB Type II muscle fibers. Although both groups increased VEGF gene expression in response to REx, the increased anti-angiogenic response in the OB group indicates a large inhibitory response to any VEGF promoted adaptations which otherwise may have occurred in OB subjects.

MVB biogenesis altered with obesity. A fundamental role of the ESCRT machinery in the formation of exosomes is to sort ubiquitinylated proteins (4). HGS and STAM are shown to be involved in this process by sequestering ubiquitinylated proteins and communicating with associated deubiquitinating proteins following sequestration (48). The present study shows a BMI specific dysregulation of HGS and VPS4a gene expression. Altered sorting (HGS) and degradation (VPS4a) of MVB's may indicate an inability in the OB group to properly sequester proteins identified for transport in exosomes. Impaired

cellular communication is a common dysfunction associated with obesity (22), which could include signals to remove proteins from the cell. Diminished VPS4a may be compensatory to decreased HGS, yet underlying problems appear to exist with obesity for protein sorting. Gene expression for exosome release (Rab family) remains intact between LN and OB groups, demonstrating evidence that differences in protein content of exosomes could exist in adults, as previously observed in youth (11).

Clathrin, part of the ESCRT-0 complex, trends towards increased gene expression 3 hr post-REx, yet no alterations of the other measured ESCRT complex components occurred. The gene expression of TSG-101 and the accessory protein alix was also unchanged, despite altered total protein changes in response to REx. CD-63 is a known member of the tetraspanin family associated with microvesicle release and exosome marker in other cell types (35) and the present study showed enhanced gene expression of CD-63 3 hr post-REx. Recent work demonstrated a relationship between specific exosome markers and unique content contained therein (27). This discovery gives insight into potential characterization of exosome surface markers and their non-coding RNA content. Based on this new information, conducting proteomic and transcriptomic analysis of exosomes based on their surface marker could provide valuable information about the response to REx in each group, such as hypertrophy or angiogenic signaling.

miRNA processing biogenesis. Increased gene expression of miRNA processing (drosha, exportin-5, and dicer) was identified 3 hr after an acute cycling bout (39) and data within our lab has observed increased miRNA machinery following acute aerobic and aerobic + REx bouts 1 hr post-exercise (data unpublished). The data displayed in the current investigation identifies no main effect of BMI at rest for miRNA processing nor in

response to REx. Although there is evidence to suggest that specific miRNA will increase following acute REx (41), and REx training will alter miRNA profiles over time (6), there was no data from the current investigation that miRNA processing in LN or OB individuals is altered by REx for the time points selected.

Exosome surface marker protein changes. As previously identified, there is a gap in the literature for the effects of REx alone on exosome biogenesis (40). The current investigation identifies reduced alix protein expression 15 min post-exercise, which remained decreased 3 hr post-exercise (Figure 10). TSG-101 protein was increased 3 hr post-exercise. CD-63 protein shows a trend for reduced protein 15 min post-exercise. Previously, evidence suggested peak exosome surface marker release on different days during myotube differentiation *in vitro* (38), and vesicles released from myotubes are known to halt proliferation and induce differentiation of myoblasts (13, 14). Therefore, altered exosome surface marker expression in response to REx could indicate alternative functions of exosomes, depending on their surface marker.

miRNA. Following a REx bout, circulating miRNA were shown to increase in human plasma samples (41). Acute REx showed decreased expression of miR-1 in young but not old subjects at 3 and 6 hours post-exercise, with no changes in miR-133 and -206 (8). miR-133 was shown to increase immediately following REx, but returned to baseline 1 hour post-exercise, with no change in miR-126 (46). The present investigation displayed no changes to miR-126, -130a, -133a, -206, or -503, but exhibit a main effect of BMI for increased expression of miR-130a, -206, and -503 (Figure 11 & 13). Capillarization is enhanced by miR130a by blocking anti-angiogenic promoters GAX and HOXA5 (3). Angiogenesis is decreased with miR-206 and miR-503 by impacting VEGF and NFκB

expression (28, 43) and inhibiting the proliferative cell cycle in endothelial cells (2). Taken together, the data shows miRNA signaling to enhance (miR-130a) and block (miR-206, -503) angiogenesis, matching the VEGF/TSP-1 signaling observed in OB subjects.

Skeletal muscle morphology and capillarization. Previously, our lab demonstrated capillary density to be reduced in OB men compared to LN with no differences in VEGF content (17). The diminished capillary density observed with obesity for Type II fibers (Figure 14) displays an inherent limitation in OB subjects to respond to chronic overload hypertrophy due to increased body mass. The anti-angiogenic response observed in the current investigation through miRNA signals at rest and the TSP-1 response to REx encourages the idea that a single bout of REx is not enough stimulus to overcome the diminished capillarization observed in OB. Consistent, regular physical activity would be required to stimulate angiogenic growth. It is unknown how many REx sessions would be required to overcome the apparent TSP-1 response in OB, yet it is well observed that an anti-angiogenic response represents the balance to keep capillarization in check (36).

Obesity and exosomes. Adipose derived exosomes from OB mice induce insulin resistance in LN wild-type counterparts, demonstrating the ability of exosomes to communicate detrimental signals in obesity (7, 22). Altered content from adipose-derived exosomes are also observed in OB children compared to LN (11). *In vitro* and *in vivo* models of c2c12 myotubes incubated with high palm oil, or mice fed a high fat diet, released skeletal muscle exosomes which transferred deleterious effects on insulin signaling (1). As research evolves with obesity and exosome signals, it would be recommended to identify exosome content differences which may alter metabolic signals and impact intercellular function in adjacent tissues.

Conclusion. Overall, altered patterns of exosome marker protein exist in response to REx, with no changes in exosome release or miRNA processing between groups. Obesity plays an inhibitory role in MVB production pathway gene expression, anti-angiogenic factors, and miRNA to both inhibit and enhance angiogenesis. Future research should focus on the content of exosomes between LN and OB subjects to determine potential therapeutic targets in an OB population.

References

1. Aswad H, Forterre A, Wiklander OP et al. Exosomes participate in the alteration of muscle homeostasis during lipid-induced insulin resistance in mice. *Diabetologia*. 2014;57(10):2155-64.
2. Caporali A, Meloni M, Völlenkle C et al. Deregulation of microRNA-503 contributes to diabetes mellitus–induced impairment of endothelial function and reparative angiogenesis after limb ischemia. *Circulation*. 2011:CIRCULATIONAHA. 110.952325.
3. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood*. 2008;111(3):1217-26.
4. Colombo M, Moita C, van Niel G et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci*. 2013;126(24):5553-65.
5. Costill D, Pearson D, Fink W. Impaired muscle glycogen storage after muscle biopsy. *Journal of Applied Physiology*. 1988;64(5):2245-8.
6. Davidsen PK, Gallagher IJ, Hartman JW et al. High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression. *Journal of applied physiology*. 2010;110(2):309-17.
7. Deng Z-b, Poliakov A, Hardy RW et al. Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes*. 2009;58(11):2498-505.
8. Drummond MJ, McCarthy JJ, Fry CS, Esser KA, Rasmussen BB. Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. *American Journal of Physiology-Endocrinology and Metabolism*. 2008;295(6):E1333-E40.
9. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *The lancet*. 2005;365(9468):1415-28.
10. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. *Nature Reviews Cancer*. 2006;6(4):259-69.

11. Ferrante SC, Nadler EP, Pillai DK et al. Adipocyte-derived exosomal miRNAs: a novel mechanism for obesity-related disease. *Pediatric research*. 2014;77(3):447-54.
12. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *American Journal of Physiology-Cell Physiology*. 2001;280(6):C1358-C66.
13. Forterre A, Jalabert A, Berger E et al. Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk? *PLoS One*. 2014;9(1):e84153.
14. Forterre A, Jalabert A, Chikh K et al. Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. *Cell cycle*. 2014;13(1):78-89.
15. Gavin T, Drew J, Kubik C, Pofahl W, Hickner R. Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta physiologica*. 2007;191(2):139-46.
16. Gavin TP, Robinson CB, Yeager RC, England JA, Nifong LW, Hickner RC. Angiogenic growth factor response to acute systemic exercise in human skeletal muscle. *Journal of applied physiology*. 2004;96(1):19-24.
17. Gavin TP, Stallings HW, Zwetsloot KA et al. Lower capillary density but no difference in VEGF expression in obese vs. lean young skeletal muscle in humans. *Journal of applied physiology*. 2005;98(1):315-21.
18. Graham T, Kahn B. Tissue-specific alterations of glucose transport and molecular mechanisms of intertissue communication in obesity and type 2 diabetes. *Hormone and metabolic research*. 2007;39(10):717-21.
19. Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of Obesity Among Adults and Youth: United States, 2015–2016.
20. Hayden MR, Patel K, Habibi J et al. Attenuation of Endocrine-Exocrine Pancreatic Communication in Type 2 Diabetes: Pancreatic Extracellular Matrix Ultrastructural Abnormalities. *Journal of the cardiometabolic syndrome*. 2008;3(4):234-43.
21. Howley ET, Bassett DR, Welch HG. Criteria for maximal oxygen uptake: review and commentary. *Medicine and science in sports and exercise*. 1995;27(9):1292-301.
22. Huang-Doran I, Zhang C-Y, Vidal-Puig A. Extracellular vesicles: novel mediators of cell communication in metabolic disease. *Trends in Endocrinology & Metabolism*. 2017;28(1):3-18.
23. Hudlicka O, Brown M, Egginton S. Angiogenesis in skeletal and cardiac muscle. *Physiological reviews*. 1992;72(2):369-417.
24. Isenberg JS, Wink DA, Roberts DD. Thrombospondin-1 antagonizes nitric oxide-stimulated vascular smooth muscle cell responses. *Cardiovascular research*. 2006;71(4):785-93.
25. Ivy JL. Role of exercise training in the prevention and treatment of insulin resistance and non-insulin-dependent diabetes mellitus. *Sports medicine*. 1997;24(5):321-36.

26. Kang K, Ma R, Cai W et al. Exosomes secreted from CXCR4 overexpressing mesenchymal stem cells promote cardioprotection via Akt signaling pathway following myocardial infarction. *Stem cells international*. 2015;2015.
27. Kaur S, Elkahloun AG, Arakelyan A et al. CD63, MHC class 1, and CD47 identify subsets of extracellular vesicles containing distinct populations of noncoding RNAs. *Scientific reports*. 2018;8(1):2577.
28. Keklikoglou I, Hosaka K, Bender C et al. MicroRNA-206 functions as a pleiotropic modulator of cell proliferation, invasion and lymphangiogenesis in pancreatic adenocarcinoma by targeting ANXA2 and KRAS genes. *Oncogene*. 2015;34(37):4867.
29. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. *Immunology letters*. 2006;107(2):102-8.
30. Kraus RM, Stallings HW, 3rd, Yeager RC, Gavin TP. Circulating plasma VEGF response to exercise in sedentary and endurance-trained men. *J Appl Physiol (1985)*. 2004;96(4):1445-50.
31. Krotkiewski M, Bylund-Fallenius AC, Holm J, Bjorntorp P, Grimby G, Mandroukas K. Relationship between muscle morphology and metabolism in obese women: the effects of long-term physical training. *Eur J Clin Invest*. 1983;13(1):5-12.
32. Lillioja S, Young AA, Culter CL et al. Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *The Journal of clinical investigation*. 1987;80(2):415-24.
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods*. 2001;25(4):402-8.
34. Malek MH, Olfert IM. Global deletion of thrombospondin-1 increases cardiac and skeletal muscle capillarity and exercise capacity in mice. *Exp Physiol*. 2009;94(6):749-60.
35. Nakamura K, Jinnin M, Harada M et al. Altered expression of CD63 and exosomes in scleroderma dermal fibroblasts. *Journal of dermatological science*. 2016;84(1):30-9.
36. Olfert IM, Birot O. Importance of anti-angiogenic factors in the regulation of skeletal muscle angiogenesis. *Microcirculation*. 2011;18(4):316-30.
37. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nature Reviews Endocrinology*. 2012;8:457.
38. Romancino DP, Paterniti G, Campos Y et al. Identification and characterization of the nano-sized vesicles released by muscle cells. *FEBS letters*. 2013;587(9):1379-84.
39. Russell AP, Lamon S, Boon H et al. Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. *The Journal of physiology*. 2013;591(18):4637-53.
40. Safdar A, Saleem A, Tarnopolsky MA. The potential of endurance exercise-derived exosomes to treat metabolic diseases. *Nat Rev Endocrinol*. 2016;12(9):504-17.
41. Sawada S, Kon M, Wada S, Ushida T, Suzuki K, Akimoto T. Profiling of circulating microRNAs after a bout of acute resistance exercise in humans. *PloS one*. 2013;8(7):e70823.

42. Simoneau J, Colberg S, Thaete FL, Kelley D. Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women. *The FASEB Journal*. 1995;9(2):273-8.
43. Stahlhut C, Suárez Y, Lu J, Mishima Y, Giraldez AJ. miR-1 and miR-206 regulate angiogenesis by modulating VegfA expression in zebrafish. *Development*. 2012;139(23):4356-65.
44. Sullivan B. Effects of Obesity and Resistance Exercise on the Regulation of Skeletal Muscle Fiber Size. Purdue University; 2017.
45. Tang K, Breen EC, Gerber H-P, Ferrara NM, Wagner PD. Capillary regression in vascular endothelial growth factor-deficient skeletal muscle. *Physiological genomics*. 2004;18(1):63-9.
46. Uhlemann M, Möbius-Winkler S, Fikenzer S et al. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. *European journal of preventive cardiology*. 2014;21(4):484-91.
47. Varma V, Yao-Borengasser A, Bodles AM et al. Thrombospondin-1 is an adipokine associated with obesity, adipose inflammation, and insulin resistance. *Diabetes*. 2008;57(2):432-9.
48. Wright MH, Berlin I, Nash PD. Regulation of endocytic sorting by ESCRT–DUB-mediated deubiquitination. *Cell biochemistry and biophysics*. 2011;60(1-2):39-46.
49. Zhou B, Ma R, Si W et al. MicroRNA-503 targets FGF2 and VEGFA and inhibits tumor angiogenesis and growth. *Cancer letters*. 2013;333(2):159-69.
50. Ziche M, Morbidelli L. Nitric oxide and angiogenesis. *Journal of neuro-oncology*. 2000;50(1-2):139-48.

Table 4. Subject Characteristics. BMI, body mass index; $\dot{V}o_{2MAX}$, maximal oxygen consumption; KE, maximal knee extensor strength; HOMA-IR, homeostasis model assessment – insulin resistance; HOMA-β, homeostatic model assessment – cell function; HDL, high density lipoprotein; LDL, low density lipoprotein. Mean ± SE. $N = 8$ LN, $N = 8$ OB. *p≤0.05

	LN	**OB**
Age, y	21.5 ± 0.5	24.8 ± 1.6
Height, m	1.7 ± 0.03	1.73 ± 0.03
Mass, kg	62.4 ± 2.0	109.9 ± 7.8*
BMI, kg/m^2	21.7 ± 0.8	36.6 ± 1.8*
$\dot{V}o_{2MAX}$, L×min^{-1}	2.34 ± 0.2	2.84 ± 0.26
$\dot{V}o_{2MAX}$, ml×kg^{-1}×min^{-1}	37.3 ± 2.7	26.1 ± 2.7*
1-RM, kg	27.8 ± 0.7	40.3 ± 4.7*
1-RM, kg/kg	0.45 ± 0.01	0.37 ± 0.04
Fasting Glucose, mg/dl	86.1 ± 4.8	89.0 ± 4.9
Fasting Insulin, µIU/ml	11.8 ± 1.3	28.3 ± 4.8*
HOMA-IR	2.46 ± 0.29	6.38 ± 1.23*
Total Cholesterol, mg/dl	162.8 ± 12.3	180.6 ± 6.0
HDL, mg/dl	58.4 ± 7.3	42.9 ± 5.4
LDL, mg/dl	88.6 ± 12.8	108.6 ± 5.2
Triglycerides, mg/dl	79.4 ± 7.7	131.6 ± 22.5*

Table 5. Gene sequences of mRNA and miRNA, obtained from the Harvard Primer Bank. Apoptotic linked gene-2 interacting protein X (Alix); Tumor Susceptibility Gene-101 (TSG-101); hepatocyte growth factor-regulated tyrosine kinase substrate (HGS); signal transducing adaptor molecule (STAM); vesicle trafficking 1 (Vta1); vacuolar protein sorting mutant (Vps4a).

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
Alix	ATGGCGACATTCATCTCGGTG	CGCTTGGGTAAGTCTGCTGG
Clathrin	ATTCTGCCAATTCGTTTTCAGGA	GCTTTCAGTGCAATTACTTTGCT
TSG-101	GAGAGCCAGCTCAAGAAAATGG	TGAGGTTCATTAGTTCCCTGGA
CD63	CAGTGGTCATCATCGCAGTG	ATCGAAGCAGTGTGGTTGTTT
Dicer	GAGCTGTCCTATCAGATCAGGG	ACTTGTTGAGCAACCTGGTTT
Exportin-5	ATCCTGGAACACGTTGTCAAG	CACTACAATTCGAGACAGAGCAT
Drosha	TGTCACAGAATGTCGTTCCAC	GGGCCTAAAGGATGGTGCT
HGS	CTCCTGTTGGAGACAGATTGGG	GTGTGGGTTCTTGTCGTTGAC
STAM	AATCCCTTCGATCAGGATGTTGA	CGAGACTGACCAACTTTATCACA
VTA1	CTCCCCGCACAGTTCAAGAG	AACGACAGTAATAAGCCACCAC
Vps4a	CCACGCTATCAAGTATGAGGC	CCGTGTTTCTCTTTGCTTCGTA
Rab11A	CAACAAGAAGCATCCAGGTTGA	GCACCTACAGCTCCACGATAAT
Rab27A	GCTTTGGGAGACTCTGGTGTA	TCAATGCCCACTGTTGTGATAAA
RAB27B	TAGACTTTCGGGAAAAACGTGTG	AGAAGCTCTGTTGACTGGTGA
RAB35	TACTGTTGCGTTTTGCAGACA	CCCCGATAATACGTGGAGGTG
Syntaxin 1A	CGAGACCGCTTCATGGATGAG	ACTTGGAACGAACTTTGTTTGC
miR-126	TCGTACCGTGAGTAATAATGCG	
miR-130a	CAGTGCAATGTTAAAAGGGCAT	
miR-133a	TTTGGTCCCCTTCAACCAGCTG	
miR-206	TGGAATGTAAGGAAGTGTGTGG	
miR-503	TAGCAGCGGGAACAGTTCTGCAG	

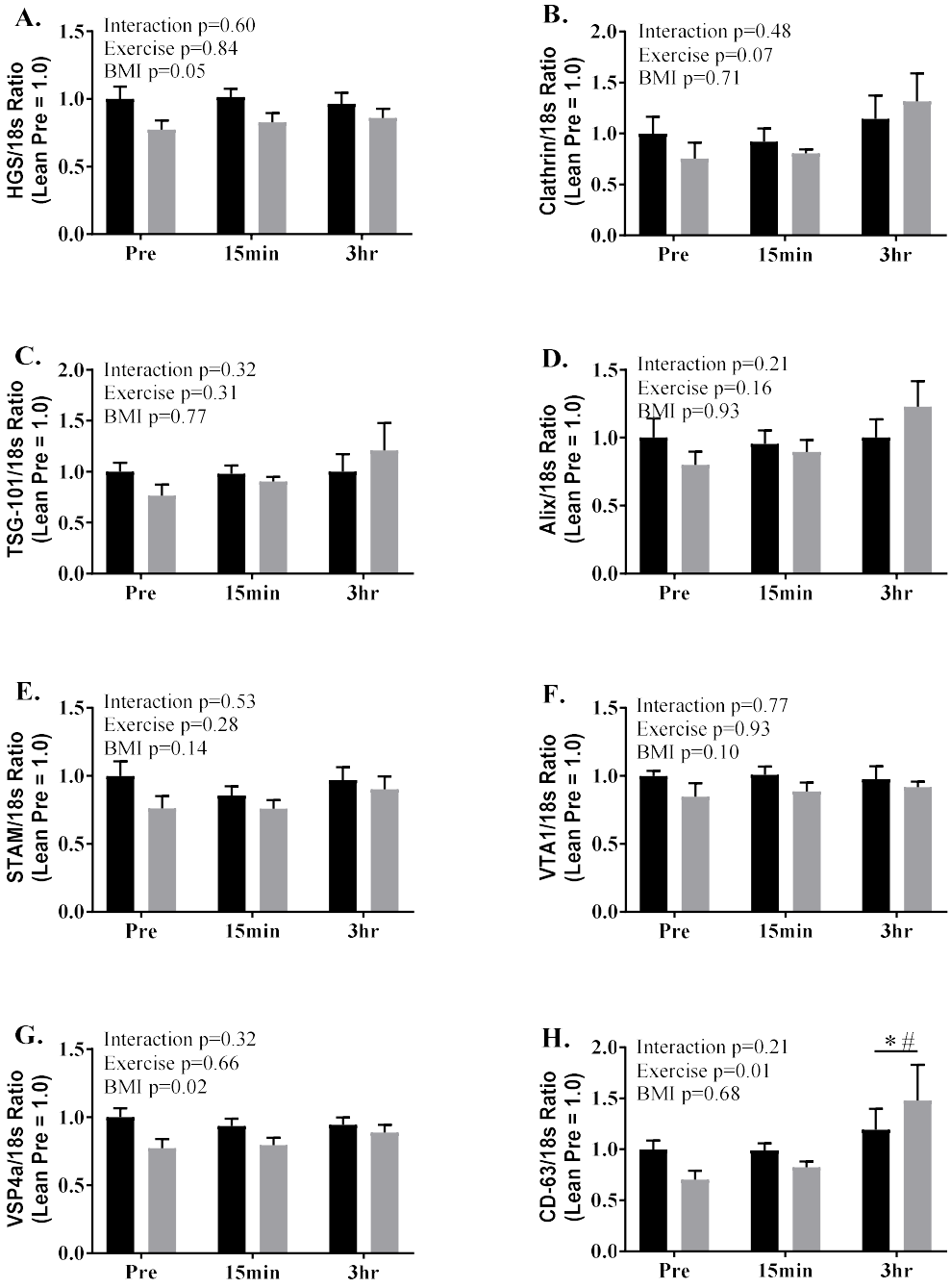

Figure 7. Gene expression analysis of MVB biogenesis/ESCRT in response to REx for LN (black) and OB (gray) subjects (n=8 each group). ESCRT-0 proteins hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) (A) and clathrin (B), ESCRT-I proteins Tumor Susceptibility Gene-101 (TSG-101) (C), Apoptotic linked gene-2 interacting protein X (Alix) (D), ESCRT-III proteins signal transducing adaptor molecule (STAM) (E), vesicle trafficking 1 (Vta1) (F), vacuolar protein sorting mutant (Vps4a) (G), and a known exosome surface marker, CD-63 (H). *p≤0.05 vs Pre; #p≤0.05 vs 15 min. Data are presented as Mean ± SE.

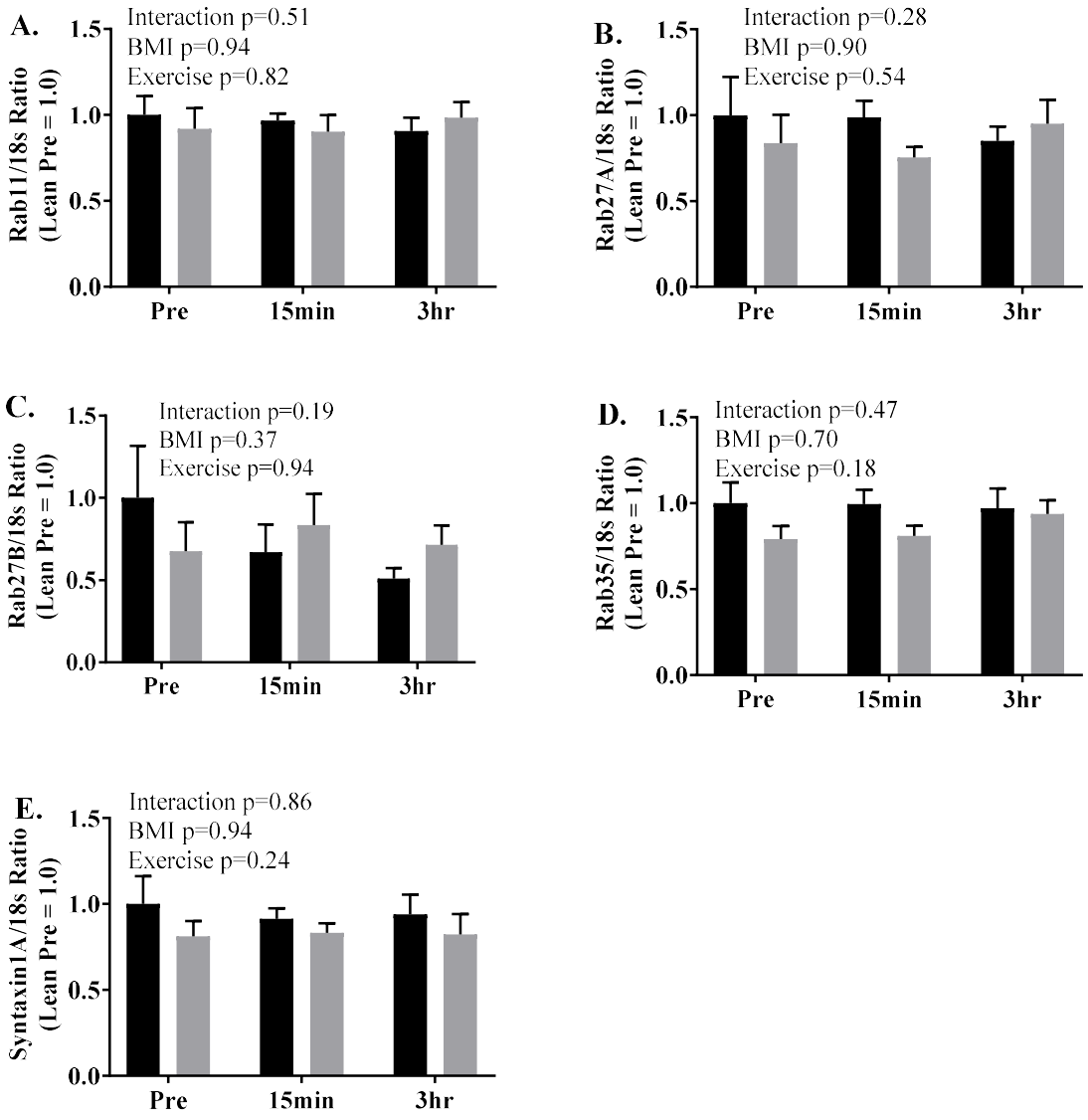

Figure 8. Gene expression analysis of MVB/exosome release for LN (black) and OB (gray) subjects (n=8 each group). Rab and syntaxin family proteins are associated with MVB fusion with the membrane, leading to exosome release. Data are presented as Mean ± SE.

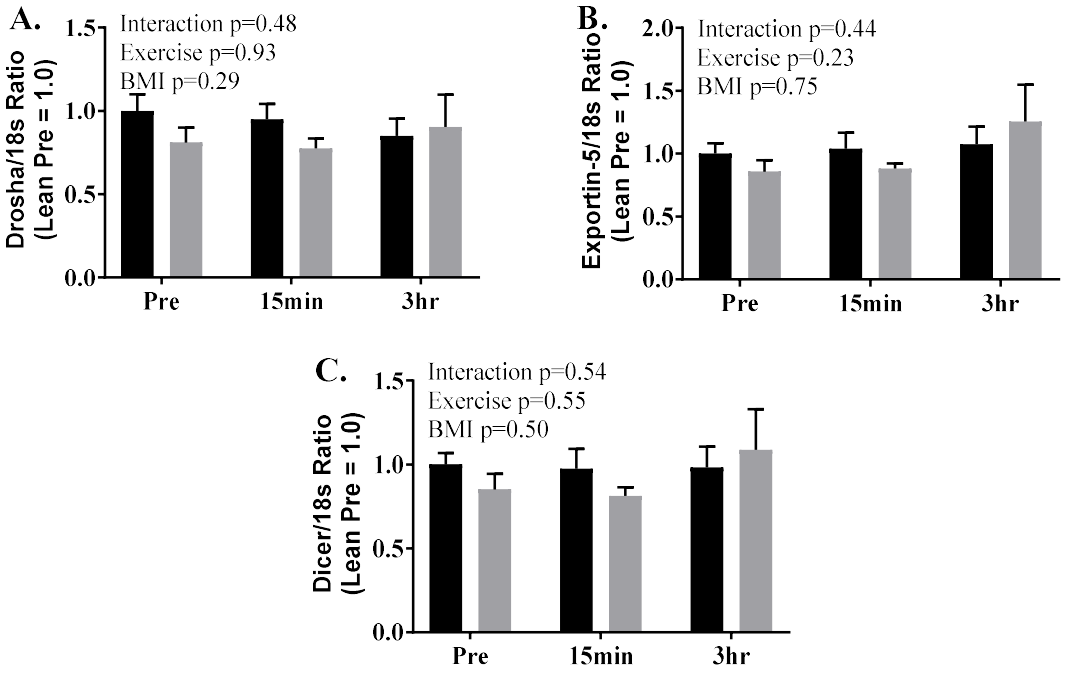

Figure 9. Gene Expression analysis of miRNA processing pathway drosha (A), exportin-5 (B), and dicer (C) for LN (black) and OB (gray) subjects (n=8 each group). Data are presented as Mean ± SE.

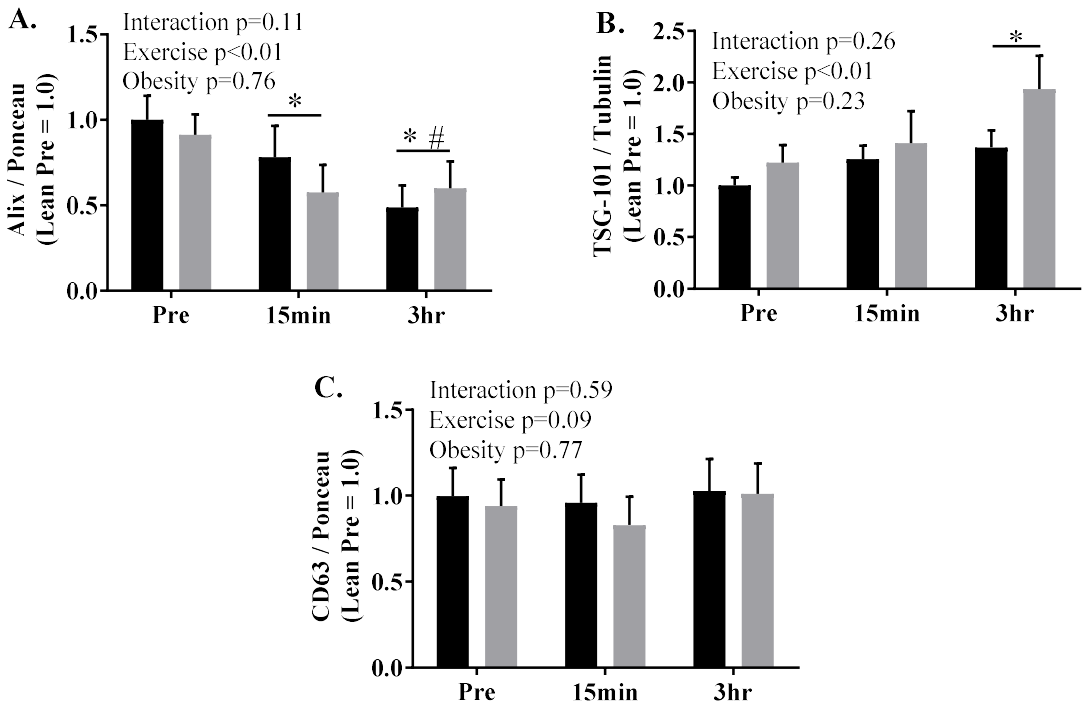

Figure 10. Western blot analysis of exosome surface markers for Alix (A), TSG-101 (B), and CD63 (C). LN (black) and OB (gray) subjects (n=8 each group). *p≤0.05 vs Pre, #p≤0.05 vs 15 min. Data are presented as Mean ± SE.

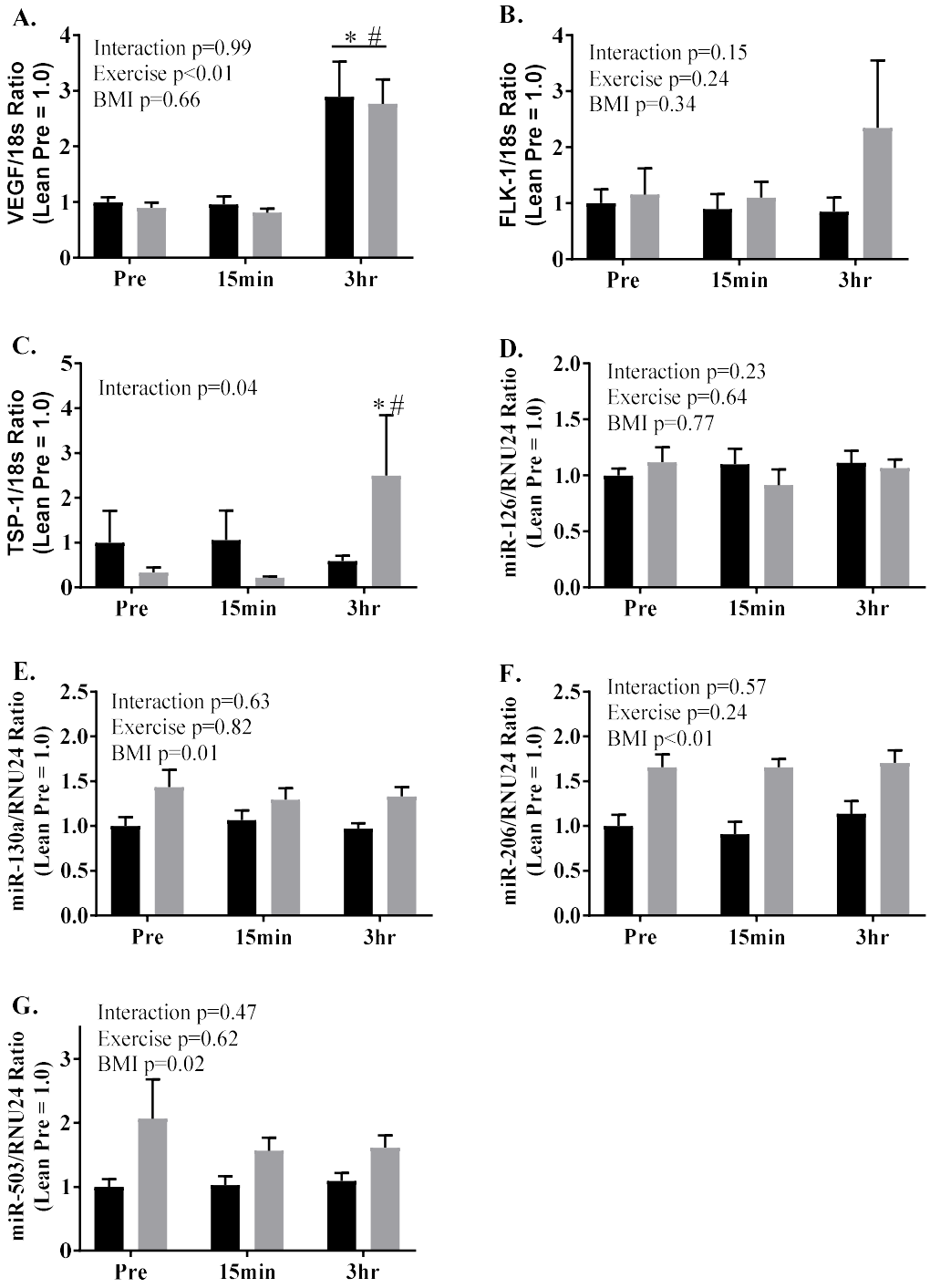

Figure 11. Gene expression analysis of angiogenic factors VEGF, TSP-1, FLK-1 and miRNA. LN (black) and OB (gray) subjects (n=8 each group). *p≤0.05 vs. Pre, #p≤0.05 vs 15 min. Data are presented as Mean ± SE.

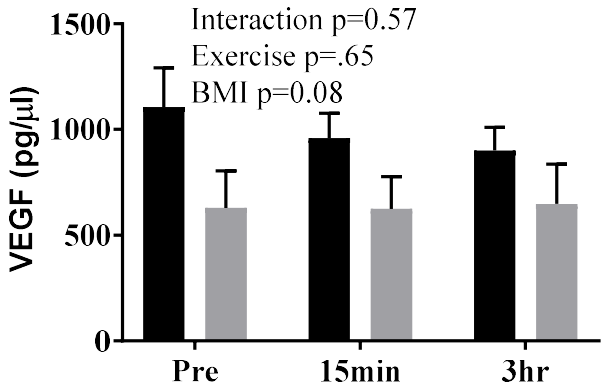

Figure 12. Vascular endothelial growth factor (VEGF) ELISA measuring total VEGF in skeletal muscle homogenates isolated in RIPA buffer. LN (Black) and OB (Gray) subjects (n=8 each group) are shown. Data are presented as Mean ± SE.

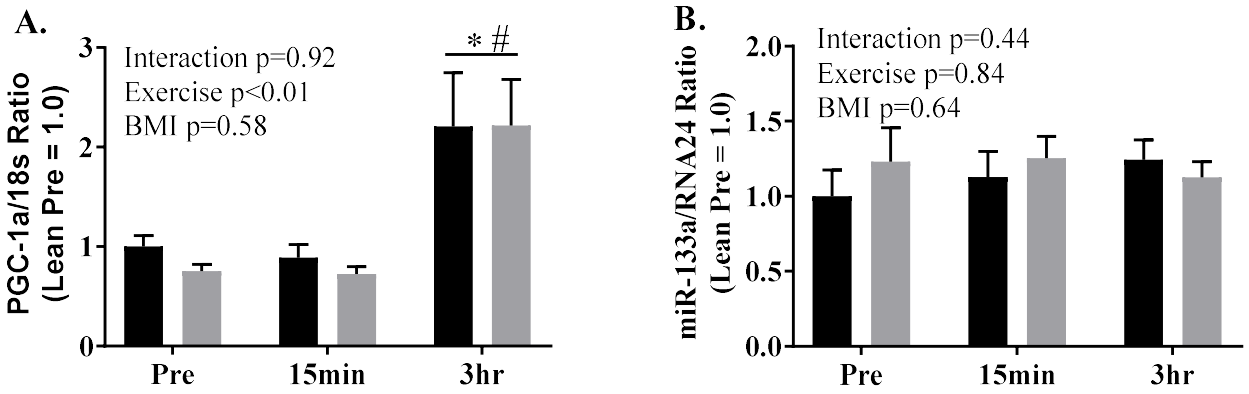

Figure 13. Gene expression analysis of mitochondrial factors PGC-1α and miR-133a. LN (black) and OB (gray) subjects (n=8 each group). *p≤0.05 vs. Pre, #p≤0.05 vs 15 min. Data are presented as Mean ± SE.

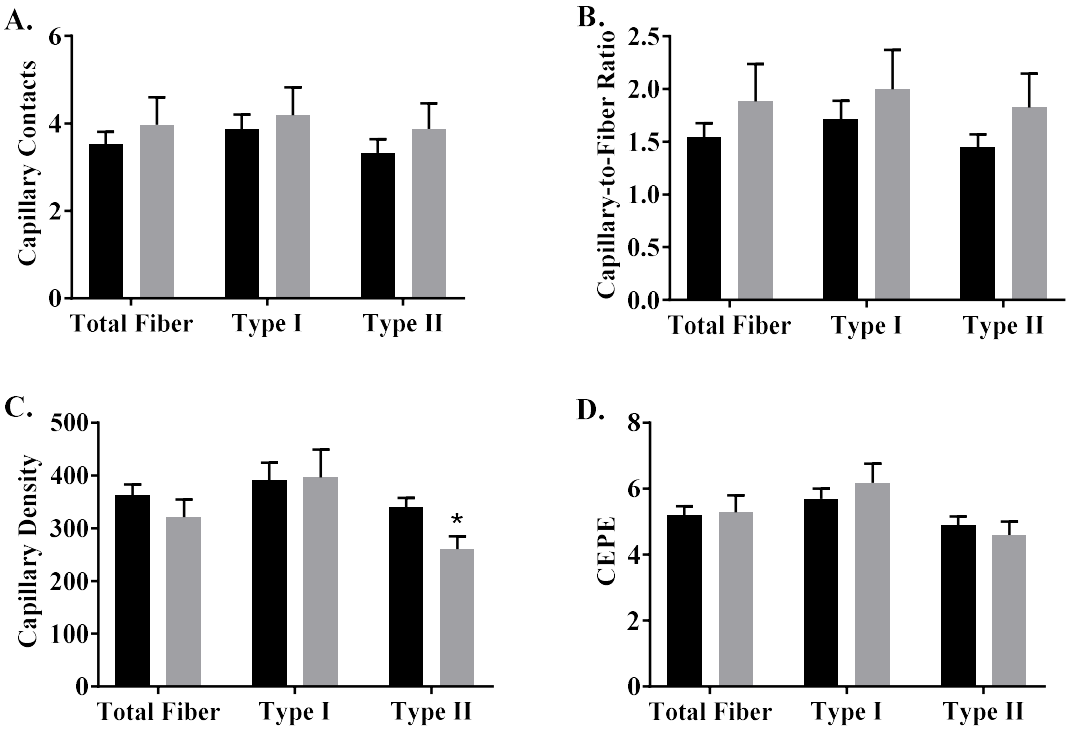

Figure 14. Muscle capillarization determined between LN (black) (n=7) and OB (gray) (n=6) subjects. Data displays capillary contacts (A), capillary-to-fiber ratio (B), capillary density (C), and capillary-to-fiber perimeter exchange index (CFPE) (D). Data are presented as Mean ± SE.

CHAPTER 4. ROLE OF CAMKII IN SKELETAL MUSCLE EXOSOME RELEASE

Ron T. Garner, Christopher K. Kargl, Yaohui Nie, Timothy P. Gavin
Purdue University, Max E. Wastl Human Performance Laboratory, West Lafayette, IN

Corresponding Author: Timothy P. Gavin, Purdue University, Health and Kinesiology Department, 800 West Stadium Avenue, West Lafayette, IN, 47906.
Email: gavin1@purdue.edu

Abstract

Introduction: Multivesicular bodies (MVB's) have generated interest in understanding intercellular communication. MVB's bud internally to form exosomes, which have potential for organ cross-talk once released. Limited data suggests exosomes are released from skeletal muscle in response to exercise. The role of skeletal muscle with exosome signaling is not well understood. Calcium is known to be involved with exosome release in other tissues, and since skeletal muscle is highly regulated by calcium signaling during muscle contraction, it is a likely pathway. One candidate is calcium-calmodulin kinase II (CaMKII), known to release other small vesicles upon activation. Electrical pulse stimulation (EPS) is an *in vitro* approach to activate differentiated skeletal muscle myotubes. We hypothesized that skeletal muscle will release exosomes, controlled in part through CaMKII, in response to EPS.

Methods: Skeletal muscle biopsies were obtained from lean, healthy, male college-aged subjects (n=8) and satellite cells isolated for use in cell culture (SkMCC). To ensure proper differentiation, SkMCC had markers pax7, Myogenin (MyoG), and myosin heavy chain (MHC) measured via Western blot on day's 0, 2, 4, 6, and 8 in a subset of cells. SkMCC were stimulated for 30 min with EPS to validate activation of *in vivo* exercise

pathways adenosine monophosphate kinase (AMPK), mitogen activated protein kinase (MAPK) and CaMKII. SkMCC underwent 24 h of EPS with and without the addition of KN-93, a chemical inhibitor of CaMKII. Quantification of isolated exosomes following EPS was accomplished by use of a NanoSight, rt-PCR, and Western blot analysis.

Results: Day 8 compared to day 6 appeared to be the ideal day for differentiation with respect to MHC protein and formation of myotubes. No differences were observed with EPS or CaMKII inhibition to alter MVB biogenesis, release, or miRNA processing pathways. There was a marked reduction of nanoparticles observed in response to EPS compared to control.

Discussion: The main finding of the current investigation was despite an identifiable change in MHC and formation of myotubes, there appears to have been minimized contractile activity *in vitro* leading to activation of metabolic pathways and MVB release. Strong trends for the phosphorylation of AMPK and clathrin gene expression, reduced gene expression of PDK4, and reduced total nanoparticles in response to EPS indicate that changes occurred, though not to the anticipated levels based on preliminary data.

Introduction

Skeletal muscle (SkM) contributes 40% of daily energy expenditure (58). As SkM is activated, myokines are released and act in paracrine or endocrine-like fashion to regulate targeted tissues (38). Aerobic exercise is a known stimulus to release myokines through contraction (38, 39). Coupled with SkM contraction is adequate calcium release from the sarcoplasmic reticulum, which leads to activation of several intracellular signaling pathways, including calcium-calmodulin kinase II (CaMKII) (1, 43, 44).

Another method for cell-to-cell communication is via exosomes, small vesicles (30-100nm) released by tissues such as mast and epithelial cells (29). These vesicles contain proteins, mRNA and miRNA which can regulate target tissues. For example, skeletal muscle myotube derived exosomes decrease myoblast proliferation by downregulating the cell cycle protein cyclin D and increase myoblast differentiation by upregulating the muscle development and repair protein myogenin (15, 16). This identifies exosomes as a potential source of paracrine signaling.

Exosome biogenesis occurs from the internal budding of multivesicular bodies (MVB) via four protein complexes called the endosomal sorting complex required for transport (ESCRT) (0, I, II, III) (12). ESCRT-0 recognizes and sequesters ubiquitinylated proteins and associates with several accessory proteins, including signal transducing adaptor molecule (STAM), hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), and tumor susceptibility gene 101 (TSG-101) (12, 53). As part of the ESCRT-I complex, TSG-101 recruits either the apoptotic linked gene-2 interacting protein X (alix) or the ESCRT-II complex (12, 25, 52). Alix or ESCRT-II will then recruit ESCRT-III, which is primarily responsible for scission of the MVB within the cell membrane (57) resulting in MVB fusion with the plasma membrane and exosome release (8, 25). MVB's not fused with the cell are degraded by a trafficking protein, vacuolar protein sorting mutant (Vps4a, Vps4b) (5).

Exosome release from the cell is a coordinated and well-documented process (4, 21, 42). Exosome release occurs through MVB fusion to the plasma membrane through soluble NSF attachment protein receptor complexes (SNARE) (4). Rab proteins are known to regulate SNARE protein function through direct or indirect contact (10, 24). Specific

members of the Rab and syntaxin family identified with exosome regulation include Rab11, 27a, 27b, 35, and Syntaxin 1A (4, 37). Increased expression of these factors indicate increased activity at the endosome, the location of MVB biogenesis (25, 37).

Exosome release can be regulated by multiple intracellular signals including altered pH, hypoxia, and oxidative stress, all of which are increased in response to exercise (42). Exosome release is increased via calcium signaling in mast cells and cortical neurons (54) as well as K-562 cells (47). Given that exercise-induced motor neuron activity leads to increases in intracellular calcium and myofiber contraction, calcium activated pathways may be important in regulating skeletal muscle exosome release. Exercise-induced activation of CaMKII has been implicated in muscle contraction (9, 49). CaMKII activation also regulates maristoylated alanine-rich C-kinase substrate (MARCKS), a membrane binding protein associated with vesicles ~100 nm in diameter (2). This association of CaMKII with membrane bound vesicles and skeletal muscle contraction make it a possible regulator for skeletal muscle exosomes.

In vivo treadmill and cycling exercise have been shown to release extracellular vesicles into the circulation, but the specific tissue of origin could not be determined (18). Using an *in vitro* modeling system allows the capture of vesicles released exclusively from skeletal muscle. Several attempts to identify skeletal muscle exosome "cross-talk" with other tissues has been accomplished in mice (3, 55) and C2C12 cell lines (15, 16). The regulation of SkM exosome release, particularly in human muscle, is poorly understood (34). As electrical pulse stimulation (EPS) has been used to induce muscle contraction of differentiated myotubes (33, 48), there is a potential to utilize EPS to enhance exosome release, which to our knowledge has not been accomplished. It was hypothesized that EPS

will stimulate the biogenesis and release of exosomes from skeletal muscle, moderated in part through CaMKII.

Methods

A multi-step approach was used to identify changes in MVB biogenesis and release in response to EPS. First, tube formation was optimized to ensure adequate growth and differentiation of primary human myoblasts into myotubes. Second, the phosphorylation of common exercise-stimulated pathways (adenosine monophosphate kinase (AMPK), mitogen activated protein kinase (MAPK), and CaMKII) in response to EPS provided validation of EPS use as an exercise mimetic. Third, myotubes were electrically stimulated for 24 hours with and without a CaMKII inhibitor to investigate regulation of exosome release.

Subjects. Subject characteristics are provided in Table 6. Lean, healthy, young (18-35 y) male subjects (n=8) volunteered for the study following informed consent approved by the University Institutional Review Board. Subjects underwent $\dot{V}O_{2Max}$ testing to characterize aerobic capacity. At least one week later, SkM biopsies from the vastus lateralis and blood draws were obtained following a 12 h fast. Sample size was calculated using a power analysis (α= 0.05, β= 0.80) from pilot work showing increased alix protein in the media from control (1.0 AU) compared to EPS (2.9 ± 1.3 AU).

Cell culture procedures. Myoblasts were isolated as previously described (7, 23). Briefly, SkM biopsies were minced and pre-plated on non-collagen coated plates for 3 h at 37˚C and 5% CO_2 to remove fibroblasts, then transferred to collagen coated plates with media changed every other day until confluence. Following isolation, myoblasts were sorted using Micro Bead CD-56$^+$ antibody (Miltenyi Biotec, Auburn, CA). Samples were

grown to confluence and transferred to six separate 6-well dishes at passage 4 beginning with approximately 75,000 cells/well.

Experimental Design. Study 1. For samples to be successfully electrically stimulated, it is essential to have myoblasts differentiate to myotubes as only myotubes express myosin heavy chain (MHC) myofilaments. Previous protocols established for differentiation of myotubes were followed (20, 31). In brief, myoblasts were grown in SkM growth media (Lonza, Basel Switzerland) to approximately 85-90% confluence on a 6-well plate, followed by serum-deprivation (2% Horse Serum in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher, Waltham, MA)). Protein was collected on day's 0, 2, 4, 6, and 8 to determine the progression of muscle differentiation markers pax7, MyoD, myogenin and MHC via western blot (6).

Immunostaining was performed to identify fusion index (FI) of myotubes on day 6 and day 8. For each sample analyzed (n=3), cells were washed 3X with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 5 min at room temperature, and washed again 3X with PBS. Cells were incubated with 100 mM glycine for 15 min, washed 3X with PBS, then incubated with blocking buffer (5% goat serum+2% BSA+ 0.2% Triton X-100 and 0.1% Sodium Azide in PBS) for 1-2 hours. Primary antibody was diluted in blocking buffer at 4^{o}C overnight with MF-20 at 1:1000 dilution (R&D, Minneapolis, MN). Secondary antibody (Alex568 IgG1, anti-mouse; Cell Signaling Technologies) and DAPI for nuclei (Cell Signaling Technologies) at 1:1000 dilution were incubated in PBS for 1 hour, then visualized by fluorescent microscopy. Myotubes which contained at least 2 nuclei were counted and FI was the total number of nuclei incorporated into MHC^{+} myotubes divided by the total number of nuclei in the field of view.

Study 2. To establish activation of known *in vivo* exercise-induced metabolic pathways, myotubes underwent control (exposure to stimulation plate without EPS activated) or EPS stimulation for 30 min at 14 V, 1 Hz, and 1 ms bursts. This intensity was determined from unpublished data in our lab using C2C12 cells to activate metabolic pathways, such as AMPK and matches protocols used in primary human SkM cultures (34). Exercise-stimulated pathways shown *in vivo* related to calcium signaling include CaMKII (1, 43, 44), AMPK (41), and MAPK (26). Following 30 min, cultures were washed with 1x PBS, collected in 100 µl RIPA buffer per well and used to identify phosphorylation changes of CaMKII, AMPK, and P-38 MAPK via western blot analysis. Combes et al. recently showed that immediately after 30 min of cycling exercise, SkM CaMKII, P38 MAPK, and AMPK were phosphorylated *in vivo* (13). A design of each plate is displayed in Figure 15.

Study 3. On Day 8 (determined in Study 1), media was changed from 2% HS DMEM to 10% exosome-free fetal bovine serum (FBS) DMEM for EPS. FBS was made exosome free via ultracentrifugation (47). Stock solution of FBS was centrifuged at 800 × g for 10 min to remove sediment, then centrifuged at 12,000 × g for 30 min to remove cellular debris. Exosomes were further isolated from the supernatant by centrifuging at 100,000 × g for 2 hours and removed.

The control condition was exposure to stimulation plate without EPS activation. EPS samples were stimulated for 24 h at 14 V, 1 Hz, 1 ms bursts, to allow an accumulation of exosomes in the media. In addition, KN-93, a chemical inhibitor of CaMKII, and KN-92 (Sigma-Aldrich, St. Louis, MO, USA), a negative control for KN-93, were added in control and EPS conditions. In total, six conditions were tested in duplicate: control (non-

stimulated) and EPS (stimulated), each with the following additions: 10 μM KN-92, 10 μM KN-93, and an equivalent volume of solvent (ddH2O) (Figure 15). Chemicals were added in 10 μM concentration, shown previously to effectively inhibit CaMKII activity in rat myotubes (36, 50). Following 24 h, SkM cells were collected in RIPA buffer (100μl per well, 50mm Tris HCL 7.4 pH, 150mm NaCl, 2mm EDTA, 0.1% SDS, 0.1% Triton x-100, 0.5% NaDeoxycholate) for Western blot analysis and Trizol (500μl per well) for RNA analysis via rt-PCR. Phosphatase inhibitors (0.2 mM Na_3VO_4, 50 mM NaF, and a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO)) were added to prevent modification of proteins. Total sample protein concentration was determined by the bicinchoninic acid method (BCA protein assay kit, Bio-Rad Laboratories, Hercules, CA). Primary outcomes identified were exosome biogenesis (STAM, HGS, TSG-101, clathrin, VSP4a), exosome release (Rab11, 27a, 27b, 35, and Syntaxin 1A), and exosome surface markers (Alix, TSG-101, CD-63).

Exosome isolation. Previous pilot data from our laboratory identified that cell culture samples measured in triplicate from the same initial number of cells produced equivalent protein and mRNA values upon isolation in RIPA or Trizol. The coefficient of variation was 7.8% for protein concentration (μg/μl) and 11.7% for RNA concentration (ng/μl). To attain adequate volume of media for exosome isolation, media was combined from individual wells in each condition. Per manufacturer's instructions, media was centrifuged at 3000 × g for 15 min to remove cellular debris and filtered with a 0.22 μm filter (Fisher Scientific, Waltham, MA) to remove large particles and additional cellular debris (3). To isolate exosomes, 4 ml of media was inverted several times in a 5X dilution of ExoQuick-Tissue Culture solution (System Biosciences, Palo Alto, CA, USA) for at least 12 hours at

4˚C. Following incubation, media was centrifuged at 5000 × g for 30 min, supernatant removed, then centrifuged at 5000 × g for 5 min. Remaining media was carefully aspirated with exosome pellet re-suspended in RIPA buffer for western blot analysis of exosome markers.

Quantitative real-time PCR. Total RNA from SkM was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). For mRNA reverse transcription, first-strand cDNA was generated by random hexamer primers with MMLV Reverse Transcriptase (Invitrogen). Real-time PCR reactions were performed with a SYBR green PCR kit in a BioRad CFX96 Real-Time System (Bio-Rad, Hercules, CA). Gene expression was determined with the $2^{-\Delta\Delta Ct}$ relative quantification method and was normalized to 18s. Primer sequences are listed in Table 7.

Skeletal muscle protein analysis. From cell culture isolations, 20-40 µg of total protein was fractionated for Western blot on SDS-polyacrylamide gels, transferred to a reduced-fluorescence PVDF membrane, and incubated with one of the following antibodies: alix (#2171s, Cell Signaling Technology), TSG-101 (#sc-7964, Santa Cruz Biotechnology), CD-63 (#sc-5275, Santa Cruz), CaMKII phospho (#12716, Cell Signaling) and pan (#3362, Cell Signaling), P38 MAPK phospho (#4511, Cell Signaling) and pan (#9512S, Cell Signaling), and AMPK phospho (#2532S, Cell Signaling) and pan (#2535S, Cell Signaling). Following primary incubation horseradish peroxide–conjugated (#7074 Anti-Rabbit, #7076 Anti-Mouse, Cell Signaling) secondary antibodies were incubated with the membrane and detected using florescence (Bio-Rad, Hercules, CA). Densitometric analysis was performed using Image Lab software (Bio-Rad). Results were normalized to alpha tubulin (#2144, Cell Signaling), or ponceau S stain (P3504, Sigma Aldrich).

Exosome quantification. For quantification of total exosomes released following EPS compared to control, exosome pellets from 1 ml of media were isolated using a 5X Exo-Quick solution, then resuspended in 300 µl PBS for quantification using the NanoSight LM10 (Malvern Panalytical, Malvern, UK). NanoSight has been demonstrated to effectively identify exosome-sized vesicles from blood plasma (28). Samples were automatically analyzed in triplicate using 60 s video clips by NanoSight tracking software using Brownian motion analysis (14).

Statistical analysis. For fusion index, differentiation markers, and preliminary exosome isolation, a paired Student's t-test was used. Otherwise, a two-way mixed-plot factorial ANOVA (group × condition) was used. Following a significant F ratio, a Fisher's LSD was used. Significance was established at $P \leq 0.05$ for all statistical sets and data reported as Mean ± SE. All data were analyzed using Prism Graphpad Software Version 7 (La Jolla, CA, USA) or Microsoft Excel 2016 (Version 1708, Redmond, WA, USA).

Results

Study 1. *SkM differentiation.* In a subset of samples (n=3), Western blot analysis identified increased MHC ~60% in day 8 compared to day 6 (Figure 16). Day 2 and day 4 had reduced MHC protein compared to day 6 and day 8. Gene expression displayed no differences between Pax3, Pax7, MyoG, and MHC between day 6 and day 8 (Table 8). SkM cultures stained for FI (n=2) visibly displayed increased myotube formation and incorporated nuclei, with multi-nucleated muscle fibers in day 8 compared to day 6 (Figure 17).

Study 2. *Experimental control between ddH_2O and KN-92*. To ensure no differences existed between control or EPS conditions with the addition of KN-92, an equal volume of

solvent (ddH_2O) was added to each plate as an experimental control. There were no differences detected for any gene expression or whole protein data analyzed by t-test between control (ddH_2O) and control (KN-92) nor in EPS (ddH_2O) and EPS (KN-92). Therefore, all data presented in the present investigation will be KN-92 and KN-93 with control (non-EPS) and EPS conditions.

Phosphorylation of in vivo pathways were not observed. There were no observed changes in phospho/pan ratio's in P38 MAPK or AMPK (Figure 18). There was a trend for an interaction effect in CaMKII phospho/pan ratio, with ~40% increased ratio in the KN-93 treatment from Control to EPS.

Study 3. *MVB biogenesis and release pathway.* There were no observed changes in MVB biogenesis pathway HGS, TSG-101, Alix, STAM, VTA1, or VPS4a, or in the exosome marker CD63 (Figure 19). However, a trending interaction for clathrin gene expression was observed in response to EPS, which displayed a ~20% decrease with KN-92 and ~20% increase with KN-93.

To determine MVB release, Rab and Syntaxin family gene expression was analyzed. There were no observed changes to Rab11A, 27A, 35, or Syntaxin1A (Figure 20). There was a trend for a main effect of CaMKII inhibition to increase Rab27B gene expression, with ~40% increase in control and ~15% increase in EPS conditions.

To determine the effect of EPS on miRNA processing, gene expression for proteins in the production of miRNA were measured. There were no differences in drosha, exportin-5, or dicer in response to EPS or CaMKII inhibition (Figure 21).

Exosome quantification and surface marker identification. To determine quantity changes, exosomes were isolated from the media for analysis as well as protein and gene

expression analysis from cell cultures. Protein isolated from cell culture for CD63 and alix remained unchanged in response to EPS or CaMKII inhibition. A notable trend with CaMKII inhibition was observed with TSG-101 and clathrin total protein, with increased expression in KN-93 for TSG-101 and diminished expression of proteins with KN-93 for clathrin (Figure 22).

No differences were observed for TSG-101 or alix when exosomes were isolated from media (Figure 22). A strong trend for CD63 with CaMKII inhibition was observed with ~30-40% increased expression in KN-93 compared to KN-92.

NanoSight tracking technology was used to determine quantity of nanoparticles detected from an isolation of exosomes from media in Control and EPS samples without CaMKII inhibition. EPS was shown to have decreased total quantity of nanoparticles 0-200 nm in size compared to control (Table 9). There was a ~30% reduction in total particle number with EPS in both the 151.5-175.5 nm and 176.5-200.5 nm ranges.

In vivo pathway expression differences. To further determine the effectiveness of EPS, gene expression analysis for well-known *in vivo* markers of muscle contraction were analyzed. A decreased expression of PDK4 was observed with CaMKII inhibition, with a trend for diminished IL-6 gene expression (Figure 23). No differences were observed in VEGF or PGC-1α in response to EPS or KN-93.

Discussion

The main overall finding of the current investigation were changes in MHC protein at day 8 compared to day 6 of differentiation, observed trends for the phosphorylation of AMPK, increased clathrin gene expression, reduced PDK4 gene expression, and reduced total nanoparticles in response to EPS. It is difficult to assess myotube contraction during

EPS, and despite identifiable MHC expression and apparent formation of myotubes, minimal contractile activity occurred compared to the anticipated preliminary data analysis previously observed with exosome surface marker changes.

In vivo phosphorylation events. As mentioned previously, evidence *in vivo* suggests 30 min of cycling sufficiently activates the phosphorylation of AMPK, CaMKII, and P38 MAPK (13). Without the ability to verify the total number of myotubes which contracted during EPS, it was not possible to determine a relative "intensity" *in vitro*. During *in vivo* exercise, the total number of muscle fibers recruited must be rigorous enough to stimulate metabolic pathways. A decrease in ATP to form AMP will stimulate AMPK (17). CaMKII is an upstream target of AMPK, and therefore, the decreased expression of AMPK in KN-93 samples may be an indication that the CaMKII inhibition was effective in the cells. The observed trend for an interaction effect with phospho/pan CaMKII could be explained by the fact that KN-93 uses competitive inhibition to block CaMKII activity, so phosphorylation still occurs with less downstream targets available (50).

MVB biogenesis, release, and miRNA processing. The interaction effect observed with clathrin gene expression gives insight to the importance of ESCRT-0 and CaMKII. Although other changes downstream of clathrin are not observed, it establishes a targeted approach for future study designs with MVB biogenesis and CaMKII interactions. The same relationship exists with MVB release as indicated by the effect of CaMKII inhibition with Rab27B. For both clathrin and Rab 27B, the inhibition of CaMKII increased their overall gene expression, suggesting CaMKII plays an inhibitory role at rest on these genes.

miRNA processing has been shown to be regulated by *in vivo* exercise (45). Data from our laboratory has also identified changes to miRNA processing drosha, exportin-5, and

dicer following aerobic exercise (unpublished data). The present investigation displayed no alterations with miRNA processing in response to EPS or CaMKII inhibition. Although calcium signaling plays a critical role in MVB release, it appears to have no effect on MVB content. If calcium signaling was involved, a reduction in miRNA processing should have been observed in non-stimulated controls at rest and diminished gene expression following EPS.

Exosome quantification and surface marker changes. Previous research has identified that extracellular vesicles released from C2C12 myotubes inhibit proliferation of myoblasts (15). With known interactions from myotubes to inhibit myoblasts through exosome signaling, a possible explanation to the reduced exosome number from the NanoSight and reduced total protein trends in the media following EPS may be explained to stimulate the proliferation of myoblasts. If skeletal muscle exosomes also diminish myoblast proliferation, then a reduced number of exosomes would allow myoblast proliferation to increase.

In vivo aerobic exercise pathway response and myotube contraction. CaMKII is known to be involved with Glut 4 transporters (35), so activation of glycolytic factors such as PDK4 would be critically important to meet energy demands with activity. A perplexing situation with the current investigation is the apparent lack of myotube contraction in response to EPS, observed by unaltered PDK4, VEGF and PGC-1α gene expression. Moreover, CaMKII inhibition reduced PDK4 gene expression.

A potential problem with calcium signaling (and therefore myotube contraction) was the use of streptomycin as an antibiotic in the culture media. This antibiotic has a known interaction to block intracellular calcium signaling, which could block the effect of calcium

release leading to myotube contractions (40). However, we have successfully completed EPS studies within our laboratory in the past in human and C2C12 myotubes (preliminary data used to calculate sample size for the present study) and other investigations commonly use antibiotics in EPS with valid results (23). A likely deterrent to myotube contraction in response to EPS is poorly differentiated tubes, as previously identified (19). An analysis of mRNA content from rt-PCR displays three of the eight subjects had 3-4 cq's lower MHC gene expression, despite optimization of the 8th day of differentiation. A 4 cq difference results in a 16-fold reduction of MHC between subjects and was a likely source of diminished myotube formation and contraction.

Alternative Hypothesis

Calcium release in SkM cells is critically important. Calcium release directly, but not via CaMKII activation, may also be a plausible cause of exosome release as has been shown in other tissue (47, 54). To fully elucidate this potential mechanism, enhanced and inhibited calcium release should also be explored in conjunction with EPS. Other calcium signaling pathways include Protein Kinase C with a calcium binding domain for activation (32), linked to MARCK activation along with CaMKII in skeletal muscle (22).

Identification of specific miRNA could play a role in furthering our understanding of the many pathways in response to CaMKII inhibition and EPS. Previous work has demonstrated that miR-107 enhances PDK4 activity (56). miR-107 is also known to increase in mice following exercise (46). Increased miR-107 would indicate an increased metabolic demand, indicating EPS in the cells was effective.

Increased oxidative stress induced through incubation of IL-4 in B cells obtained from mice increased exosome release (30). If oxidative stress were induced through EPS, one

potential mechanism to increase vesicle release is the phosphorylation of P38 MAPK, shown to increase endocytosis through a guanylnucleotide dissociation inhibitor (GDI)-Rab5 complex (11). Further analysis of oxidative stress need to be explored to determine this potential mechanism.

Limitations

A KN-93 concentration of 10 μM has been identified to reduce CaMKII activity in rat myotubes, but not properly established in human muscle cells. Ideally, a dose-response should be established with primary human cells to determine an effective inhibition. To combat this, downstream targets of CaMKII would also need to be explored to determine the effectiveness of inhibition, such as Glut-4 expression (35) or cAMP response element binding protein (51).

Although calcium could be an important regulator of exosome release, there was no measure of the total amount of calcium *in vitro*. To explore the effect of intracellular calcium on exosome biogenesis and release independent of EPS, it would be prudent to stimulate calcium using an ionophore such as A-23187 (27). Stimulation of calcium release without EPS would help to determine the full effect of EPS conditions compared to calcium alone.

Conclusion

The future direction of human SkM exosomes isolated *in vitro* will enable treatment of other cellular environments to determine "cross talk" potential. To our knowledge, primary human SkM exosomes have not been used to identify changes in other cells types (3, 34). One therapeutic target of interest is endothelial cells (EC). Preliminary data from our lab

has shown enhanced proliferation and migration of EC's in response to treatment with exosomes isolated from C2C12 SkM cultures. Other potential targets related to exosomes isolated from EPS would be cardiomyocytes, hepatocytes, and alveolar cells. The heart, liver, and lungs receive a large amount of blood during aerobic exercise, making them ideal locations for SkM exosomes to interact *in vivo*.

References

1. Al-Shanti N, Stewart CE. Ca2+/calmodulin-dependent transcriptional pathways: potential mediators of skeletal muscle growth and development. *Biological Reviews*. 2009;84(4):637-52.
2. Arbuzova A, Murray D, McLaughlin S. MARCKS, membranes, and calmodulin: kinetics of their interaction. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*. 1998;1376(3):369-79.
3. Aswad H, Forterre A, Wiklander OP et al. Exosomes participate in the alteration of muscle homeostasis during lipid-induced insulin resistance in mice. *Diabetologia*. 2014;57(10):2155-64.
4. Azmi AS, Bao B, Sarkar FH. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer and Metastasis Reviews*. 2013;32(3-4):623-42.
5. Babst M, Sato TK, Banta LM, Emr SD. Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *The EMBO journal*. 1997;16(8):1820-31.
6. Bentzinger CF, Wang YX, Rudnicki MA. Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor perspectives in biology*. 2012;4(2):a008342.
7. Berggren JR, Tanner CJ, Houmard JA. Primary cell cultures in the study of human muscle metabolism. *Exercise and sport sciences reviews*. 2007;35(2):56-61.
8. Booth AM, Fang Y, Fallon JK, Yang J-M, Hildreth JE, Gould SJ. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. *J Cell biol*. 2006;172(6):923-35.
9. Braun AP, Schulman H. The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annual review of physiology*. 1995;57(1):417-45.
10. Cai H, Reinisch K, Ferro-Novick S. Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. *Developmental cell*. 2007;12(5):671-82.
11. Cavalli V, Vilbois F, Corti M et al. The stress-induced MAP kinase p38 regulates endocytic trafficking via the GDI: Rab5 complex. *Molecular cell*. 2001;7(2):421-32.

12. Colombo M, Moita C, van Niel G et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci*. 2013;126(24):5553-65.
13. Combes A, Dekerle J, Webborn N, Watt P, Bougault V, Daussin FN. Exercise-induced metabolic fluctuations influence AMPK, p38-MAPK and CaMKII phosphorylation in human skeletal muscle. *Physiological reports*. 2015;3(9):e12462.
14. Dragovic RA, Gardiner C, Brooks AS et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2011;7(6):780-8.
15. Forterre A, Jalabert A, Berger E et al. Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk? *PLoS One*. 2014;9(1):e84153.
16. Forterre A, Jalabert A, Chikh K et al. Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. *Cell cycle*. 2014;13(1):78-89.
17. Friedrichsen M, Mortensen B, Pehmøller C, Birk JB, Wojtaszewski JF. Exercise-induced AMPK activity in skeletal muscle: role in glucose uptake and insulin sensitivity. *Molecular and cellular endocrinology*. 2013;366(2):204-14.
18. Fruhbeis C, Helmig S, Tug S, Simon P, Kramer-Albers EM. Physical exercise induces rapid release of small extracellular vesicles into the circulation. *J Extracell Vesicles*. 2015;4:28239.
19. Fujita H, Nedachi T, Kanzaki M. Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes. *Experimental cell research*. 2007;313(9):1853-65.
20. Gaster M, Kristensen S, Beck-Nielsen H, Schrøder H. A cellular model system of differentiated human myotubes. *Apmis*. 2001;109(11):735-44.
21. Guay C, Regazzi R. Exosomes as new players in metabolic organ cross-talk. *Diabetes, Obesity and Metabolism*. 2017;19(S1):137-46.
22. Hartwig J, Thelen M, Resen A, Janmey P, Nairn A, Aderem A. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium–calmodulin. *Nature*. 1992;356(6370):618.
23. Hinkley JM, Zou K, Park S, Turner K, Zheng D, Houmard JA. Roux-en-Y gastric bypass surgery enhances contraction-mediated glucose metabolism in primary human myotubes. *American Journal of Physiology-Endocrinology and Metabolism*. 2017;313(2):E195-E202.
24. Hutagalung AH, Novick PJ. Role of Rab GTPases in membrane traffic and cell physiology. *Physiological reviews*. 2011;91(1):119-49.
25. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. *Immunology letters*. 2006;107(2):102-8.
26. Kramer HF, Goodyear LJ. Exercise, MAPK, and NF-κB signaling in skeletal muscle. *Journal of applied physiology*. 2007;103(1):388-95.
27. Kubis H-P, Hanke N, Scheibe RJ, Meissner JD, Gros G. Ca 2+ transients activate calcineurin/NFATc1 and initiate fast-to-slow transformation in a primary skeletal muscle culture. *American Journal of Physiology-Cell Physiology*. 2003;285(1):C56-C63.

28. Li M, Rai AJ, DeCastro GJ et al. An optimized procedure for exosome isolation and analysis using serum samples: Application to cancer biomarker discovery. *Methods*. 2015;87:26-30.
29. Lotvall J, Valadi H. Cell to cell signalling via exosomes through esRNA. *Cell adhesion & migration*. 2007;1(3):156-8.
30. Momen-Heravi F, Bala S, Bukong T, Szabo G. Exosome-mediated delivery of functionally active miRNA-155 inhibitor to macrophages. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2014;10(7):1517-27.
31. Muoio DM, Way JM, Tanner CJ et al. Peroxisome proliferator-activated receptor-α regulates fatty acid utilization in primary human skeletal muscle cells. *Diabetes*. 2002;51(4):901-9.
32. Newton AC. Protein kinase C: poised to signal. *American Journal of Physiology-Endocrinology And Metabolism*. 2010;298(3):E395-E402.
33. Nikolić N, Bakke SS, Kase ET et al. Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. *PloS one*. 2012;7(3):e33203.
34. Nikolić N, Görgens SW, Thoresen GH, Aas V, Eckel J, Eckardt K. Electrical Pulse Stimulation of Cultured Skeletal Muscle Cells as a Model for in vitro Exercise–Possibilities and Limitations. *Acta Physiologica*. 2016.
35. Ojuka EO, Goyaram V, Smith JA. The role of CaMKII in regulating GLUT4 expression in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*. 2012;303(3):E322-E31.
36. Ojuka EO, Jones TE, Nolte LA et al. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca2+. *American journal of physiology-endocrinology and metabolism*. 2002;282(5):E1008-E13.
37. Ostrowski M, Carmo NB, Krumeich S et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature cell biology*. 2010;12(1):19-30.
38. Pedersen BK, Åkerström TC, Nielsen AR, Fischer CP. Role of myokines in exercise and metabolism. *Journal of applied physiology*. 2007;103(3):1093-8.
39. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nature Reviews Endocrinology*. 2012;8:457.
40. Redman R, Silinsky E. Decrease in calcium currents induced by aminoglycoside antibiotics in frog motor nerve endings. *British journal of pharmacology*. 1994;113(2):375-8.
41. Richter EA, Ruderman NB. AMPK and the biochemistry of exercise: implications for human health and disease. *Biochemical Journal*. 2009;418(2):261-75.
42. Roma-Rodrigues C, Fernandes AR, Baptista PV. Exosome in tumour microenvironment: overview of the crosstalk between normal and cancer cells. *BioMed research international*. 2014;2014.
43. Rose AJ, Hargreaves M. Exercise Increases Ca2+–Calmodulin-Dependent Protein Kinase II Activity in Human Skeletal Muscle. *The Journal of physiology*. 2003;553(1):303-9.
44. Rose AJ, Kiens B, Richter EA. Ca2+–calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise. *The Journal of physiology*. 2006;574(3):889-903.

45. Russell AP, Lamon S, Boon H et al. Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. *The Journal of physiology*. 2013;591(18):4637-53.
46. Safdar A, Abadi A, Akhtar M, Hettinga BP, Tarnopolsky MA. miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice. *PloS one*. 2009;4(5):e5610.
47. Savina A, Furlán M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *Journal of Biological Chemistry*. 2003;278(22):20083-90.
48. Scheler M, de Angelis MH, Al-Hasani H, Häring H-U, Weigert C, Lehr S. Methods for proteomics-based analysis of the human muscle secretome using an in vitro exercise model. *Proteomic Profiling: Methods and Protocols*. 2015:55-64.
49. Soderling T, Jung V, Jones C et al. Protein kinases. Regulation by autoinhibi-tory domains. *J Biol Chem265*. 1990:1823-6.
50. Sumi M, Kiuchi K, Ishikawa T et al. The newly synthesized selective Ca2+ calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochemical and biophysical research communications*. 1991;181(3):968-75.
51. Sun P, Enslen H, Myung PS, Maurer RA. Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes & development*. 1994;8(21):2527-39.
52. Sundquist WI, Schubert HL, Kelly BN, Hill GC, Holton JM, Hill CP. Ubiquitin recognition by the human TSG101 protein. *Molecular cell*. 2004;13(6):783-9.
53. Tamai K, Tanaka N, Nakano T et al. Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. *Biochemical and biophysical research communications*. 2010;399(3):384-90.
54. Urbanelli L, Magini A, Buratta S et al. Signaling pathways in exosomes biogenesis, secretion and fate. *Genes*. 2013;4(2):152-70.
55. Whitham M, Parker BL, Friedrichsen M et al. Extracellular Vesicles Provide a Means for Tissue Crosstalk during Exercise. *Cell Metabolism*. 2018;27(1):237-51.e4.
56. Wilfred BR, Wang W-X, Nelson PT. Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. *Molecular genetics and metabolism*. 2007;91(3):209-17.
57. Wollert T, Wunder C, Lippincott-Schwartz J, Hurley JH. Membrane scission by the ESCRT-III complex. *Nature*. 2009;458(7235):172-7.
58. Zurlo F, Larson K, Bogardus C, Ravussin E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *Journal of Clinical Investigation*. 1990;86(5):1423.

Table 6. Subject characteristics. BMI, body mass index; $\dot{V}O_{2MAX}$, maximal oxygen consumption; HOMA-IR, homeostasis model assessment – insulin resistance; HOMA-β, homeostatic model assessment – cell function; HDL, high density lipoprotein; LDL, low density lipoprotein. Mean ± SE. *N* = 8.

	Mean ± SE	Range (Min-Max)
Age, y	24.1 ± 1.4	19-32
BMI, kg/m^2	22.4 ± 0.8	18.2-25.9
$\dot{V}O_{2MAX}$, ml×kg^{-1}×min^{-1}	39.7 ± 2.0	34.9-49.1
Fasting Glucose, mg/dl	92.4 ± 3.1	81-109
Fasting Insulin, µIU/ml	8.4 ± 0.9	5-12
HOMA-IR	1.9 ± 0.2	1.1-2.6
HOMA-β	106.0 ± 16.6	62.8-180.8
Total Cholesterol, mg/dl	164.6 ± 9.6	140-217
HDL, mg/dl	48.0 ± 4.1	34-67
LDL, mg/dl	98.9 ± 10.2	73-161
Triglycerides, mg/dl	88.9 ± 11.0	51-143

Table 7. Gene sequences of mRNA and miRNA, obtained from the Harvard Primer Bank. Apoptotic linked gene-2 interacting protein X (Alix); Tumor Susceptibility Gene-101 (TSG-101); hepatocyte growth factor-regulated tyrosine kinase substrate (HGS); signal transducing adaptor molecule (STAM); vesicle trafficking 1 (Vta1); vacuolar protein sorting mutant (Vps4a); Myogenin (MyoG); myosin heavy chain (MHC).

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
Alix	ATGGCGACATTCATCTCGGTG	CGCTTGGGTAAGTCTGCTGG
Clathrin	ATTCTGCCAATTCGTTTTCAGGA	GCTTTCAGTGCAATTACTTTGCT
TSG-101	GAGAGCCAGCTCAAGAAAATGG	TGAGGTTCATTAGTTCCCTGGA
CD63	CAGTGGTCATCATCGCAGTG	ATCGAAGCAGTGTGGTTGTTT
Dicer	GAGCTGTCCTATCAGATCAGGG	ACTTGTTGAGCAACCTGGTTT
Exportin-5	ATCCTGGAACACGTTGTCAAG	CACTACAATTCGAGACAGAGCAT
Drosha	TGTCACAGAATGTCGTTCCAC	GGGCCTAAAGGATGGTGCT
HGS	CTCCTGTTGGAGACAGATTGGG	GTGTGGGTTCTTGTCGTTGAC
STAM	AATCCCTTCGATCAGGATGTTGA	CGAGACTGACCAACTTTATCACA
VTA1	CTCCCCGCACAGTTCAAGAG	AACGACAGTAATAAGCCACCAC
Vps4a	CCACGCTATCAAGTATGAGGC	CCGTGTTTCTCTTTGCTTCGTA
Rab11A	CAACAAGAAGCATCCAGGTTGA	GCACCTACAGCTCCACGATAAT
Rab27A	GCTTTGGGAGACTCTGGTGTA	TCAATGCCCACTGTTGTGATAAA
RAB27B	TAGACTTTCGGGAAAAACGTGTG	AGAAGCTCTGTTGACTGGTGA
RAB35	TACTGTTGCGTTTTGCAGACA	CCCCGATAATACGTGGAGGTG
Syntaxin 1A	CGAGACCGCTTCATGGATGAG	ACTTGGAACGAACTTTGTTTGC
Pax3	AGCTCGGCGGTGTTTTTATCA	CTGCACAGGATCTTGGAGACG
Pax7	ACCCCTGCCTAACCACATC	GCGGCAAAGAATCTTGGAGAC
MyoG	GGGGAAAACTACCTGCCTGTC	AGGCGCTCGATGTACTGGAT
MHC	ATTGCTTCGTGGTGGACTCAA	GGCCATGTCTTCGATCCTGTC

Table 8. Gene expression differences between day 6 and day 8 for muscle differentiation markers Pax3, Pax7, Myogenin (MyoG) and myosin heavy chain (MHC). Data were controlled to Beta Actin. N=4.

	Day 6	Day 8	P value
Pax3	1.00 ± 0.13	0.90 ± 0.08	0.55
Pax7	1.00 ± 0.11	1.32 ± 0.64	0.71
MyoG	1.00 ± 0.01	0.88 ± 0.04	0.13
MHC	1.00 ± 0.15	0.60 ± 0.04	0.07

Table 9. NanoSight average counts, separated by each 25 nm range. *p≤0.05. N=8.

Range (nm)	Control (1.0) ± SE	EPS (Fold Change) ± SE	P value
0-75.5	No Counts	No Counts	N/A
76.5-100.5	1.00 ± 0.51	0.28 ± 0.15	0.18
101.5-125.5	1.00 ± 0.44	0.54 ± 0.24	0.40
126.5-150.5	1.00 ± 0.24	0.64 ± 0.22	0.16
151.5-175.5	1.00 ± 0.15	0.61 ± 0.16	0.05*
176.5-200.5	1.00 ± 0.09	0.69 ± 0.14	0.03*
0-200.5 (total)	1.00 ± 0.11	0.66 ± 0.14	0.04*

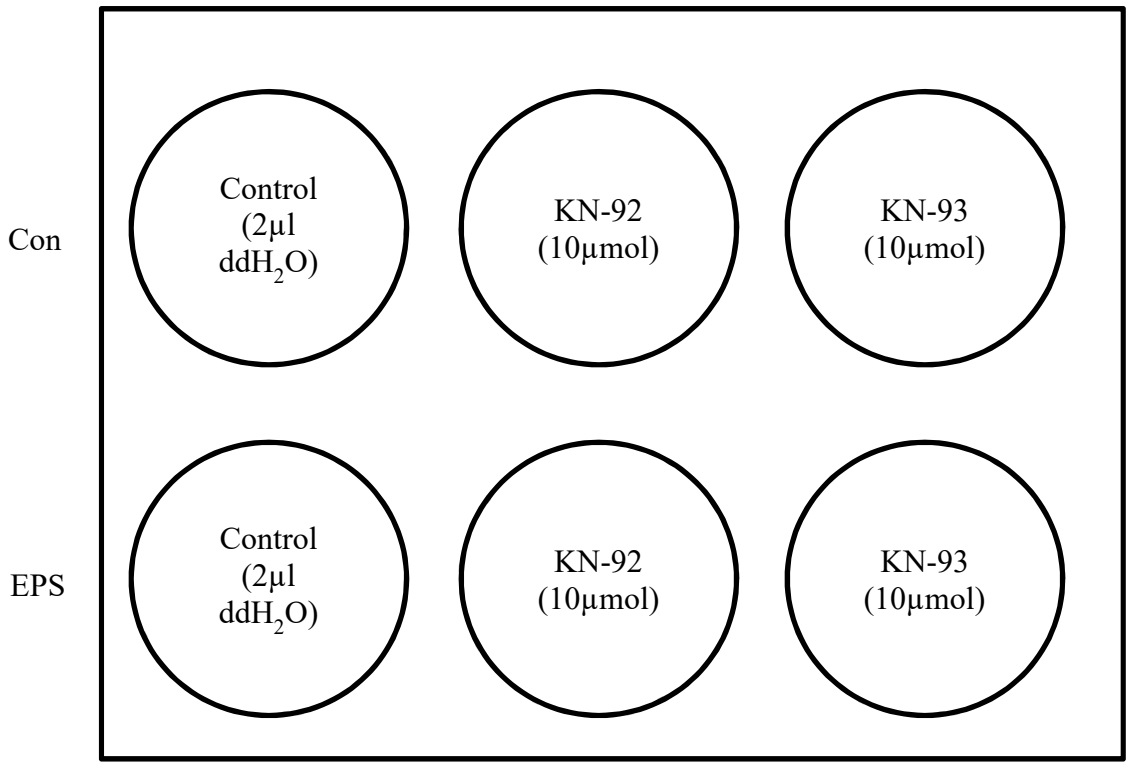

Figure 15. Representation of the six conditions tested for each cell culture plate. Six identical dishes were placed per subject (~75,000 cells/well) with 1 Con and 1 EPS plate collected for 1) 30-min stimulation to validate EPS effectiveness, 2) 24-hour stimulation for Western Blot analysis, and 3) 24-hour stimulation for rt-PCR analysis. Media was also collected from each plate with 24-hour treatments, 8 ml/condition, for exosome isolation.

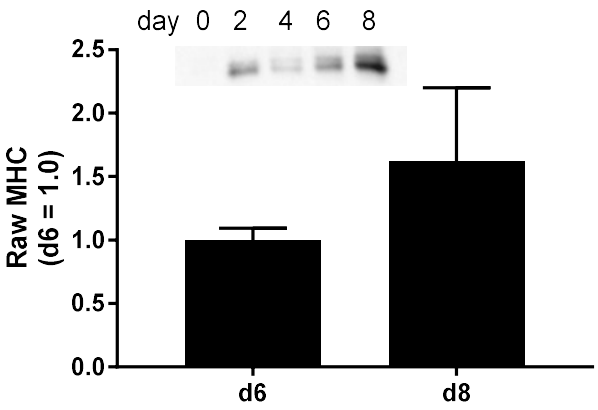

Figure 16. Raw myosin heavy chain protein expression observed at day 6 and day 8 following differentiation. N=4.

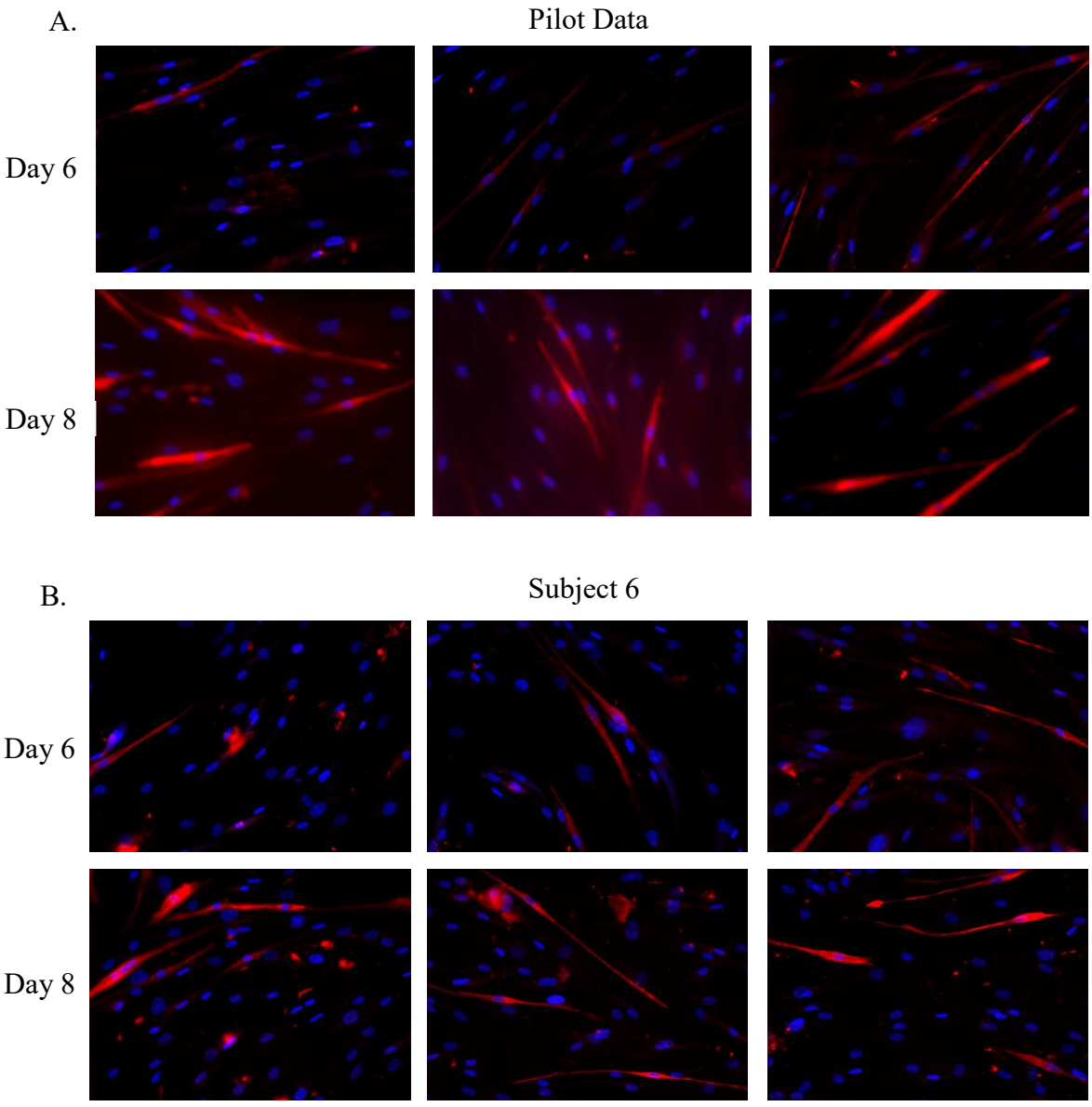

Figure 17. Cell Stains *in vitro* to test differentiation of day 6 compared to day 8. Clear myotubes were formed during preliminary findings from previous human subjects on day 8 vs. day 6 (A). and with the present investigation (B). MF-20 (Red) and DAPI (Blue).

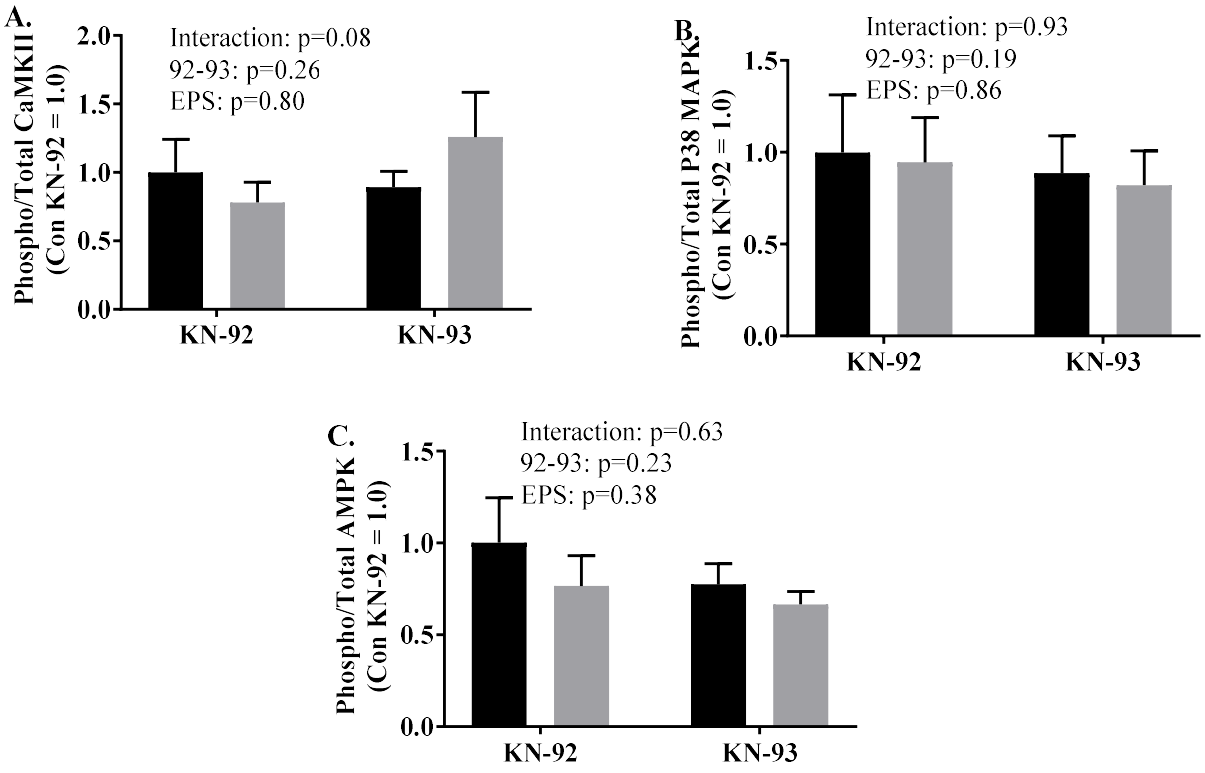

Figure 18. Phosphorylation of common *in vivo* exercise pathways following 30 min of EPS. Calcium-calmodulin kinsase II (n=6) (A), P38 mitogen activated protein kinase (n=5) (B), and adenosine monophosphate kinase (n=7) (C). Con (black) and EPS (gray). Data presented represent Mean ± SE.

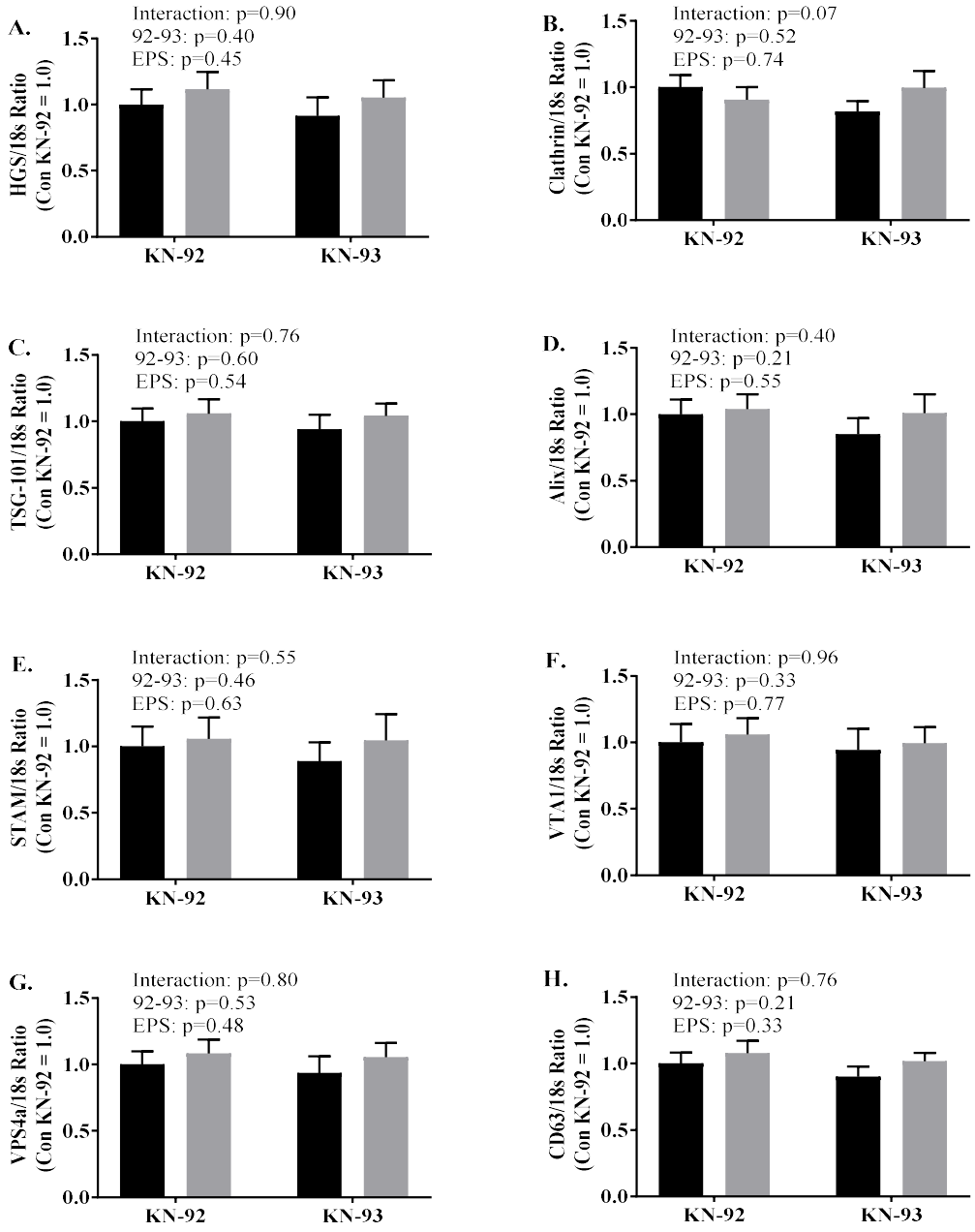

Figure 19. Gene expression analysis of MVB biogenesis/ESCRT in control (black) and EPS (gray) conditions. ESCRT-0 proteins hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) (A) clathrin (B), and signal transducing adaptor molecule (STAM) (E), ESCRT-I proteins Tumor Susceptibility Gene-101 (TSG-101) (C), accessory protein apoptotic linked gene-2 interacting protein X (Alix) (D), ESCRT-III protein vesicle trafficking 1 (Vta1) (F), and vacuolar protein sorting mutant (Vps4a) (G), and the tetraspanin CD-63 (H). Data presented represent Mean ± SE.

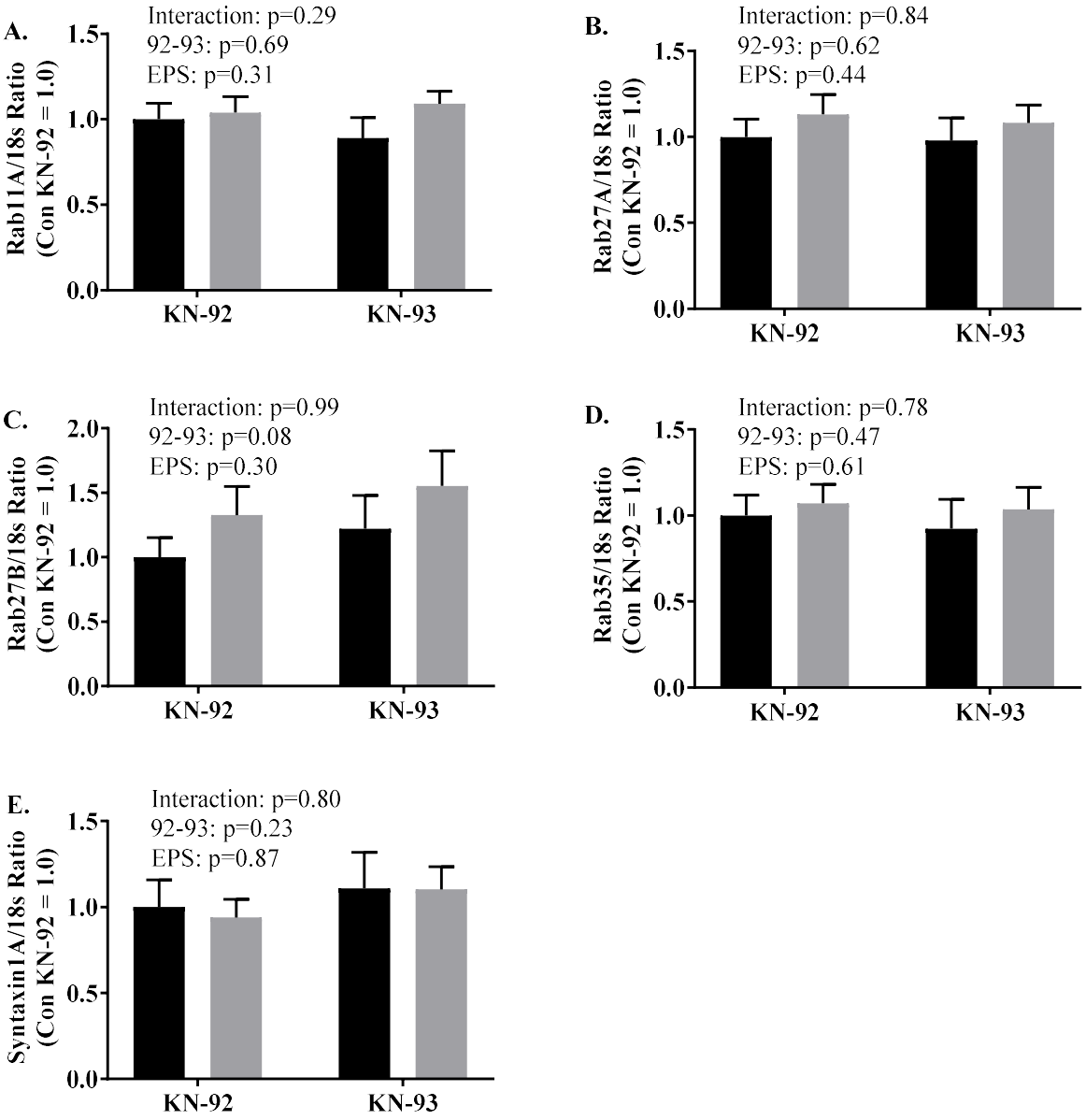

Figure 20. Gene expression analysis of MVB/exosome release for Con (black) and EPS (gray) subjects. Rab and syntaxin family proteins are associated with MVB fusion with the membrane, leading to exosome release. Data presented represent Mean ± SE, n=8.

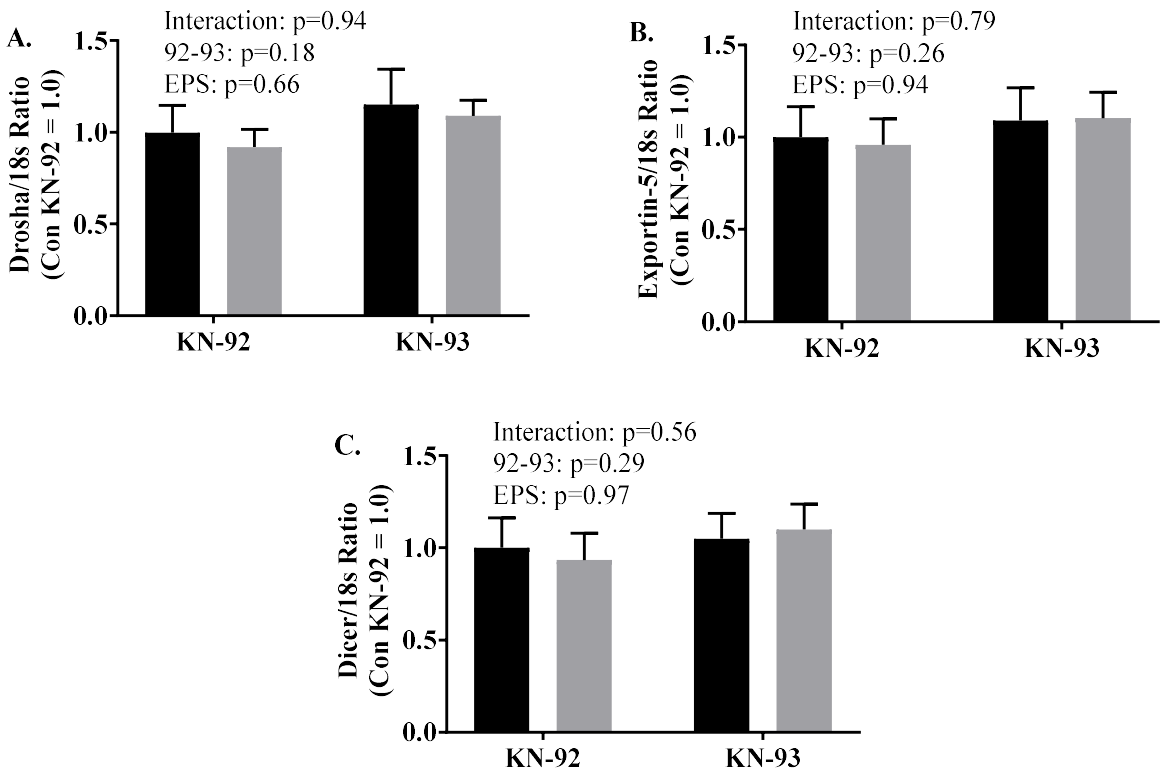

Figure 21. Gene Expression analysis of the miRNA processing pathway drosha (A), exportin-5 (B), and dicer (C). Con (black) and EPS (gray). Data presented represent Mean ± SE, n=8.

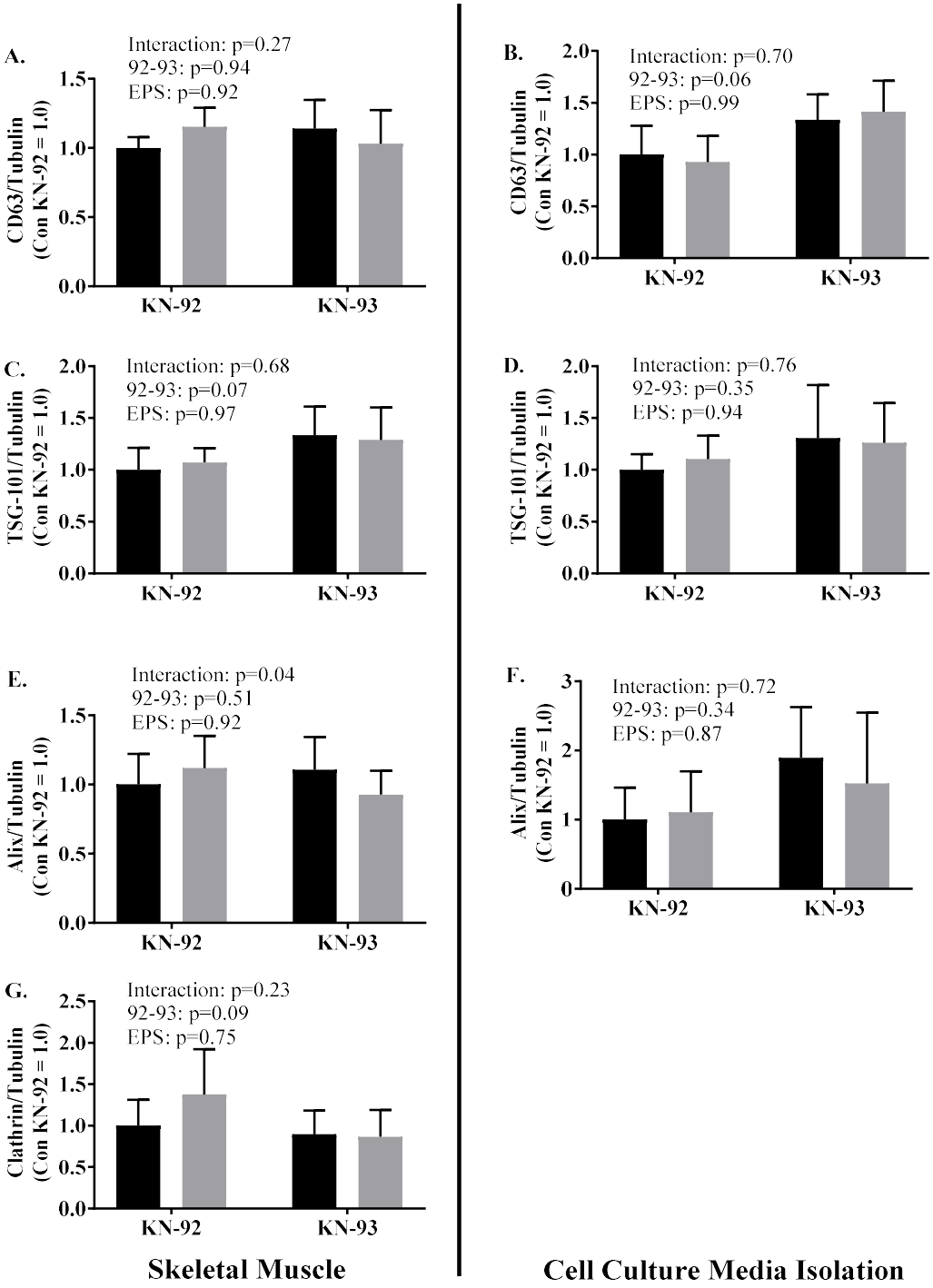

Figure 22. Western blot analysis of exosome surface markers after 24 h in skeletal muscle (Left) and exosomes isolated in the media (Right). Markers include CD-63 (A,B), TSG-101 (C,D), Alix (E,F). and clathrin (G, undetected in exosome isolation). Con (black) and EPS (gray). Data presented represent Mean ± SE, n=8.

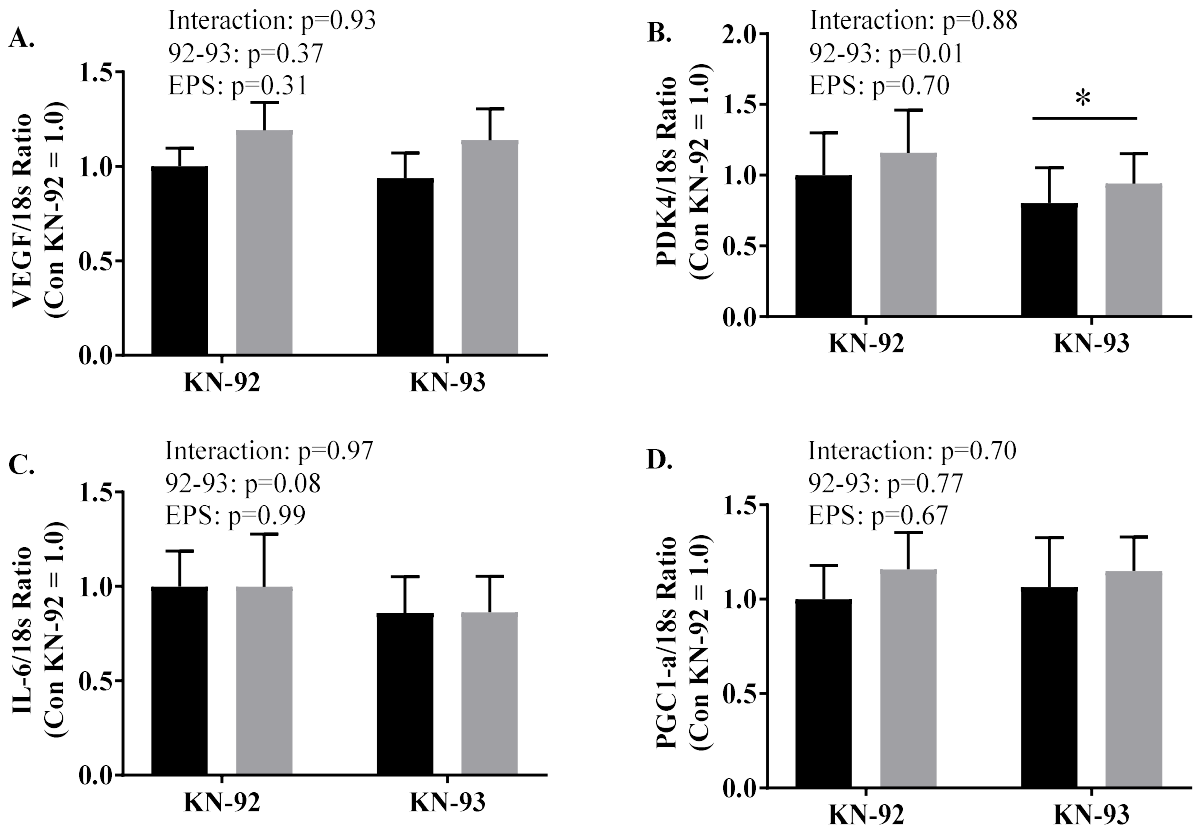

Figure 23. Gene Expression analysis of common *in vivo* pathways to identify exercise response. Vascular endothelial growth factor (A), pyruvate dehydrogenase kinase 4 (B), interleukin-6 (C), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (D). Con (black) and EPS (gray). Data presented represent Mean ± SE, n=8.

CHAPTER 5. CONCLUSION

Summary

In summary, the present work demonstrates several changes in response to acute exercise in whole skeletal muscle: 1) MVB biogenesis gene expression is impaired at rest with obesity, but able to increase in response to A+REx in LN and REx in LN and OB, 2) miRNA processing gene expression changes in response to AEx and A+REx at 1 hr post-exercise in LN, but no change in response to REx immediately and 3 hr post-exercise in LN and OB, 3) VEGF and PGC-1α gene expression increases in response to AEx, A+REx, and REx immediately following, 1 hr, and 3 hr post-exercise in LN and OB, 4) in OB, anti-angiogenic signals are also increased in response to REx 3 hr post-exercise, 5) miRNA related to angiogenesis are impaired at rest in OB, potentially leading to the diminished vascularization associated with obesity, and are unchanged in response to moderate intensity AEx, A+REx, and REx, and 6) exosome surface markers remain unchanged in response to AEx and A+REx, with varying effects based on protein marker in response to REx alone. Differences in skeletal muscle MVB pathway analysis *in vitro* via EPS and CaMKII inhibition include: 1) CaMKII inhibition decreased PDK4 gene expression and trended to an interaction effect to increase clathrin gene expression, 2) no changes in MVB biogenesis, release, or miRNA processing with EPS or CaMKII inhibition, 3) trends for increased and decreased exosome surface markers were observed with CaMKII inhibition, and 4) quantification of exosomes isolated in the media were reduced in response to EPS compared to control.

Limitations and Future Directions

Project 1 – Acute AEx and A+REx. Limitations of project 1 include the timing of biopsies post-exercise. As each subject served as their own control and biopsies were removed from each leg at the same time point, we wanted to reduce the total number of biopsies in a single day to three. Due to this, we selected 1 hr post-exercise as a time to monitor changes to enzyme activity, from an initial project design regarding metabolic enzyme activities following acute exercise. Future directions related to MVB biogenesis and release should expand the type, time, and intensity of well-known interventions leading to exercise adaptations to identify the potential role that MVB/exosomes play in acute exercise bouts and training over time. To enable more time points, an emphasis should be placed on one exercise modality, such as cycling.

Project 2 – LN and OB REx. Future directions for project 2 include a focus on the content of exosomes between LN and OB subjects to determine potential therapeutic targets in an OB population. Additionally, conducting proteomic and transcriptomic analysis of exosomes based on surface marker could provide valuable information about the response to REx in each group, such as hypertrophy or angiogenic signaling. Several studies could be designed to confirm MVB/exosome release from whole human muscle, starting with a time-course release following acute exercise. Ideally, immunohistochemistry changes in response to acute exercise should be performed to demonstrate a reduction of exosome surface markers, as previously observed in mice (52). Samples should be mounted prior to and immediately following exercise to identify this change. Differences between LN and OB would be likely, based on the altered signaling identified in our study with MVB biogenesis and various miRNA related to angiogenesis.

Project 3 – CaMKII inhibition and exosome release. Several limitations occurred during project 3, including poorly differentiated cells, questionable use of antibiotics which may block calcium signaling, and unknown effective doses of CaMKII inhibition with KN-93 in human SkMCC.

Future research *in vitro* should investigate a time-course in human SkMCC response to EPS related to MVB biogenesis and optimal MVB/exosome release from myoblasts and myotubes separately (36). The future direction of human SkM exosomes isolated *in vitro* will enable treatment of other cellular environments to determine "cross talk" potential. To our knowledge, primary human SkM exosomes have not been used to identify changes in other cells types (2, 36). One therapeutic target of interest is endothelial cells (EC). Preliminary data from our lab has shown enhanced proliferation and migration of EC's in response to treatment with exosomes isolated from C2C12 SkM cultures. Other potential targets related to exosomes isolated from EPS would be cardiomyocytes, hepatocytes, and alveolar cells. The heart, liver, and lungs receive a large amount of blood during aerobic exercise, making them ideal locations for SkM exosomes to interact *in vivo*. It is also suggested to improve EPS protocols to enhance contraction of primary human skeletal muscle cell lines. Identifying limiting factors for individual myotube contraction, such as the use or disuse of antibiotics (43), or the optimal day of differentiation to fully formed myotubes.

BIBLIOGRAPHY

1. Al-Shanti N, Stewart CE. Ca2+/calmodulin-dependent transcriptional pathways: potential mediators of skeletal muscle growth and development. *Biological Reviews*. 2009;84(4):637-52.
2. Aswad H, Forterre A, Wiklander OP et al. Exosomes participate in the alteration of muscle homeostasis during lipid-induced insulin resistance in mice. *Diabetologia*. 2014;57(10):2155-64.
3. Azmi AS, Bao B, Sarkar FH. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer and Metastasis Reviews*. 2013;32(3-4):623-42.
4. Baar K, Esser K. Phosphorylation of p70 S6k correlates with increased skeletal muscle mass following resistance exercise. *American Journal of Physiology-Cell Physiology*. 1999;276(1):C120-C7.
5. Babst M, Sato TK, Banta LM, Emr SD. Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *The EMBO journal*. 1997;16(8):1820-31.
6. Balducci S, Zanuso S, Nicolucci A et al. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. *Nutrition, Metabolism and Cardiovascular Diseases*. 2010;20(8):608-17.
7. Booth AM, Fang Y, Fallon JK, Yang J-M, Hildreth JE, Gould SJ. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. *J Cell biol*. 2006;172(6):923-35.
8. Caporali A, Meloni M, Völlenkle C et al. Deregulation of microRNA-503 contributes to diabetes mellitus–induced impairment of endothelial function and reparative angiogenesis after limb ischemia. *Circulation*. 2011:CIRCULATIONAHA. 110.952325.
9. Carlson SA, Fulton JE, Pratt M, Yang Z, Adams EK. Inadequate physical activity and health care expenditures in the United States. *Progress in cardiovascular diseases*. 2015;57(4):315-23.
10. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood*. 2008;111(3):1217-26.
11. Colombo M, Moita C, van Niel G et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci*. 2013;126(24):5553-65.
12. Dirks NA, Leeuwenburgh C. Sarcopenia: the role of apoptosis and modulation by caloric restriction. *Exercise and sport sciences reviews*. 2008;36(1):19-24.
13. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *The lancet*. 2005;365(9468):1415-28.
14. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. *Nature Reviews Cancer*. 2006;6(4):259-69.

15. Ferrante SC, Nadler EP, Pillai DK et al. Adipocyte-derived exosomal miRNAs: a novel mechanism for obesity-related disease. *Pediatric research*. 2014;77(3):447-54.
16. Forterre A, Jalabert A, Berger E et al. Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk? *PLoS One*. 2014;9(1):e84153.
17. Forterre A, Jalabert A, Chikh K et al. Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. *Cell cycle*. 2014;13(1):78-89.
18. Fruhbeis C, Helmig S, Tug S, Simon P, Kramer-Albers EM. Physical exercise induces rapid release of small extracellular vesicles into the circulation. *J Extracell Vesicles*. 2015;4:28239.
19. Graham T, Kahn B. Tissue-specific alterations of glucose transport and molecular mechanisms of intertissue communication in obesity and type 2 diabetes. *Hormone and metabolic research*. 2007;39(10):717-21.
20. Guay C, Regazzi R. Exosomes as new players in metabolic organ cross-talk. *Diabetes, Obesity and Metabolism*. 2017;19(S1):137-46.
21. Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of Obesity Among Adults and Youth: United States, 2015–2016.
22. Hannon GJ. RNA interference. *Nature*. 2002;418(6894):244-51.
23. Hawley John A, Hargreaves M, Joyner Michael J, Zierath Juleen R. Integrative Biology of Exercise. *Cell*. 2014;159(4):738-49.
24. Hayden MR, Patel K, Habibi J et al. Attenuation of Endocrine-Exocrine Pancreatic Communication in Type 2 Diabetes: Pancreatic Extracellular Matrix Ultrastructural Abnormalities. *Journal of the cardiometabolic syndrome*. 2008;3(4):234-43.
25. Keklikoglou I, Hosaka K, Bender C et al. MicroRNA-206 functions as a pleiotropic modulator of cell proliferation, invasion and lymphangiogenesis in pancreatic adenocarcinoma by targeting ANXA2 and KRAS genes. *Oncogene*. 2015;34(37):4867.
26. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. *Immunology letters*. 2006;107(2):102-8.
27. Kramer HF, Goodyear LJ. Exercise, MAPK, and NF-κB signaling in skeletal muscle. *Journal of applied physiology*. 2007;103(1):388-95.
28. Lotvall J, Valadi H. Cell to cell signalling via exosomes through esRNA. *Cell adhesion & migration*. 2007;1(3):156-8.
29. Martins AR, Nachbar RT, Gorjao R et al. Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. *Lipids in health and disease*. 2012;11(1):30.
30. Mercier V, Laporte MH, Destaing O et al. ALG-2 interacting protein-X (Alix) is essential for clathrin-independent endocytosis and signaling. *Scientific reports*. 2016;6.
31. Myers J, Prakash M, Froelicher V, Do D, Partington S, Atwood JE. Exercise capacity and mortality among men referred for exercise testing. *New England Journal of Medicine*. 2002;346(11):793-801.

32. Naguibneva I, Ameyar-Zazoua M, Polesskaya A et al. The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. *Nature cell biology*. 2006;8(3):278.
33. Nie Y, Sato Y, Wang C, Yue F, Kuang S, Gavin TP. Impaired exercise tolerance, mitochondrial biogenesis, and muscle fiber maintenance in miR-133a–deficient mice. *The FASEB Journal*. 2016;30(11):3745-58.
34. Nielsen S, Scheele C, Yfanti C et al. Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle. *The Journal of physiology*. 2010;588(20):4029-37.
35. Nikolić N, Bakke SS, Kase ET et al. Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. *PloS one*. 2012;7(3):e33203.
36. Nikolić N, Görgens SW, Thoresen GH, Aas V, Eckel J, Eckardt K. Electrical Pulse Stimulation of Cultured Skeletal Muscle Cells as a Model for in vitro Exercise–Possibilities and Limitations. *Acta Physiologica*. 2016.
37. Ostrowski M, Carmo NB, Krumeich S et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature cell biology*. 2010;12(1):19-30.
38. Pedersen BK, Åkerström TC, Nielsen AR, Fischer CP. Role of myokines in exercise and metabolism. *Journal of applied physiology*. 2007;103(3):1093-8.
39. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nature Reviews Endocrinology*. 2012;8:457.
40. Petriz BA, Gomes CP, Almeida JA et al. The Effects of Acute and Chronic Exercise on Skeletal Muscle Proteome. *J Cell Physiol*. 2016.
41. Poirier P, Giles TD, Bray GA et al. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. *Circulation*. 2006;113(6):898-918.
42. Pratley R, Nicklas B, Rubin M et al. Strength training increases resting metabolic rate and norepinephrine levels in healthy 50-to 65-yr-old men. *Journal of Applied Physiology*. 1994;76(1):133-7.
43. Redman R, Silinsky E. Decrease in calcium currents induced by aminoglycoside antibiotics in frog motor nerve endings. *British journal of pharmacology*. 1994;113(2):375-8.
44. Rejeski WJ, Mihalko SL. Physical activity and quality of life in older adults. *The Journals of Gerontology Series A: Biological sciences and medical sciences*. 2001;56(suppl_2):23-35.
45. Richter EA, Ruderman NB. AMPK and the biochemistry of exercise: implications for human health and disease. *Biochemical Journal*. 2009;418(2):261-75.
46. Roma-Rodrigues C, Fernandes AR, Baptista PV. Exosome in tumour microenvironment: overview of the crosstalk between normal and cancer cells. *BioMed research international*. 2014;2014.
47. Rose AJ, Hargreaves M. Exercise Increases Ca2+–Calmodulin-Dependent Protein Kinase II Activity in Human Skeletal Muscle. *The Journal of physiology*. 2003;553(1):303-9.

48. Rose AJ, Kiens B, Richter EA. Ca2+–calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise. *The Journal of physiology*. 2006;574(3):889-903.
49. Ross R, Dagnone D, Jones PJ et al. Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-induced weight loss in menA randomized, controlled trial. *Annals of internal medicine*. 2000;133(2):92-103.
50. Russell AP, Lamon S, Boon H et al. Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. *The Journal of physiology*. 2013;591(18):4637-53.
51. Safdar A, Abadi A, Akhtar M, Hettinga BP, Tarnopolsky MA. miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice. *PloS one*. 2009;4(5):e5610.
52. Safdar A, Saleem A, Tarnopolsky MA. The potential of endurance exercise-derived exosomes to treat metabolic diseases. *Nat Rev Endocrinol*. 2016;12(9):504-17.
53. Scheler M, de Angelis MH, Al-Hasani H, Häring H-U, Weigert C, Lehr S. Methods for proteomics-based analysis of the human muscle secretome using an in vitro exercise model. *Proteomic Profiling: Methods and Protocols*. 2015:55-64.
54. Schiaffino S, Mammucari C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. *Skeletal muscle*. 2011;1(1):4.
55. Schild M, Ruhs A, Beiter T et al. Basal and exercise induced label-free quantitative protein profiling of m. vastus lateralis in trained and untrained individuals. *Journal of proteomics*. 2015;122:119-32.
56. Stahlhut C, Suárez Y, Lu J, Mishima Y, Giraldez AJ. miR-1 and miR-206 regulate angiogenesis by modulating VegfA expression in zebrafish. *Development*. 2012;139(23):4356-65.
57. Sundquist WI, Schubert HL, Kelly BN, Hill GC, Holton JM, Hill CP. Ubiquitin recognition by the human TSG101 protein. *Molecular cell*. 2004;13(6):783-9.
58. Tamai K, Tanaka N, Nakano T et al. Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. *Biochemical and biophysical research communications*. 2010;399(3):384-90.
59. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nature Reviews Immunology*. 2009;9(8):581-93.
60. Thompson PD, Buchner D, Piña IL et al. Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease. *Circulation*. 2003;107(24):3109-16.
61. Uhlemann M, Möbius-Winkler S, Fikenzer S et al. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. *European journal of preventive cardiology*. 2014;21(4):484-91.
62. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology*. 2007;9(6):654-9.
63. van Solingen C, Seghers L, Bijkerk R et al. Antagomir-mediated silencing of endothelial cell specific microRNA-126 impairs ischemia-induced angiogenesis. *Journal of cellular and molecular medicine*. 2009;13(8a):1577-85.

64. Wilmore JH. Increasing physical activity: alterations in body mass and composition. *The American journal of clinical nutrition*. 1996;63(3):456S-60S.
65. Wollert T, Wunder C, Lippincott-Schwartz J, Hurley JH. Membrane scission by the ESCRT-III complex. *Nature*. 2009;458(7235):172-7.
66. Zamboni M, Rossi AP, Fantin F et al. Adipose tissue, diet and aging. *Mechanisms of ageing and development*. 2014;136:129-37.
67. Zhang J, Li S, Li L et al. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics, proteomics & bioinformatics*. 2015;13(1):17-24.
68. Zhang Y, Yu M, Tian W. Physiological and pathological impact of exosomes of adipose tissue. *Cell proliferation*. 2016;49(1):3-13.
69. Zhou B, Ma R, Si W et al. MicroRNA-503 targets FGF2 and VEGFA and inhibits tumor angiogenesis and growth. *Cancer letters*. 2013;333(2):159-69.
70. Zurlo F, Larson K, Bogardus C, Ravussin E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *Journal of Clinical Investigation*. 1990;86(5):1423.

VITA

Ron T. Garner

Education

Doctor of Philosophy, Purdue University, West Lafayette, IN Anticipated, Aug 2018
Health and Kinesiology
Research Area: Effects of Acute Exercise on Skeletal Muscle Exosomes
Advisor: Tim Gavin, PhD
Specialization: Exercise Physiology

Master of Science, Utah State University, Logan, UT 2013
Health and Human Movement
Thesis: Lactate Threshold: Land versus Water Treadmill Running Comparison
Committee Chair: Dennis Dolny, PhD
Specialization: Exercise Physiology

Bachelor of Science, Brigham Young University–Idaho, Rexburg, ID 2010
Exercise and Sports Science
Undergraduate Research Project: Time to Exhaustion at the Lactate Threshold
Mentor: Eli Lankford, PhD
Specialization: Exercise Physiology

Professional Experience

Purdue University, West Lafayette, IN
Graduate Teaching Assistant 2013-14; 2016-Current
Graduate Research Assistant 2015-2016
Graduate Lecturer (4x4) 2014-2015

Utah State University, Logan, UT
Graduate Teaching Assistant 2011-2013
Graduate Research Assistant 2011-2012

Brigham Young University–Idaho, Rexburg, ID
Adjunct Faculty 2010-2011; 2013

Teaching Experience

Courses Taught (Purdue University)

- PES 111 (Online): Lifetime Fitness
- HK 266: Intro to Health and Fitness Programs
- HK 368: Exercise Physiology (Lecture & Laboratory)
- HK 421: Health Screening and Fitness Evaluation (Lecture & Laboratory)
- HK 422: Basic Concepts in Exercise Program Design
- HK 424: Health and Fitness Program Management
- HK 469: Exercise Testing and Prescription in Special Populations

Courses Taught (BYU–Idaho)

- ESS 375: Exercise Physiology (and ESS 375L)
- HS 391: Research Methods and Program Evaluation

Courses Taught (Utah State University)

- PEP 4100: Exercise Physiology Lab
- PEP 4400: Evaluation in Physical Education (Statistics)
- PEP 5100: Fitness Assessment
- PEP 5430: History & Philosophy of Physical Education and Sport

Research Experience

PhD Student, Purdue University, West Lafayette, IN 2013-Current

Skills acquired during my PhD training include:

- Subject recruitment, experimental design, metabolic data collection
- Assistance with human skeletal muscle biopsies
- Primary human and mouse cell culture lines
- Skeletal muscle analysis including Western Blots, RNA isolation and quantitative RT-PCR (including microRNA analysis), and enzyme activities (spectrophotometer)
- Immuno-staining from whole muscle cross-sections and *in vitro* samples
- Exosome isolation from whole muscle and *in vitro* samples
- Graduate RA position (2015-2016)
- Supervise / train several undergraduate (3) and Master's students (3)

Graduate Research Assistant, Purdue University, West Lafayette, IN 2015–2016

Funded by the HK Department through the Purdue Research Foundation

- Skeletal muscle exosomes – aged cell-culture model *in vitro* – metabolic changes in acute aerobic vs concurrent exercise – metabolic changes in short- and long-duration type 2 diabetic tissue

Graduate Research Assistant, *Utah State University, Logan, UT* 2011–2012

Effect of Milk Fat Globule Membrane on Gut Barrier Protection in Runners 2011
(Nutrition and Health Physical Education, and Recreation department), Dr. Eadric Bressel (HPER), Dr. Kory Hintze (NDFS), Dr. Robert Ward (NDFS)
Funded by United Dairymen of Idaho

- Metabolic data – body composition – data analysis

Motorized versus Regular Elliptical Energy Comparison 2012
(Health, Physical Education, and Recreation department), Dr. Edward Heath
Funded by ICON Health and Fitness

- Metabolic data – recruited subjects – schedule management – Data Analysis

Publications

Peer-Reviewed Articles:

Gavin, T.P., Ernst, J.M., Kwak, HB., Caudill, S.E., Reed, M.A., **Garner, R.T**., Nie, Y., Weiss, J.A., Pories, W.J., Dar, M., Lin, CT, Hubal, M.J., Neufer, P.D., Kuang, S., & Dohm, G.L. High incomplete skeletal muscle fatty acid oxidation explains low muscle insulin sensitivity in poorly controlled T2D. *The Journal of Clinical Endocrinology & Metabolism*, 2017.

Garner, R., Wagner, D., Bressel, E., & Dolny, D., (2014). Land versus water treadmill running: Lactate threshold. *International Journal of Aquatic Research and Education,* 8, 9-19.

Garner, R., Wagner, D., (2013). Validity of certified trainer-palpated and exercise-palpated post-exercise heart rate. *Journal of Exercise Physiology Online,* 16, 31-38.

Manuscripts Submitted:

Solfest, J.S., Nie, Y., Weiss, J.A., **Garner, R.T.**, Kuang, S., Stout, J., & Gavin, T.P. Skeletal muscle metabolic enzyme responses to acute concurrent aerobic and resistance exercise.

Manuscripts in Preparation:

Garner, R.T., Nie, Y., Solfest, J.S., Stout, J., Gavin, T.P. Exosome and Exosome-Related Pathway Responses to Acute Exercise.

Garner, R.T., Weiss, J.A., Nie, Y., Gavin, T.P. Effects of Obesity and Acute Resistance Exercise on Skeletal Muscle Intercellular Communication Pathways.

Peer-Reviewed Abstracts:

Garner, R.T., Nie, Y., & Gavin, T.P., (2018). Effect of acute exercise on skeletal muscle exosome biogenesis. *Medicine and Science in Sports and Exercise,* 50 (5S):552.

Sullivan, B.P., Weiss, J.A., **Garner, R.T.**, Nie, Y., & Gavin, T.P. (2018). Altered Skeletal Muscle IGF-1 and miR-206 at Rest and Following Resistance Exercise in Obese Humans. *Medicine and Science in Sports and Exercise,* 50(5S):116.

Kargl, C.K., Nie, Y., **Garner, R.T**., & Gavin T.P. (2018). Effects of Obese Skeletal Muscle Cells on Endothelial Cell Angiogenesis. *Medicine & Science in Sports & Exercise.* 50(5S):543.

Hettinger, Z.R., Nie, Y., **Garner, R.T**., Kargl, C.K., Patel, S., & Gavin, T.P. (2018). Serial Passaging Reduces Replication and Fusion Capacity of Primary Human Skeletal Muscle Satellite Cells. *Medicine & Science in Sports & Exercise.* 50(5S):552.

Garner, R.T., Ernst, J.M., Kehe, S.E., Reed, M.A., Nie, Y., Dohm, G.L., Pories, W.J., Dar, M., & Gavin, T.P., (2015). Glycemic control, skeletal muscle insulin sensitivity, and skeletal muscle mitochondrial complex I-V content in type 2 diabetics. *Medicine and Science in Sports and Exercise,* 47, S452.

Garner, R., Gengler, J., Robertson, C., Lym, B., Rindlisbacher, J., Schreher, N., & Lankford, D.E., (2012). A new method for determining exercise intensity to meet cardiovascular needs. *Medicine and Science in Sports and Exercise, 44, S439.*

Conference Presentations

Garner, R.T., Nie, Y., & Gavin, T.P. Effect of acute exercise on skeletal muscle exosome biogenesis. *National ACSM, May 2018,* Minneapolis, MN.

Kargl, C.K., Nie, Y., **Garner, R.T.**, Evans, S., Hettinger, Z.R., Sullivan, B.P., Gavin T.P. Effects of obese skeletal muscle cells on endothelial cell angiogenesis. *HHS Fall Research Day, November 2017*, West Lafayette, IN.

Hettinger, Z.R., Nie, Y., **Garner, R.T**., Kargl, C.K., Patel, S.H., Kuang, S., Gavin, T.P. Serial passaging reduces replication and fusion capacity of primary human skeletal muscle satellite cells. *HHS Fall Research Day, November 2017*, West Lafayette, IN.

Sullivan, B.P., Weiss, J.A., **Garner, R.T.**, Nie, Y., Gavin T.P. Altered skeletal muscle IGF-1 and miR-206 at rest and following resistance exercise in obese humans. *HHS Fall Research Day, November 2017*, West Lafayette, IN.

Garner, R.T., Weiss, J.A., Nie, Y., Gavin, T.P. Response of skeletal muscle exosome markers to acute resistance exercise in lean and obese humans. *Integrative Biology of Exercise VII, November 2016,* Phoenix, AZ.

Sullivan, B.P., Weiss, J.A., **Garner, R.T.**, Nie, Y., Gavin T.P. Effects of obesity and resistance exercise on skeletal muscle Acetyl CoA Carboxylase. *MW ACSM, November 2016,* Fort Wayne, IN.

Garner, R.T., Weiss, J.A., Nie, Y., Solfest, J.S., Gavin, T.P. Skeletal muscle exosome responses to acute aerobic and aerobic + resistance exercise. *Muscle Biology Conference, January 2016,* Gainesville, FL.

Garner, R.T., Weiss, J.A., Nie, Y., Gavin, T.P. 3-L-hydroxyacyl Co-A dehydrogenase enzyme activity is increased in poorly controlled type 2 diabetes. *MW ACSM, November 2015,* Fort Wayne, IN.

Weiss J.A., **Garner, R.T.**, Nie, Y., Solfest, J.S., Gavin, T.P. Skeletal Muscle Enzyme Protein Responses to Acute Aerobic and Aerobic + Resistance Exercise. *MW ACSM, November 2015*, Fort Wayne, IN.

Garner, R.T., Ernst, J.M., Kehe, S.E., Reed, M.A., Nie, Y., Dohm, G.L., Pories, W.J., Dar M., Gavin, T.P. Glycemic control, skeletal muscle insulin sensitivity, and skeletal muscle mitochondrial complex I-V Content in Type 2 Diabetics. *National ACSM, May 2015,* San Diego, CA.

Garner, R., & Dolny, D. Lactate threshold: land versus water treadmill running comparison. *Intermountain Graduate Research Symposium,* Apr. 2013, Logan, UT.

Louder T., Flynn, D., **Garner R**. Effect of acute static stretching of agonists vs antagonists on vertical jump performance. *Intermountain Graduate Research Symposium,* Apr. 2012, Logan, UT.

Garner, R., Gengler, J., Robertson, C., Lym, B., Schrerer, N., & Lankford, D.E., (2012). A new method for determining exercise intensity to meet cardiorespiratory needs. *National ACSM, May 2012,* San Francisco, CA.

Garner, R., Gengler, J., Robertson, C., Lym, B., Schrerer, N., & Lankford, D.E., (2012). A new method for determining exercise intensity to meet cardiorespiratory needs. *Southwest American College of Sports Medicine, Oct 2011,* Reno, NV.

Grants

Funded:

Purdue University, Purdue Research Foundation Grant 2015-2016 Academic Year
Aging and Metabolic Flexibility
Health and Kinesiology Department, West Lafayette, IN, $25,000

Not Funded:

ACSM Foundation Doctoral Student Research Grant Jan 2017
Skeletal Muscle Exosomes & Type II Diabetes
American College of Sports Medicine, Indianapolis, IN, $5,000

Purdue University, Purdue Research Foundation Grant 2016-2017
Type II Diabetes Mellitus and Angiogenesis
Health and Kinesiology Department, West Lafayette, IN, $25,000

Utah State University, *Research and Projects Grant* 2012
Lactate Threshold: Land vs. Water Treadmill Running
Student Involvement Office, Logan, UT, $1000

Invited Talks

Health and Kinesiology Department Colloquia, Purdue University March 2017
Acute Exercise and Skeletal Muscle Exosomes

Professional Affiliations

American Physiological Society, National Member 2016–Current
American College of Sports Medicine, National member 2011–Current
American College of Sports Medicine, Midwest Chapter member 2015–2016
American College of Sports Medicine, Southwest Chapter member 2011–2012
American College of Sports Medicine, Northwest Chapter member 2010–2011

Service

To the Profession:

Abstract Reviewer, Northwest Chapter of ACSM Fall 2014

To the Community:

Scoutmaster, Boy Scouts of America 2010 & 2012
Service Mission, Denver, CO 2006–2008
Volunteer Medical Assistant CHASQUI Organization, Cusco, Peru 2006

Awards and Recognition

Donald L. Corrigan Grant, $550, Spring 2018
Health and Kinesiology Department, Purdue University, West Lafayette, IN

HHS Compton Graduate Research Travel Award, $500, Fall 2016
College of Health and Human Sciences, Purdue University, West Lafayette, IN

Teaching Academy Graduate Teaching Award Spring 2016
Health and Kinesiology Department, Purdue University, West Lafayette, IN

PhD Poster Presentation Winner, $150, Fall 2015
Midwest American College of Sports Medicine, Fort Wayne, IN

A.A. Annarino Award for Outstanding Graduate Teaching, $200, Spring 2015
Health and Kinesiology Department, Purdue University, West Lafayette, IN

HK Barrett Memorial Scholarship, $150, Spring 2015
Health and Kinesiology Department, Purdue University, West Lafayette, IN

Dale Hanson Award, $500, Spring 2015
Health and Kinesiology Department, Purdue University, West Lafayette, IN

Donald L. Corrigan Grant, $300, Fall 2014
Health and Kinesiology Department, Purdue University, West Lafayette, IN

Graduate Teaching Assistant of the Year 2012-2013
Health, Phys Ed, and Rec Dept., Utah State University, Logan, UT

Winner of Poster Session, Hum, Arts, Soc Sci, and Edu category, $300, April 2013
Intermountain Graduate Research Symposium, Utah State University, Logan, UT

Department Private Scholarship, $500, 2010
Exercise and Sports Science, BYU–Idaho, Rexburg, ID

Leadership Scholarship, $350 2005
BYU–Idaho, Rexburg, ID 2004

Eagle Scout Award 2004
Boy Scouts of America, Fruitland, ID