MTOR: A MECHANISTIC TARGET OF MUSCLE AND CANCER CROSSTALK

A Dissertation

by

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ABSTRACT

In the United States, the number of colon and breast cancer cases that are attributed to physical inactivity lead the exercise-associated cases of heart disease and type II diabetes. For the millions of people in the United States burdened with breast cancer, there is a noted substantial risk reduction with increased physical activity. Previous research has investigated skeletal muscle's endocrine-like potential on inflammation and cancer metabolism; however there is limited investigation into exercise-facilitated suppression of cancer's major anabolic pathway, the mechanistic target of rapamycin (mTOR) pathway. While previous cancer research has established that mTOR's activity is dysregulated in cancer, little is known about the impact of exercise on the regulation of anabolic/proliferative features of breast cancer cells, nor has exercise been evaluated as a mediator of muscle and cancer crosstalk. This project's objective is to determine how exercise is affecting the biological regulation of tumorgenesis (a critical component of treatment innovation) via the mTOR pathway, and how that regulation is mediated by skeletal muscle contraction.

While research efforts and analyses about the mTOR pathway have led to key insights into its regulation of apoptotic and autophagic signaling in cancer, these efforts do not capture the complete profile of mTOR control on cell growth and survival, nor address preliminary data indicating that cellular proliferation rates are significantly reduced in breast cancer cells treated with excretion factors arising from contracting skeletal muscle ("exercise").

The work presented here-in utilizes a research approach consisting of cell culture and animal models to investigate key mechanistic foundations that underlie the biological regulation of breast cancer in individuals who partake in exercise. Specifically, the MCF7 epithelial breast

cancer cell line, a hemicorpus hind limb perfusion (HHLP) surgery, and pharmacological interventions allow for evaluation of skeletal muscle's endocrine ability, global protein synthesis, signal transduction and gene expression. The investigators' consideration of muscle-cancer crosstalk via exercise lays the foundation for future evaluation of muscle-derived biomolecules (ie. microRNA) as a potential crosstalk mediators.

This project's successful completion proposes key mechanistic foundations that underlie the biological regulation of breast cancer, contributing greatly to science's efforts towards novel translational investigation of the beneficial relationship between muscle and cancer crosstalk. Establishing a casual role for exercise as primary cancer prevention would have major translational impact in cancer prevention and patient survivorship, with even a small reduction in incidence of cancer resulting in multi- billion dollar health care savings.

DEDICATION

To my loving parents, George and Caroline, whose encouragement and unwavering support have guided me throughout this journey, and to my brother, Kyle, who continues to inspire me with his pursuit of knowledge, commitment to ethics, and his courage to venture into the unknown.

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Its contents are solely the responsibility of the authors and do not necessarily represent the official views of any of the previously mentioned awarding offices.

NOMENCLATURE

MTOR	Mechanistic target of rapamycin
DEPTOR	DEP domain target of rapamycin
HHLP	Hemicorpus Hind Limb Perfusion
Non-Stim	Non-Electrical Stimulation
E-Stim	Electrical Stimulation
Post-Stim	Post-Electrical Stimulation
KB	Krebs Buffer
CC	Cell Control
VC	Vehicle Control
RAP	Rapamycin
TOR1	Torin1
ACTD	ActinomycinD
CYX	Cycloheximide
DMSO	Dimethyl sulfoxide
(p)	Phosphorylated
mirRNA	microRNA
myomiR	muscle released microRNA
mRNA	messenger RNA
MCF7	Human Breast Cancer Cell Line (Michigan Cancer Foundation)

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1. INTRODUCTION

The burden of breast cancer affects over 2.8 million women in the United States, with an estimated 268,600 *new cases* in 2019 alone, thus accounting for 30% of all new cancer diagnoses in women alone [1]. However, this burden can be reduced with exercise. Exercise has been shown to elicit beneficial effects in the treatment and prevention of breast cancer in addition to improved immune response with evidence of decreased risk with increased physical activity [2, 3]. The role of exercise as a key player in reduction of tumor growth has been noted since the late 1960s [4, 5], yet most research has focused on the *efficacy* of physical activity in cancer prevention, leaving the physiological mechanisms less understood, even in rodent models [2, 6, 7]. Centrally, proposed mechanisms behind a noted 20-40%% reduction in cancer risk [2, 8, 9] include alterations in whole body conditions such as body composition and improved metabolism, in addition to circulating adipokines, hormones and inflammatory makers [10-18]. While these systemic modifications undoubtedly *contribute* to improved prognosis, they can not explain the association of isolated muscle contractions to decrease tumor growth noted in animals models. Investigations into tissue specific contributions to improved prognosis provide mechanistic insights and thusly propose means of tissue and cancer crosstalk.

1.1. Skeletal Muscle As An Endocrine Organ

Underappreciated by many is skeletal muscle's ability to release molecules during repetitive contractions into the systemic circulation. These molecules, termed "myokines", are known to alter signaling pathways, associated with homeostasis and inflammation, both of which are prominent in cancer patients [8, 19-22]. F-substance ("fatigue" substance) and growth retarding

tumoricidal substance isolated from both *in vivo* and *ex vivo* approaches have demonstrated delayed mitotic rate and inhibited tumor progression *in vitro* and *in vivo* [4, 21, 23, 24]; however isolated cellular mechanisms pertaining to cell growth have not been identified. Using perfusate collected from our lab, Westerlind et al [25] demonstrated increased rates of apoptosis concomitant with unaltered mitotic capacity (ability of non-apoptosed cells to daughter) in perfusate treated MCF7 cells. The increase in apoptosis is consistent with other investigators who also noted decreased proliferations rates both in *culture* and in tumor bearing animals treated with perfusate either by subcutaneous injection [25] or with exercise intervention [4, 21, 24, 26].

Advancement in biochemical and detection technologies have allowed the identification of microRNA (miRNA), a class of small, non-coding RNAs ~22 nucleotides in length. The miRNA system is an endogenous mechanism of gene regulation, controlling 30% of overall gene expression primarily through translational repression [27]. Skeletal muscle is a potent reservoir of these small molecules. Gene expression studies have identified hundreds of miRNA that are dysregulated in disease states. In cancer cells, control of oncogenes and tumor suppressors have been demonstrated by functional studies [28, 29]

1.2. Cellular Anabolism

Anabolism is a key physiological process contributing to cell growth**,** and yet, in relation to cancer and alternative chemotherapeutic approaches, it remains poorly utilized. While the majority of basic science experimental evidence supports an inhibition of mammary tumorigenesis with exercise [30-36], these investigations can only provide insight into cell survival and programmed cell death. With the knowledge that cell cycles are highly integrated

(Figure1), it is significant to note that there has been neglect in evaluating changes in the anabolic state of a cancer cell.

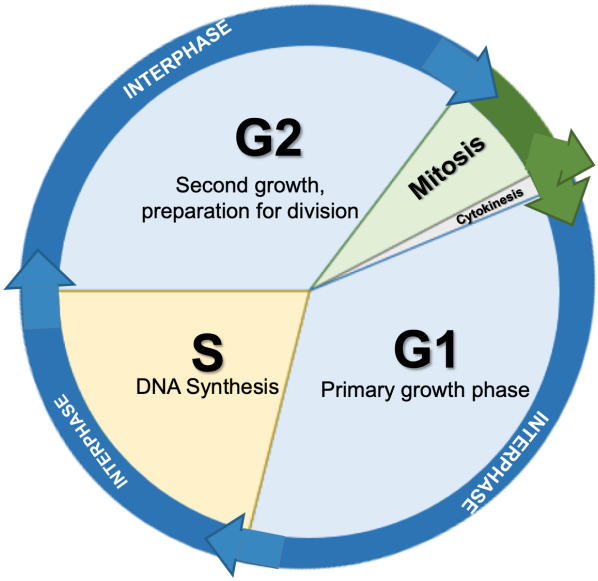

Figure 1. Cell Cycle Schematic

Changes in mitotic capacity and proliferation can both be attributed to changes in anabolism and global protein synthesis; thus, it is proposed that muscle-perfusate treated cells/tumors may have delayed growth rates (ie. global protein synthesis) which significantly contributes a delayed attainment of sufficient cellular mass required to mitose. As such, the comparison of both cellular physiological processes associated with mitosis and proliferation provide a more complete picture of exercise's impact on breast cancer. Both cell growth and proliferation are regulated by signaling pathways sensitive to anabolic stimuli, such as nutrients, oxygen availability, growth factors and environmental stimulants. As such, understanding the activation and signal transduction within these pathways provides a logical starting point to investigate exercise's impact of breast cancer.

mTOR

The mechanistic target of rapamycin (mTOR) signaling pathway (Figure 2) is central to multiple cell cycle and survival mechanisms. The mTOR kinase coordinates environmental stimuli and intrinsic feedback loops to regulate cell growth, cellular proliferation and survival, all of which are vital for normal cell function. However when this pathway is dysregulated or mTOR activity is aberrant, such as in cancer, it can lead to the hyperactivation of mTOR signals that serve to both promote cellular proliferation and protect against apoptosis [37-44].

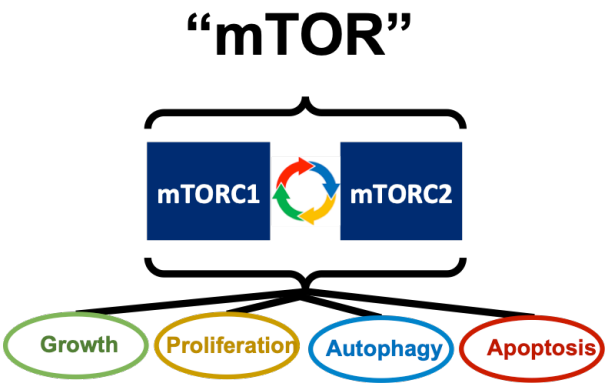

Figure 2. General mTOR Signaling Pathway

The mTOR protein associates with other proteins to define its complex, and ultimately its downstream effectors. The mTORC1 is a heterodimer protein kinase defined by its associated proteins Regulatory-Associated Protein of mTOR (RAPTOR) and Mammalian Lethal with SEC13 protein 8 (mLST8). The complex is further accessorized with inhibitory proteins PRAS40, and DEP domain-containing mTOR-interacting protein (DEPTOR). The mTORC2 complex is defined by mTOR's association with Rictor, the Rapamycin insensitive counterpart.

This complex also is accessorized by DEPTOR and mLST8, in addition to Protein Associated with Rictor (PROTOR) and stress-activated protein kinase-interacting protein-1 (mSINI).

Signal integration by mTOR is primarily through three nodes: 1) Tuberosclerosis complex (TSC) integrating growth factors, stressors, and energy, 2) RAPTOR and PRAS40 integrating energy and growth factor, respectively, and 3) lysosomal membrane association using the RHEB axis for nutrient interaction [40]. While mTORC1 activity is potently anti-apoptotic, most investigation into its role in the biological regulation of cancer has been directed towards apoptotic and autophagic signals [23, 25, 45-47] with minimal efforts directed at cellular anabolism occurring concomitantly or separately. Active mTORC1 modulates two separate downstream translational regulators, ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF-4E)-binding protein (4E-EBP1) [48]. The S6K pathway is activated upon phosphorylation, and thusly functions in RNA processing and protein biogenesis to increase cellular size, which is the limiting factor for cell division [49-52]. Additionally S6K is a proponent of multiple feedback loops targeting upstream PI3K-Akt and Rictor-Akt signaling [53, 54] thus contributing to cross-complex interplay.

Although first discovered in the early 1990s, the much of what is known about serine/threonine mTOR has come through investigations of a canonical inhibitor, Rapamycin (also known as sirolimus). Discovered in the early 1970s, this macrolide fungicide was originally developed for use as a clinical immunosuppressant [55]. It exerts inhibition through allosteric binding at the FKP 12 domain of the cell-cycle-specific kinase, TOR (Target of Rapamycin) to generally slow proliferation and reduce cell size. Notably, response to rapamycin varies widely amongst cell types. Moreover, its effect on mTORC1 substrates S6K1 and 4E-BP1 vary in ability to suppress the downstream effectors' respective contribution to cap-dependent translation.

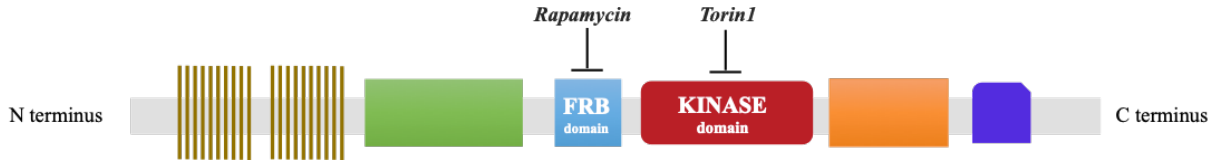

Figure 3. mTOR Inhibition via Pharmacology
A schematic representation of the mTOR gene domain and proposed domain targets of Rapamycin and Torin 1 inhibition. Adapted from "Commentary: Overcoming mTOR resistance mutations with a new-generation mTOR inhibitor." M. Renna, 2016, Frontiers in Pharmacology 7(431). Copyright © 2016 Renna.

While S6K1 activity is completely inhibited by rapamycin treatment, the more intricate co-ordination of multiple 4E-BP1 phosphorylation sites results in only partial and transient inhibition, mediating some rapamycin resistant function [56, 57]. As investigations into the interplay between the two complexes continues to provide pro-survival feedback loops and sources of self-regulation (Figure 4), biochemical innovation continues into second and tertiary generations of rapalogs and mTOR inhibitors in hopes or success in future clinical trials.

Emerging as a key protein in the mTOR signaling pathways, DEPTOR is endogenous regulator of mTOR activity. DEPTOR is an mTOR binding protein which inhibits the mTORC1 and mTORC2 activity, and whose expression is low in most cancers [37, 40, 43, 58, 59]. Investigation of protein has gained momentum over the last ten years, primarily focusing efforts into understanding the mTOR-DEPTOR interaction and implications on cell growth and proliferation *in vitro*. These efforts have mainly addressed apoptotic and autophagy pathways [29, 60-69]. Unlike the mTOR kinase, which aberrant hyperactivity is consistent through out most cancers, the mTOR-DEPTOR relationship is less predictable. Specifically in breast cancer, DEPTOR expression is known to be low and is a defining characteristic of metastatic and invasive breast cancers such as

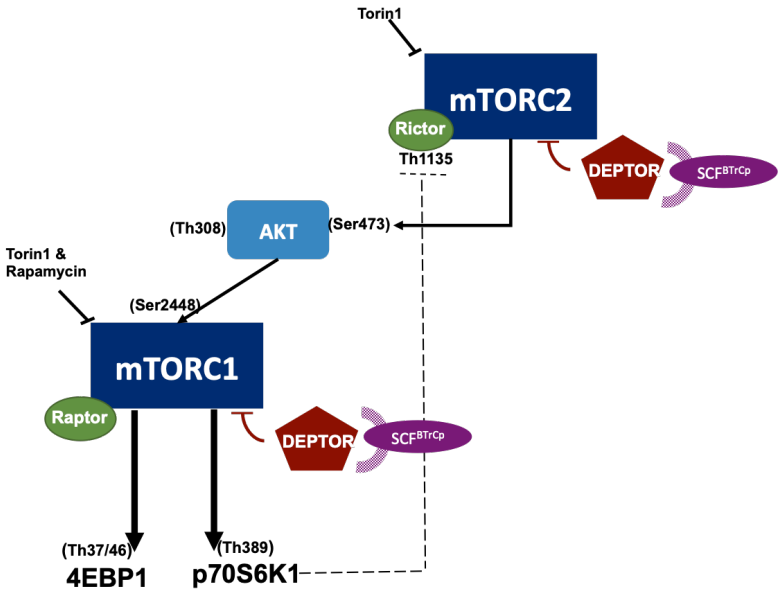

Figure 4. Dynamic mTOR cross-complex feedback signaling

the MDA-MD-231 cell line [70]. To date, research efforts has focused on induced DEPTOR-suppression during re-sensitization to apoptotic stimuli [40] , upregulated DEPTOR in aggressive cancer cells [59, 70], DEPTOR degradation process evaluation [58, 59] and understanding the feedback loops in signals arising from DEPTOR-mTOR interaction [37, 38, 41, 42, 44, 71]. However, mTOR-DEPTOR interaction in epithelial MCF7 cells in direct response to exercise or contraction medium is not fully understood, nor its relation to cellular anabolism and protein synthesis of a breast cancer cell.

Protein Synthesis

Anabolism is the coordinated metabolic activity that allows cells to produce macromolecules [72]. Protein synthesis rate is an indicator of metabolic activity and thusly contributes to a cell's anabolic profile. The mTORC1 downstream network highly regulates protein synthesis by co-coordinating aspects of cap-dependent translation, translation elongation,

mRNA biogenesis and ribosome biogenesis (Figure 5). Cumulative protein synthesis is resultant from both *de novo* (i.e. nutrients) and recycled amino acid incorporation [73], of which cell growth is highly dependent upon [57, 72, 74, 75]. To allow for the dynamics of protein synthesis, an appropriate method to assess global fractional synthesis rate (FSR) in a cancer cell exposed to simulated exercise is the deuterium oxide (^2H_2O) methodology. This method allows for the incorporation of a stable isotope over physiologically relevant conditions, such as 24h (daily FSR value), thus assessing long-term biosynthesis of macromolecules and accounting for fluctuations in cellular activity [73, 76-80]. Furthermore, with the objective to maintain the translational application of study findings, use of ^2H_2O as stable isotope tracer accounts for the dynamic changes in both skeletal muscle behavior and tumor microenvironment with exercise (i.e. nutrient availability, hormone circulation, mechanical stimuli) in patients existing under free-living conditions. To our knowledge, cumulative fractional protein synthesis (24h FSR [80]) of a cancer cell following exercise or pharmacological intervention has not been directly assessed. As such, elucidating the integration of signaling events that contribute to protein synthetic response with both illustrate and contribute to a complete anabolic profile of breast cancer cells exposed to simulated exercise.

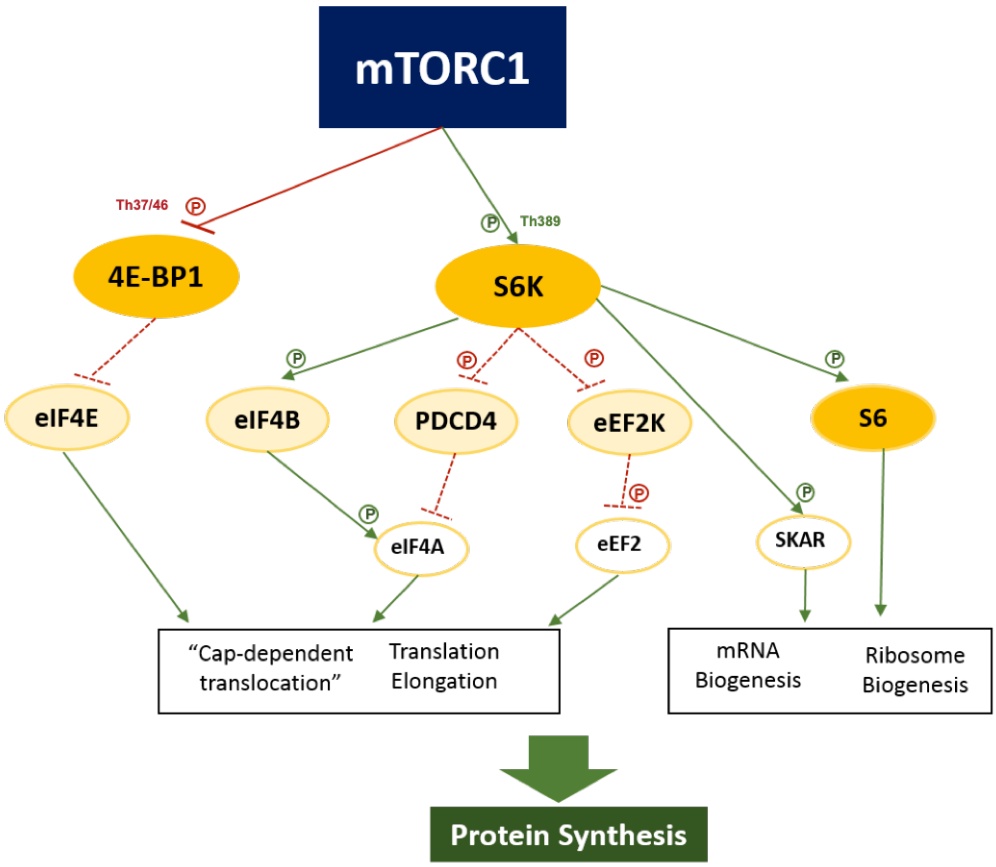

Figure 5. mTORC1 protein synthesis signaling

1.3. Endogenous Regulation of Gene Expression

Proteins dictate cell function, but what proteins are found in our cells are dictated by the messaging transcripts that code for their production. The amounts and types of those coding transcripts, known to scientists as *messenger RNA* (mRNA), dictate protein levels and ultimately reflect cellular function. When the transcription rate of certain mRNA molecules fluctuates and/or the translation of those mRNA into target proteins becomes irregular, cellular homeostasis can become dysregulated. This process, known as *Gene* Expression, is an area of high importance when investigating causes of human diseases, such as breast cancer. Researchers are diligently investigating how exercise may alter tumor gene expression, limiting how and if the

cancer cells proliferate and tumors grow. Using pharmacological and animal models of exercise, scientists can manipulate the transcription-translation process within breast cancer cells, evaluating changes in protein-signaling pathways vital to growth and normal cell function. Like the mTOR pathway that can influence selective translation of transcripts, gene expression takes coordination from a lot of moving parts from delicate cellular machinery to ideal environmental conditions, making many possible check points between transcription and translation available for therapeutic intervention.

1.4. Purpose

The purpose of the work described in the following dissertation was to establish a potential mechanistic basis for muscle and breast-cancer crosstalk with exercise.

Our long-range goal is to explore the therapeutic mechanisms of exercise associated with protein turnover/cell growth in normal vs cancerous cells. Our objective for this proposal is to characterize exercise's impact on breast cancer anabolism and investigate the mTOR-pathway in epithelial breast cancer in vitro. Our rationale underlying this research was that once the mechanistic basis for muscle and cancer crosstalk is known, targeted pharma-, chemo-and exercise therapeutic strategies may be used to protect at-risk populations.

Each of the following studies are in manuscript format, complete with a brief introduction, methods specific to the study, results and discussion. A general conclusion of the combined studies can be found in the last chapter. The underlying theme of the work here-in is

that contracting skeletal muscle exerts its influence on the regulation of mTOR signaling in cancer cells.

The first study in this dissertation was divided into two portions. The first portion of the study established that the hemicorpus hind limb preparation protocol used during studies 1 and 3 was sufficient to suppress rates of protein synthesis in breast cancer cells exposed to muscle released factors for 24h. This assessment was made using an established deuterium (^2H_2O) isotope tracer method [81, 82], modified for in culture. The second portion of the study attempted to elucidate whether skeletal muscle contraction imparted regulation on the mTOR signaling pathway in MCF7 breast cancer cells. If so, then one may suspect that this finding could have important implications for exercise prescriptions currently advocated by physicians and provide insight into biological mechanisms responsible for noted decreases in tumor growth and improved prognosis in physically active patients. Our colleagues addressed the potential for altered mTOR activity via apoptosis in response to exercise with contraction perfusate collected in our lab [25]. Westerlind et al. demonstrated not only increased apoptosis in cells treated in vitro with perfusate of stimulated muscles [25], but increased tumor time to latency and tumor growth retardation with moderate exercise training in vivo [23]. The protein regulating cellular apoptosis is the same protein that regulates cell growth and proliferation, and methods used by Westerlind et al. do not address changes in the anabolic activity, nor capacity, of the cancer cells. Currently, no studies exist regarding the impact of exercise on the anabolic response on breast cancer and whether specific proliferative pathways are altered leading to suppressed cell growth and/or induction of apoptosis.

The second study in this dissertation was divided into two portions. The first portion established that pharmacological administration of mTOR inhibitors suppress protein synthesis

and consequent anabolic signaling, with a rescue of DEPTOR protein content. This assessment was made using MCF7 breast cancer cells treated with known mTOR kinase inhibitors Rapamycin and Torin1 [83]. Assessment of rates of cellular protein synthesis was made using the same modified deuterium (^2H_2O) isotope tracer method used in study 1. The second portion of the study attempted to disclose whether discrepancies in protein expression between study 1 muscle-perfusate treated cells and study 2 mTOR inhibitor treated cells was due to bioavailability of messenger RNA transcript encoding mTOR growth and proliferative factors. By using gene expression inhibitors ActinomycinD (transcription inhibitor) [84] and Cycloheximide (translation inhibitor) [85], this investigator could observe whether mRNA transcription and/ translation was dependent on mTOR activity. No studies exist regarding the dependency of mTOR proliferative factors' transcription- translation on mTOR kinase activity in breast cancer cells.

The third and last study in this dissertation was divided into two portions. The first portion established that the bilateral hemicorpus hind limb perfusion was a sufficient model of skeletal muscle contraction, which isolated contracting skeletal muscle as direct contributor to cancer crosstalk. The second portion examined miRNome profile of perfusate released from contracting skeletal muscle and the ability of myomiRs to enter systemic circulation. The ability of skeletal muscle to secret factors into systemic circulation to exert para- and autocrine functions within the body are well documented [8, 19-22]. Recent studies have shown that skeletal muscle is potent reservoir of miRNA (myomiR), and possesses the ability to release myomiR into circulation passively and actively. The mechanisms associated with myomiR delivery to breast cancer cells is not completely understood, but may contribute to altered gene expression and anabolic activity of breast cancer cells exposed to muscle-contraction medium.

Given breast cancer's prevalence in the United States, and the recognized benefit of exercise in breast cancer patients, *there is a critical need to translate how exercise impacts breast cancer proliferation in order to vertically move the field's understanding of muscle and breast cancer crosstalk, and* of *the biological regulation on breast cancer*.

1.5. Specific Aims

Study 1

1. To determine if breast cancer anabolism is altered by simulated exercise.

Study 2

2. To identify whether the altered proliferation of breast cancer cells is mediated by cellular signal transduction of specific proliferative pathways.
3. To determine if cellular proliferation of breast cancer cells is mediated by changes of cellular expression of proliferative factors.

Study 3

4. To profile the ability of muscular contraction to facilitate the release of endogenous microRNA into systemic circulation

This project's successful completion provides key mechanistic foundations that underlie the biological regulation of breast cancer, vertically moving the field towards novel translational investigation of the beneficial relationship between exercise and cancer.

1.6. Hypotheses

Given our preliminary data demonstrating decreased proliferation of MCF7s following exercise exposure, *our central hypothesis is that the impact of exercise on breast cancer is by directly influencing mTOR and its associated pathway.*

The following hypotheses are stated in the Null format.

Study 1

1. H_o: Anabolic activity of breast cancer cells will not be affected by exposure to exercise perfusate medium.

Study 2

2. H_o: Pharmacologically altered mTOR activity will not result in altered anabolic activity and expression of inhibitory proteins compared to control cells.
3. H_o: mTOR activity does not affect the expression of inhibitory proteins and activation of downstream anabolic signals in breast cancer cells.

Study 3

4. H_o: Endogenous skeletal muscle microRNA secretion will not be different with and without electrical-simulated contraction.

1.7. Significance and Innovation

Significance

In the United States, breast cancer is the most common cancer in women [86] with more than 2.8 million women with a history of breast cancer and an estimated 238,130 *new cases* 268, 600 *new cases* in 2019 alone [1]. Exercise has been shown to elicit beneficial effects in the treatment and prevention of cancer, in addition to decreased cancer risk with increased physical activity [2, 3]. The role of exercise as a key player in reduction of tumor growth has been noted since the late 1960s [4, 5], yet most research has focused on the *efficacy* of physical activity in cancer prevention, leaving the biological mechanisms of exercise-associated 20-40%% reduction in breast cancer risk less understood [2, 8, 9], even in rodent models [2, 6, 7].

Skeletal muscle has established a positive association with regard to enhanced activities of daily living and improved quality of life, and its protective effect on multiple metabolic disease states [37, 57, 87]. Underappreciated however, is that skeletal muscle exhibits an endocrine-like behavior during exercise where exercise-induced 'hormones' are released into circulation in both rodents and humans post muscular contraction [19, 20, 35, 88]. Specifically, contracting muscle releases "myokines" (hormones), which are molecules that effect signaling pathways involved with muscle homeostasis, inflammation, and colon cancer, amongst others [19, 22, 89, 90]. The earliest established connection between myokines, previously "fatigue substance", and inhibited tumor progression dates back to the early 1960s. Extract released from rat muscle during passive electrical-stimulated contraction resulted in significant tumor growth inhibition when administered via subcutaneous injections into tumor-bearing rodents [4, 21, 24]. Recently, our lab has contributed to the field's current findings that *in situ* produced myokines significantly inhibits breast cancer proliferation and increase rates of apoptosis when cells are treated with the

contraction medium *in vitro* [22, 26, 91, 92]. As such, we hypothesize that impact of exercise on breast cancer is by directly influencing mTOR activity, which subsequently alters downstream anabolic signaling, anabolic activity and attenuates cell proliferation rates, forcing cells into senescence/apoptosis. The cellular mechanism between exercise and suppressed proliferation, growth and anabolic magnitude appears to be facilitated via the inhibition of the mTOR pathway. The field has demonstrated direct crosstalk between muscle and breast cancer via an mTOR regulated cellular state, apoptosis [23, 25]; however the mTOR's anabolic activity, regulation of cellular proliferation, and survival feedback loops remain unacknowledged in the physiological paradigm. While our proposed investigation into the mTOR regulation in cancer is not entirely novel, the context of exercise as a physiological mechanism to exert control over mTOR's anabolic activity as a means of therapeutic control on cell survival *is*.

Currently there is an evolving appreciation for exercise-combined therapies advocated by clinicians [93-96]. Given breast cancer's prevalence in the United States, and the recognized benefit of exercise in breast cancer patients [93-96], identifying and understanding mechanistic impact of exercise on cancer will advance research and may lead to new treatments. Establishing a casual role for exercise as primary cancer prevention would have major translational impact in cancer prevention and patient survivorship, with even a small 1% reduction in incidence of cancer resulting in $500 billion in health care savings. We believe that our investigation of mTOR and determination of its role in breast cancer cell signaling will vertically advance the field's conceptualization of muscle, exercise and explain noted improved prognosis in exercising breast cancer patients.

Innovation

Huge challenges exist to identify and investigate biological regulation of exercise to noted medical benefits. One of the factors hampering the translation of knowledge from preclinical studies to the clinic has been the limitations of *in vitro* diseases models [97]. In this project, we are moving beyond the status quo of mTOR regulation of cell survival in cancer [37, 42, 44, 47, 98] by exposing the potential chemotherapeutic significance of exercise-altered mTOR signaling. We are hypothesizing that *altered mTOR activity via exercise and pharmacologically will be concomitant with diminished anabolic activity within epithelial breast cancer cells, attenuating proliferation rates, and forcing cells into senescence/apoptosis*. **The scientific premise of this study rests on the lack of integration between skeletal muscle's purported endocrine function that considers the impact of exercise-induced molecules on metabolic diseases and the strong literature indicating that perfusate collected from contracting skeletal muscle has a chemotherapeutic effect on breast cancer growth and proliferation.** To our knowledge, the existing body of literature has not addressed impact of molecules arising from muscle during contraction on other cell types, like cancer, nor considered exercise as a mediator of muslce-cancer crosstalk. Thus, the contribution of the prosed project is expected to be a delineation of the potential mechanisms by which exercise improves cancer prognoses and the cellular control by directly influencing a possible culprit for uncontrolled proliferation in cancerous cells. This contribution will be significant because it will directly determine how exercise is affecting the biological regulation of tumorgenesis (a critical component of treatment innovation), and how that regulation is mediated by skeletal muscle contraction.

2. MTOR GENE EXPRESSION IS A TARGET OF MUSCLE-BREAST CANCER CROSSTALK WITH EXERCISE

2.1. Summary

Proteins dictate cell function, but what proteins are found in cells are dictated by the transcription and translation of the coding message transcripts. The mechanistic target of rapamycin (mTOR) is a heterotrimeric protein kinase and its signaling pathway is vital for normal cell function, which has the ability influence selective protein translation. Comprised mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2), both the downstream and upstream effectors of this protein regulate growth and cellular proliferation. When this pathway is dysregulated, such as in cancer, it can lead to hyperactivation of signals which promote cell proliferation and protect against cell death. In this study, the anabolic profile of epithelial breast cancer cells was evaluated to identify changes in growth and proliferation mediated by muscular contraction and pharmacology. Hemicorpus Hind Limb-Perfusion (HHLP) preparation with electrical stimulation of rats served as a model of resistance exercise. Through a combination of cell counts, immunoblotting, and gas chromatography-mass spectrometry, mTOR was identified as a key mechanistic protein whose anabolic activity may be influenced by exercise facilitated muscle and breast cancer crosstalk. When MCF-7 cancer cells were treated with perfusate collected during muscle contraction, a significant inhibition of proliferation was noted alongside diminished mTOR activity and decreased protein synthesis rates. Given breast cancer's prevalence in the United States, and the recognized benefit of exercise in breast cancer patients, identifying and understanding the mechanistic impact of exercise on cancer will advance research and may lead to new treatments.

2.2. Introduction

In the United States, breast cancer is the most common cancer in women [86] with more than 2.8 million women with a history of breast cancer and an estimated 268,600 *new cases* in 2019 alone [1, 99].

Exercise has been shown to elicit beneficial effects in the treatment and prevention of cancer in addition to improved immune response with evidence of decreased risk with increased physical activity [2, 3]. The role of exercise as a key player in reduction of tumor growth has been noted since the late 1960s [4, 5], yet most research has focused on the *efficacy* of physical activity in cancer prevention, leaving the biological mechanisms of exercise-associated 20-40%% reduction in breast cancer risk less understood [2, 8, 9],even in rodent models [2, 6, 7].

Mechanistic Target of Rapamycin (mTOR)

The mechanistic target of rapamycin (mTOR) signaling pathway is centric to multiple cell cycle and survival mechanisms. The mTOR kinase coordinates environmental stimuli and intrinsic feedback loops to regulate cell growth, cellular proliferation and survival, all of which are vital for normal cell function. The mTOR protein associates with other proteins to define its complexes (mTORC1 and mTORC2), and ultimately influencing metabolic and cellular changes. The mTORC1 is a heterodimer protein kinase defined by its associated proteins regulatory-associated protein of mTOR (RAPTOR) and mammalian lethal with SEC13 protein 8 (mLST8). The complex is further accessorized with inhibitory proteins PRAS40, and DEP domain-containing mTOR-interacting protein (DEPTOR), The mTORC2 complex is defined by mTOR's association with Rictor, the Rapamycin insensitive counterpart. This complex also is accessorized by DEPTOR and mLST8, in addition to protein associated with Rictor (PROTOR)

and stress-activated protein kinase-interacting protein-1(mSINI). When the mTOR kinase is active, it positively regulates many anabolic processes of the cell, and its stimulation can lead to both positive and negative intrinsic feedback mechanisms which can result in altered activation [100]. For example, when the mTOR pathway is dysregulated, such as in cancer, it can lead to the hyperactivation of signals that serve to both promote cellular proliferation and protect against apoptosis [37-44]. mTORC1 activity is potently anti-apoptotic and anti-autophagic; its activation stimulates amino acid uptake and protein synthesis [101]while suppressing autophagy [102]. Its downstream targets (Figure 6), most notably ribosomal protein S6 kinase (S6K1/$p70^{s6k}$) and the translational repressor eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), are indicators of mTORC1 activity and sources of negative feedback signals [45, 48, 53, 103-110]. DEPTOR is an mTOR binding protein which inhibits the mTORC1 and mTORC2 activity, and whose expression is low in most cancers [37, 40, 43, 58, 59].

To date, research efforts have focused on induced DEPTOR-suppression during re-sensitization to apoptotic stimuli [40] , upregulated DEPTOR in aggressive cancer cells [59, 70], DEPTOR degradation process evaluation [58, 59] and understanding the feedback loops in signaling arising from DEPTOR-mTOR interaction [37, 38, 41, 42, 44, 71]. However, mTOR-DEPTOR interaction in MCF7 cells in direct response to exercise is not fully understood.

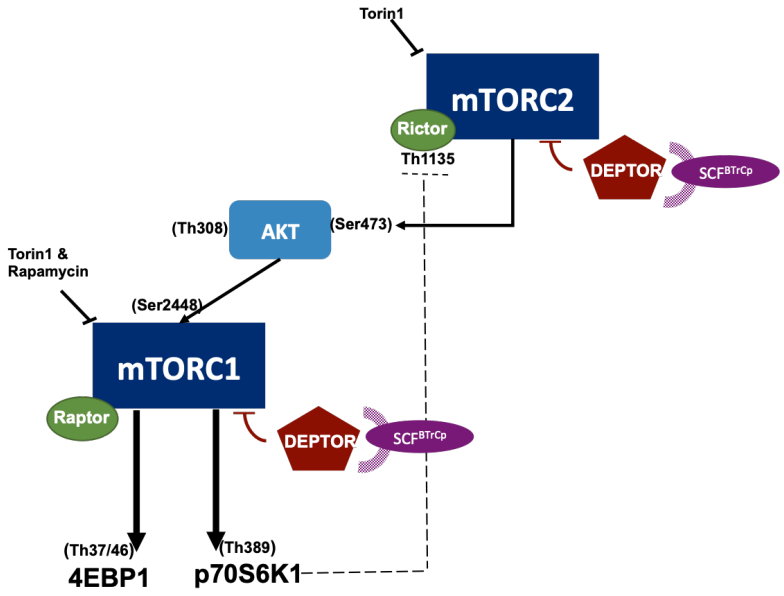

Figure 6. Dynamic mTOR cross-complex feedback signaling

Anabolism is the coordinated metabolic activity that allows cells to produce macromolecules [72]. mTOR is a master growth regulator that promotes anabolism [107]. Moreover, the mTOR kinase is a master switch for a cells induction from quiescence into growth, whose activity facilitates the direction of post-transcriptional programming of growth-specific genes [111]. While both mTORC1 and mTORC2 function in growth control, signals controlling protein synthesis machinery and the production of proteins are primarily transmitted through complex 1. mTORC1 is tightly linked to several steps of protein synthesis including ribosome biogenesis, translation initiation and elongation [100]. Specific mTORC1 active-site inhibitors have been shown to significantly reduce overall rates of protein synthesis in proliferating cells [56, 107, 112]. To date, no studies exist that have systematically assessed rates of global protein synthesis in breast cancer cells, nor how that anabolic function is affected by mTOR activity.

Pharmalogical Control of mTOR Activity

The anabolic process of protein synthesis is energetically expensive, thus if the cell is in a state of survival, the anabolic process may be suppressed allowing the cell to senesce and protect itself to current apoptotic injury. Recent studies support that the two distinct mTOR complexes, although different in their biochemical and functional design, share common upstream signals [100]. Early work unveiled that mTOR catalytic activity can be altered via pharmacology. Rapamycin, an allosteric inhibitor, mostly affects mTORC1 activity and the phosphorylation and activation of its downstream target S6K1 and 4E-BP1, thus primarily inhibiting cell growth, cell proliferation and cell cycle progression [45, 113, 114]. While rapamycin and its analogues are well tolerated by patients, only a minority of patient, including breast cancer patients, show positive response to treatment. Its inefficiency at suppressing translation in mammalian cells via 4E-BP1 [45, 57, 83, 115] has led to second generation inhibitors which are ATP competitive, such as Torin1 [56, 83, 115, 116]. Torin1 has the ability to inhibit mTORC2 activity. This is significant in the context of cancer, for without suppression of mTORC2 activity, the complex's "pro survival function" via feedback signaling is maintained, and mTORC1 can remain transiently active, leading to continued proliferation [57, 83]. As such, the established overlap of cell cycle regulation and integration of feedback signals associated with anabolic activity provide urgency for investigation of mTOR as a centric mechanism for the biological regulation of breast cancer.

Pharmalogical Control of Gene Expression

Proteins dictate cell function, but what proteins are found in our cells are dictated by the messaging transcripts that code for their production. Like the mTOR pathway that can influence

selective translation of transcripts, gene expression takes coordination from a lot of moving parts from delicate cellular machinery to ideal environmental conditions, making many possible check points between transcription and translation available for therapeutic intervention.

ActinomycinD and Cycloheximide are antibiotics which have previously been used to inhibit precursor incorporation into macromolecules, thusly allowing the investigators to study the role of RNA and protein synthesis [117-119]. ActinomycinD is a transcriptional inhibitor which blocks the progression of RNA polymerases in eukaryotic cells. Cycloheximide is a potent protein synthesis inhibitor which, in MCF7s, has been found to minimize cell death whilst suppressing global protein synthesis [120, 121]. In combination, these compounds are key tools in evaluating whether there are changes in specific transduction intermediates associated with changes in anabolic proteins. Furthermore, they are conducive to identifying whether changes in anabolism are due to *de novo* protein synthesis of the other cellular processes such as ubiquination and protein degradation. Overall, understanding the transcriptional-translational regulation of altered proliferative factors within the mTOR cascade will highlight the potential for endogenous sources of proliferation regulation in cancer cells, such as microRNA.

The purpose of these directed studies is to develop an experimental approach to determine if cellular proliferation of breast cancer cells is mediated by changes of cellular expression of proliferative factors, using an *in vitro* pharmacology model. We are hypothesizing that altered mTOR activity via pharmacologically will be concomitant with lowered gene expression of intermediates controlling cellular proliferation and growth of breast cancer cells.

2.3. Methods

Hemicorpus Hind Limb Perfusion

Female Wistar rats (N=10) 8-12 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). All procedures were approved by the Institutional Anima Care and Use Committee at Texas A&M University. Animals were housed two-rats per cage under standard 12h photoperiod, provided with normal Rat Chow with water *ad libitum.* Following a two day acclimation period after arrival, animals underwent a non-survival Hemi Corpus Hind limb Perfusion preparation (HHLP) (Figure 7) as described previously [91, 92, 122-125]. Briefly, midline to caudal end of the animal was surgically prepared so both hind limb limbs could be perfused with an oxygenated Krebs-Heinseliet Buffer during electrically stimulated muscle contraction. Electrical stimulation was administered using a stimulator (Grass Instruments, West Warwick, RI) and a force transducer (Warner Instruments, Harvard Bioscience Inc., Holliston, MA) at a surgically exposed sciatic nerve on a single hind limb of the animal. Perfusate medium was maintained at 31.7°C using a bipolar temperature controller (Model #CL-100, Warner Instruments, Harvard Bioscience Inc., Holliston, MA) and administered at a flow rate of 12ml/min, by peristaltic pump (MPL 8-Channel) (Watson Marlow, Marlo, United Kingdom).

Perfusion sample collection is depicted in Figure 8. During perfusion, medium was collected on ice in 50ml sterile conical tubes (Corning Inc., Corning, NY) before electrical stimulation (Non-Stim, NS), during electrical stimulation (E-Stim, ES), and following electrical stimulation (Post-Stim, PS). Following collection, samples was centrifuged at 2500 rpm at 4°C to remove red blood cells and the supernate was then stored at -80°C until analysis.

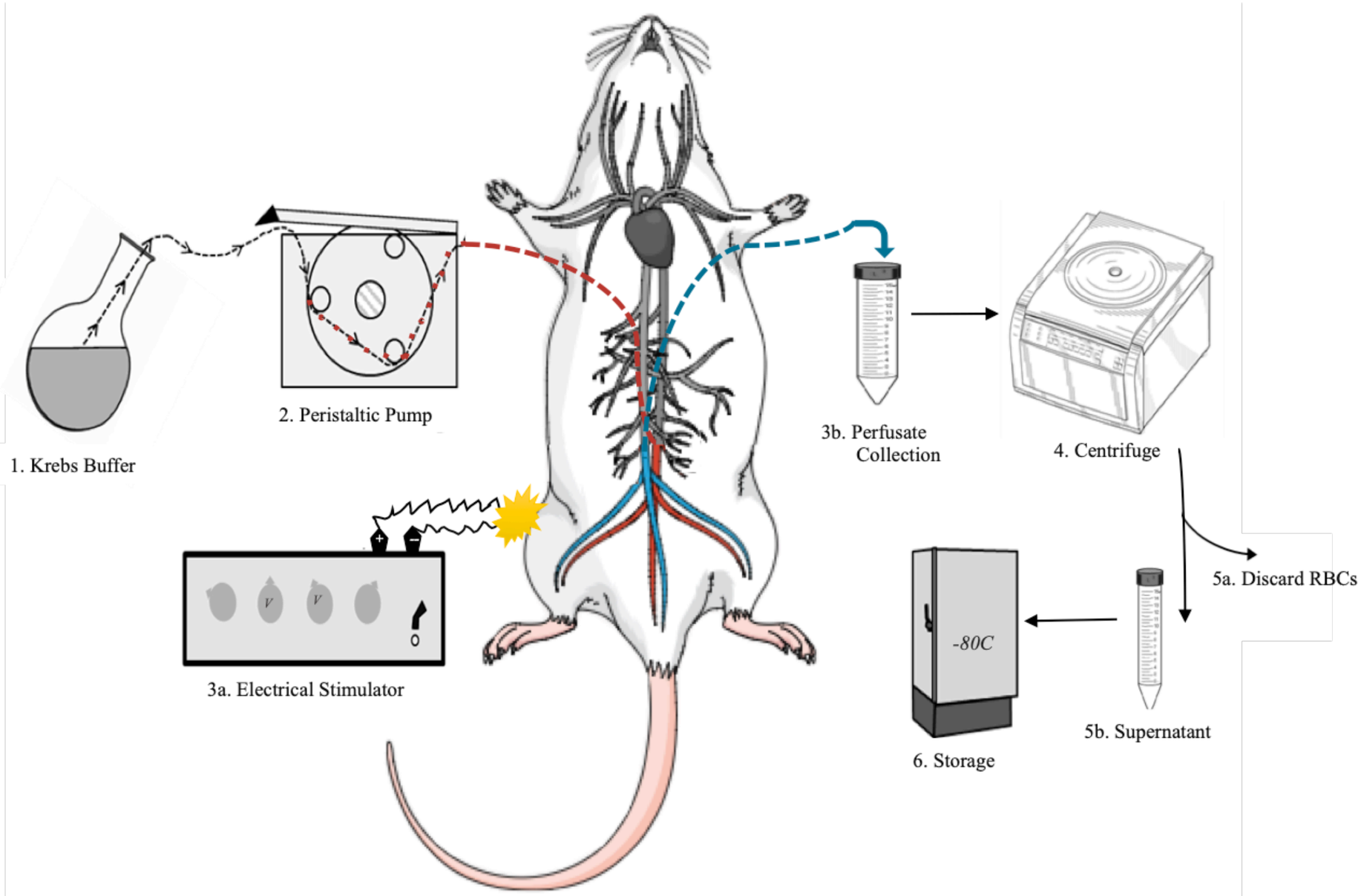

Figure 7. Hemicorpus Hindlimb Perfusion Schematic.

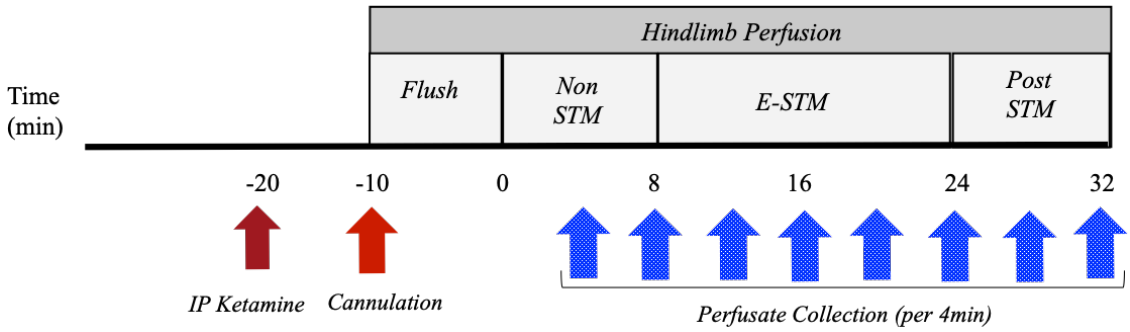

Figure 8. Hemicorpus Perfusion Preparation Timeline

Cell Culture

Human MCF7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in 20mmcm dishes (Corning Inc., Corning, NY) with 10ml growth media (GM) containing Dulbecco's Modification of Eagle Medium (DMEM) (Corning, Mediatech INC., Manassas, VA), supplemented with 5%(v/v) Fraction V Fetal Bovine Serum (FBS) (VWR International, Randor, PA) and 1% (v/v) Penicillin/Streptomycin (BioVision, Milpitas, CA). Cells were maintained GM at 37°C in a humidified atmosphere containing 5% CO2 until 60% confluent.

Cell Harvest Protocol. Growth medium from plates allocated for western blot or RT-qPCR analysis was aspirated and 3 ml of cold Phosphate Buffer Solution (PBS) (VWR International, Randor, PA) was applied to each plate. Growth medium from plates allocated for protein synthesis analysis was collected into 2ml microcentrifuge tubes (VWR International, Randor, PA) and snap frozen into liquid nitrogen and then transferred to storage at -80°C until analysis. All cells were collected using cell scrapers (VWR International, Randor, PA) and transferred to 2ml microcentrifuge tubes, and centrifuged at 130xg for 8 minutes to separate cell pellet from the PBS supernate solution. Supernate was discarded from each vial and cell pellet

samples were then snap frozen into liquid nitrogen and then transferred to storage at -80°C until analysis.

For muscle perfusate (Figure 9), 10ml GM was supplemented with 10% exercise perfusate media collected during hind limb perfusion surgeries during non-electrical stimulation (NS), electrical stimulation (ES) or post-electric stimulation (PS) as previously described.

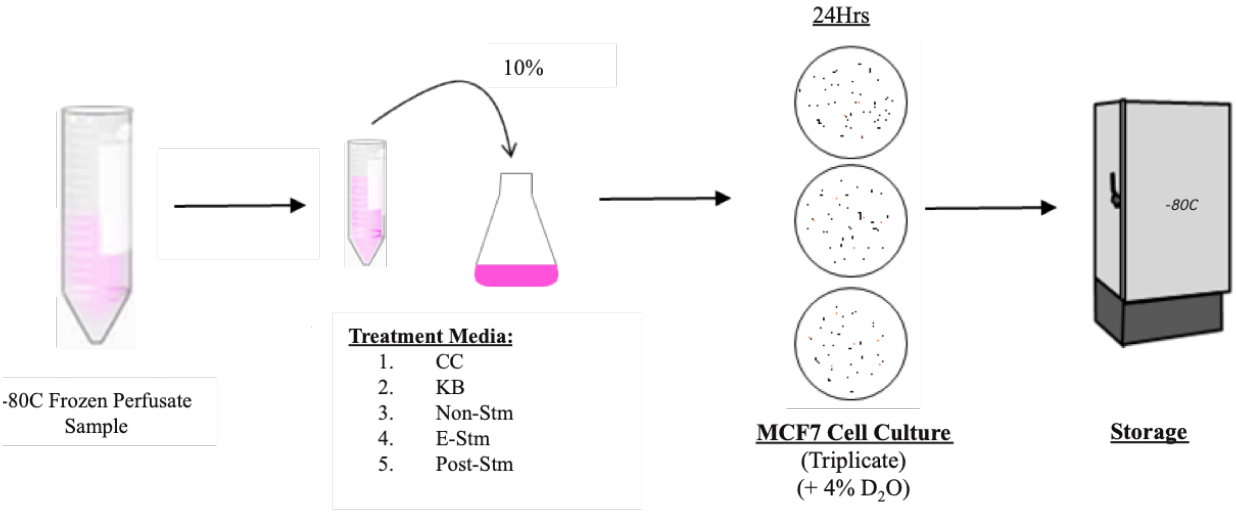

Figure 9. Muscle Perfusate Experimental Workflow

Cells incubated in respective media for 24h (Figure 10). All cells allocated for protein synthesis analysis were additionally treated with heavy water (^2H_2O) to yield a 4% final volume of 10ml perfusate media.

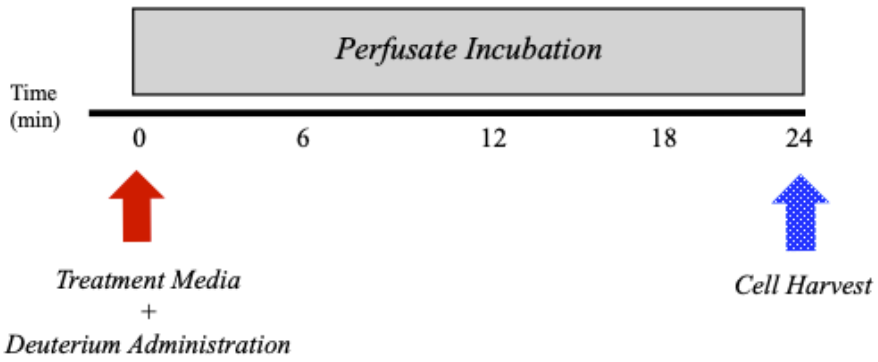

Figure 10. Exercise Perfusate Cell Culture Timeline

For mTOR pharmacological inhibitor experiments (Figure 11), 10ml GM received addition of either Rapamycin (RAP) (CAT #2353, BioVision, Milpitas, CA), Torin1 (TOR1) (CAT #2353, BioVision, Milpitas, CA), or both (RAP+TOR1) to yield concentrations of 100nM, or 250nM respectively.

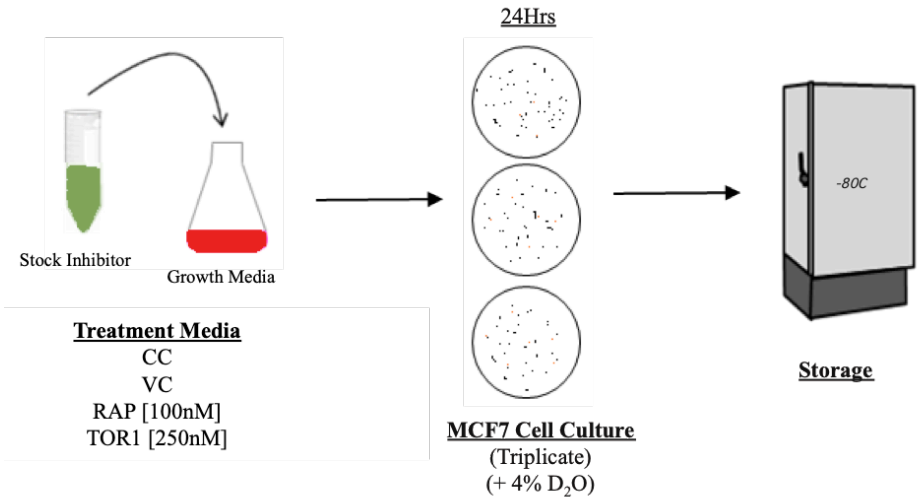

Figure 11. mTOR Pharmacology Experimental Workflow

All treatment groups received equal dosing of dimethyl sulfoxide (DMSO) to serve as a vehicle control (VC) and incubated in respective media for 24h (Figure 12). All cells allocated for protein synthesis analysis were additionally treated with heavy water (^2H_2O) to yield a 4% final volume of 10ml perfusate media.

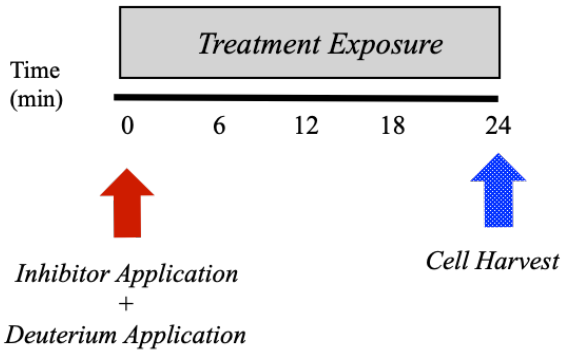

Figure 12. mTOR Pharmacology Cell Culture Timeline

For gene expression pharmacological experiments (Figure 13), cells were randomly assigned to treatment group first by mTOR inhibitor treatments of Rapamycin (Rap, 100nM) or Torin1 (Tor1, 250nM), and then to transcription-translation inhibitor treatments of ActinomycinD-D (1ug/ml) (AdipoGen Life Sciences, San Diego, CA), Cycloheximide (25ug/ml) (Sigma-Aldrich, St. Louis, MO), or both, with 10ml GM reflecting these doses

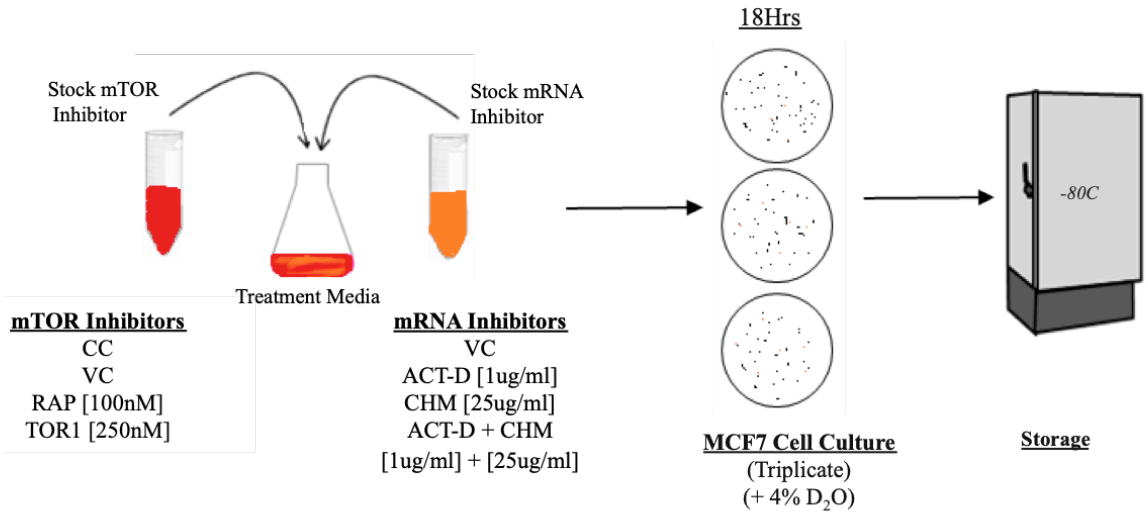

Figure 13. Gene Expression Pharmacology Experimental Workflow

Cell control and a vehicle control (DMSO) plates were cultured alongside experimental groups for an incubation time of 18hrs (Figure 14).

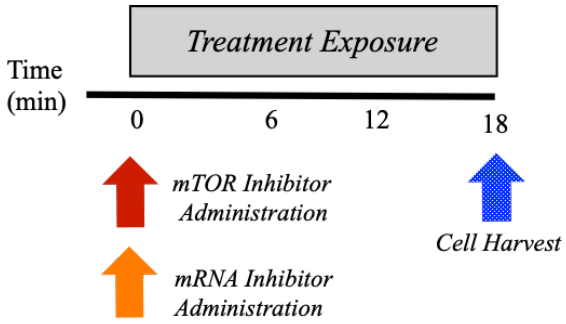

Figure 14. Gene Expression Pharmacology Cell Culture Timeline

Western Blotting

To assess expression and activation of individual proteins within the mTOR signaling cascade, the following primary antibodies were used at a 1:1000 antibody/buffer ratio: DEPTOR

(Cell Signaling #11816), phospho-P70S6K1^{Th389} (Cell Signaling #2114), p70S6K1 (Cell Signaling #2114), phospho-AKT^{Ser473} (Cell Signaling #2114), phospho-AKT^{Th308} (Cell Signaling #2114), AKT (Cell Signaling #2114), phospho-Rictor^{Thr1135} (Cell Signaling #3806), Rictor (Cell Signaling #2114), and appropriate HRP-linked secondary antibodies at 1:2000 antibody/buffer ratio. Cell pellets were homogenized and applied to gels in conjunction with molecular weight ladder (Lonza #193837) to verify size, separated and transferred onto blotting membranes as previously described. Membranes were imaged using a FlouroCHem SP imaging system (Alpha Innotech, San Leandro, CA, USA) and optical density of protein bands was determined using the Alphaease FC software (Alpha Innotech). All bands were normalized to Ponceau S stains and expressed as arbitrary units.

Deuterium Method

The validity of a deuterium approach has been demonstrated by its ability to accurately measure muscle protein synthesis (MPS) in free-living subjects over longer periods of time in order to better replicate physiological relevant conditions. The method used to assess fractional synthesis rates (FSR) in MCF7 cells was modified from the gas chromatography-mass spectroscopy (Agilent 7890 GC/5975 VL MSD, Agilent Technologies, Santa Clara, CA) method previously described [126].

$^{2}H_20$ enrichment of water soluble proteins/cell media. Briefly, cell media collected at harvest following 24-h of isotopic exchange between $^{2}H_20$ enrichment of cell media samples and acetone, and frozen at -80C were thawed on ice for 15minutes. Alongside calibration standards (0 –5% 2 H2 O, prepared by mixing naturally labeled water with 99.9% 2 H2 O), 20ul were aliquotted into 2ml microcentrifuge tubes and incubated for 24-h at room temperature with 2ul of

10N NaOH and 4ul of a 5% (vol/vol) solution of acetone:acetonitrile. Procedural steps from this point on were consistent as previously described [126]. All plasma samples were measured twice with separate preparations, and an average value of the two runs were used for calculations.

[^{2}H] alanine enrichment in MCF7 cells. Briefly, frozen cell samples (1 microcentrifuge tube = 1x 10cm plate, 70-80% confluence) were thawed on ice for 15mins before homogenization in 300ul of iced 10% TCA (Cl_3CCOOH). Samples were vortexed for ~10s before centrifugation at 3,000 rpm for 10mins to remove unbound amino acids. Following, supernate was decanted, and the remaining cell pellet placed on ice. TCA treatment, centrifugation and decanting were repeated for a total of 3 spins before proceeding in accordance with previously described details procedures [126]. Fractional synthesis rates (FSR) of mixed proteins were calculated using the equation:

$$E_A \text{ X } [E_{CM} \text{ X } 3.7 \text{ X t (h)}]^{-1} \text{ X } 100$$

where E_A represents amount of protein-bound [^{2}H]alanine (mole% excess), E_{CM} is the quantity of ^2H_2O in cell media (mole% excess), and 3.7 represents the exchange of ^{2}H between cell media and alanine (e.g., 3.7 of 4 carbon-bound hydrogen of alanine exchange with water [127]). The novel use of primed-constant exposure method, with the use of a deuterium oxide (^2H_2O) as a tracer, allows for assessment cumulative protein synthesis of a mixed proteins in cancer cells exposed various treatments. This assessment contributes to generating a complete anabolic profile of MCF7 breast cancer cells.

Gene Expression

RNA Isolation. Total RNA was manually extracted. Briefly, samples were manually extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and 1-Bromo-3-chloropropane

(Biomedical Research Centre) protocol and precipitated in isopropanol. Following, RNA was treated with a series of ethanol washes to prevent downstream salt inhibition, and then re-suspended in 50ul of nuclease free water. RNA concentration was the quantified by a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) according to manufacturers protocol. RNA integrity (RIN) was assessed on a 4200 TapeStation System (Agilent Technologies, Santa Clara, CA), which assigned a RINe value on a scale of 1 to 10. Purity and quality are summarized in Table 4 (Appendix A). RNA samples with RINe quality values >7.5 were included in RT-qPCR assays.

Reverse Transcription (cDNA Synthesis). Reverse transcription for cDNA synthesis was performed using Superscript VILO cDNA Synthesis Kit (Applied Biosystems, Carlsbad, CA) with 200 ng total RNA in reactions of 20ul according to manufacturers instructions. mRNA expression of target genes were quantified with real time PCR using Applied Biosystems 7900HT Fast Real-Time PCR System (Carlsbad, CA). TaqMan Gene Expression assays (Applied Biosystems, Carlsbad, CA) for mTOR (Hs00234508_m1), DEPTOR (Hs00961900_m1), BTRC1 (Hs00182707_m1), FBXW11 (Hs00362667_m1), RPS6KB1 (Hs00177357_m1), RPS6KB2 (Hs00177689_m1), 4EBP1 (Ha0060705_m1) were handled according to the manufacturer's instructions. Assay details are referenced in Appendix A-Table 5. Quantitative PCR was performed in triplicates of 20ul reactions using 1X TaqMan Universal Master Mix II, no UNG (Applied Biosystems, Carlsbad, CA) with pooled sample reactions without reverse transcriptase (-RT), as well as no template controls (NTC). Thermal conditions were as follows: 10min at 95°C, and 15s at 95°C with 1min at 60°C for 40 cycles. Six genes have been identified as potential reference genes for expression normalization using a review of available literature for cell type, treatment type, co-treatment and RT-qPCR design (Appendix A-

Table 6). Expression of target genes will be normalized to the most stable endogenous control using methods described by Dawes et al [64]. All data analysis will be conducted with Sequence Detection Software v 2.2.2 (Applied Biosystems, Carlsbad, CA).

Statistical Analysis

Muscle Perfusate Experiments. The effects of electrical stimulation and perfusate on expression and synthesis of proteins was assessed by one-way ANOVA followed by Fisher LSD post hoc test (SigmaStat 3.5; Systat Software Inc., San Jose, CA,USA).

mTOR Pharmacology Inhibitor Experiments. The effects of pharmacological inhibition on expression and synthesis of proteins was assessed by one-way ANOVA followed by Fisher LSD post hoc test (SigmaStat 3.5; Systat Software Inc., San Jose, CA,USA).

2.4. Results

Muscle Perfusate Experiments

Results of immunoblotting analysis are illustrated in (Figure 15). Expression of phosphorylated-p70S6K1^{Thr389} and ratio of phosphor-to-total p70S6K1 protein was significantly decreased in all treatment groups compared to CC, while ES was significantly different from NS and PS treated cells ($P<0.05$). There was no difference in total p70S6K1 protein across all treatment groups. Expression of phosphorylated-4EBP1$^{Thr37/46}$ and ratio of phosphor-to-total 4EBP1 protein was significantly decreased in ES and PS groups compared to CC, while ES was significantly different all other treatment groups ($P<0.05$). There was no difference in total 4EBP1 protein across all groups. A significant increase in total DEPTOR protein expression was observed in ES treated cells compared to all other treated groups ($p<0.05$). Protein synthesis

analyses (Figure 16) showed a significant decrease in absolute daily FSR in both E-stim and Post-Stim treatment groups, compared to CC and Non-Stim groups ($p<0.05$) (19.3, 18.2- vs 28.1, 31.8 %/d respectively). A significant decrease in relative % daily FSR in both E-Stim and Post-Stim treatment groups relative to CC ($p<0.05$).

mTOR Pharmacology Inhibitor Experiments.

Cell counts (Figure 17) showed a significant decrease in absolute proliferation in TOR1 and RAP+TOR1 treated cells. Immunoblot analysis (Figure 18) showed decrease in phosphorylated-mTOR^{Ser2448} and phospho-to-total mTOR in both RAP and TOR1 compared to VC treated cells. Expression of phosphorylated-p70^{Th389} and phospho-to-total p70S6K1 was decreased in both RAP and TOR1 compared to VC treated cells. There was a significant increase in DEPTOR protein expression in both RAP ($p=0.005$) and TOR1 ($p=0.001$) treated cells compared to VC. Additionally, phosphorylated-AKT^{Ser473} and phospho-to-total AKT was higher in RAP treated cells compared to VC and TOR1, respectively. Phosphorylated- Rictor^{Thr1135} and phospho-to-total Rictor was decreased in both RAP and TOR1 compared to VC. Protein synthesis analyses (Figure 19) showed decreased in absolute daily FSR in both RAP and TOR1 treated cells compared to VC (14.41,9.09 vs 19.63 %/d respectively) over 24hrs.

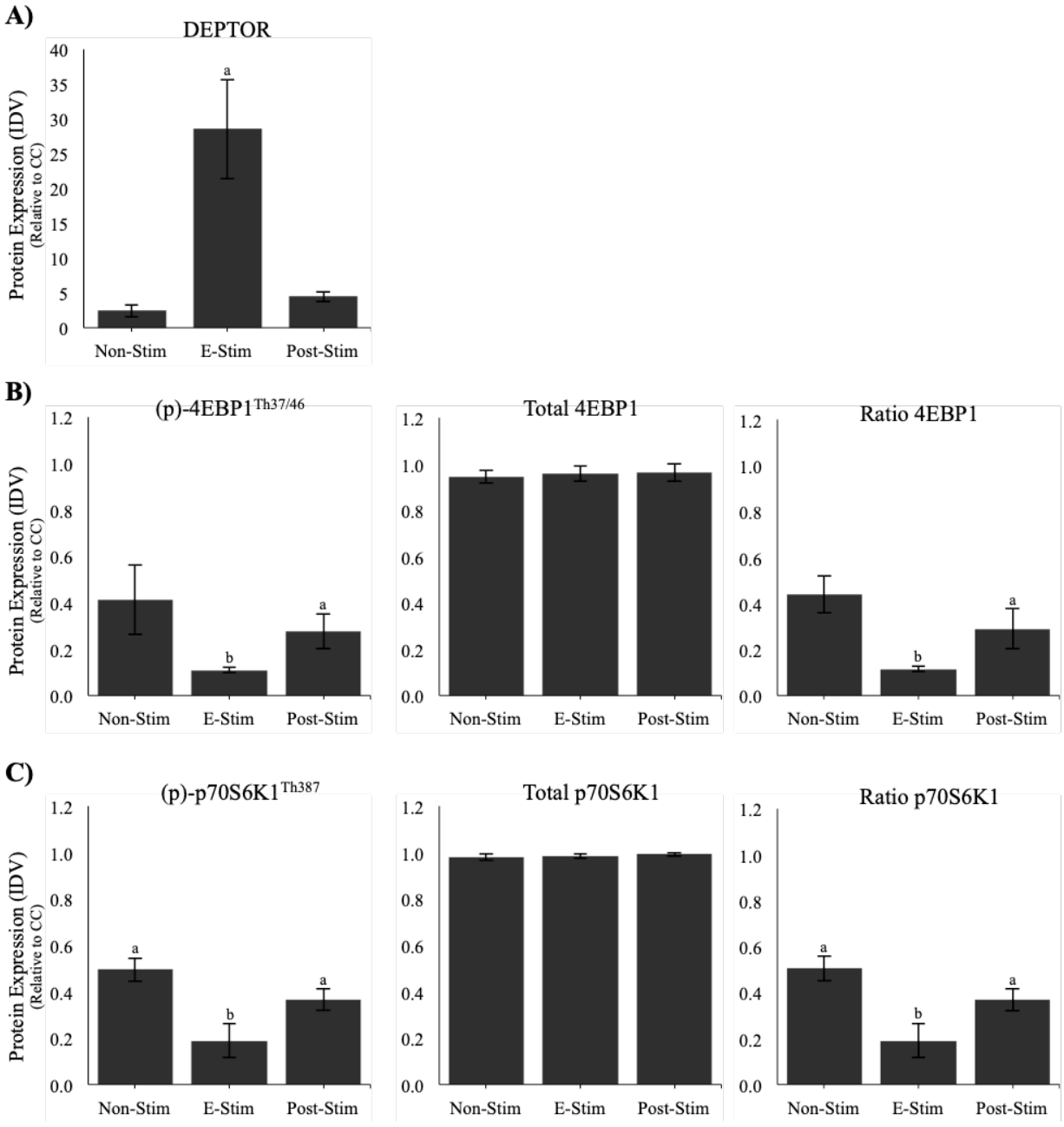

Figure 15. Muscle Perfusate Experiment, Immunoblotting Results

Protein expression data are shown in Integrated Density Units (IDV) with standard error of the mean. A) DEPTOR protein, B) 4EBP1 protein phosphorylated, total, phospho-to-total ratio, C) p70S6K1 protein phosphorylated, total, phospho-to-total ratio. Statistical significance: a= different from CC, b= different from Non-Stim where p=0.05

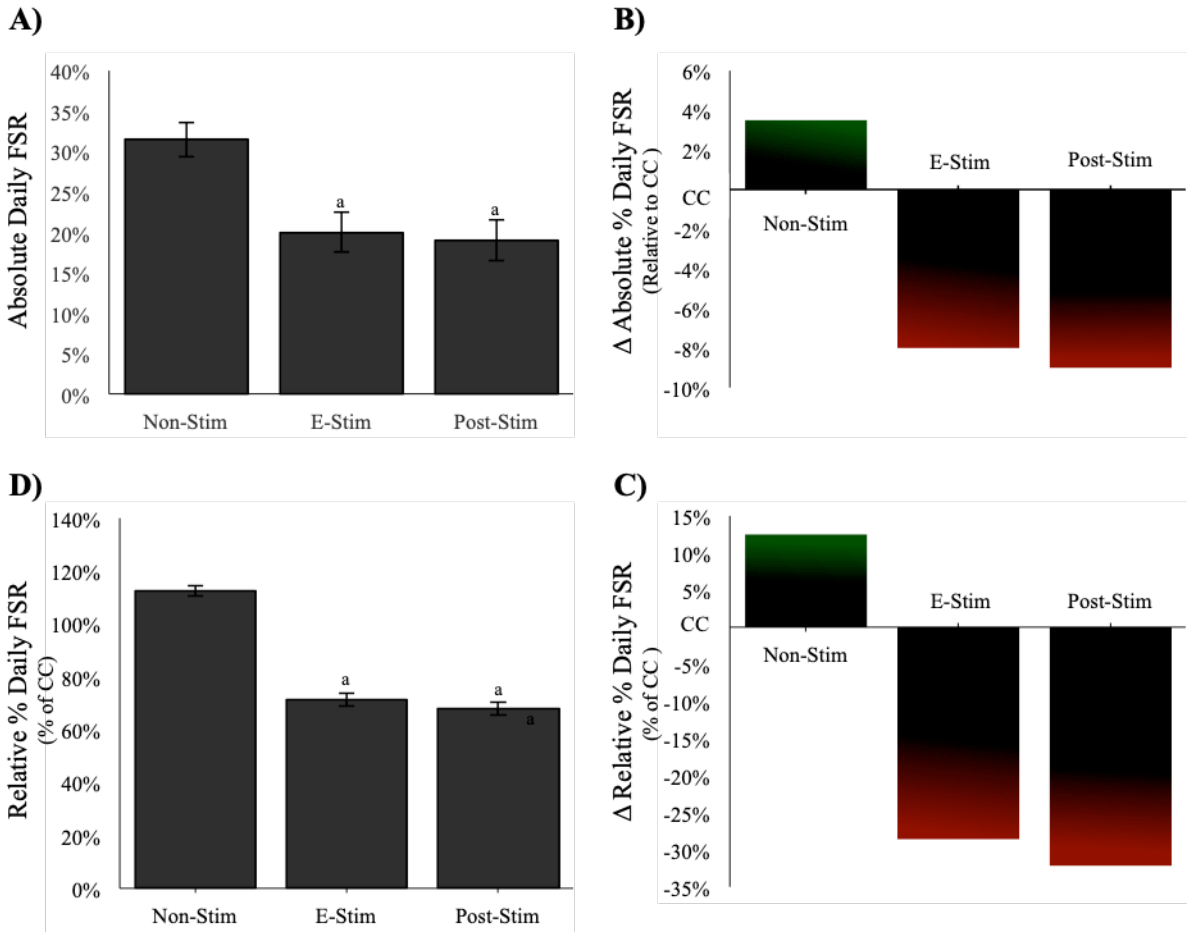

Figure 16. Exercise Perfusate Experiment, 24H Protein Synthesis Results
Data are presented at percent of daily fraction synthesis rate (FSR) with standard error of the mean. Statistical significance: a= different from CC, b= different from Non-Stim where p=0.05

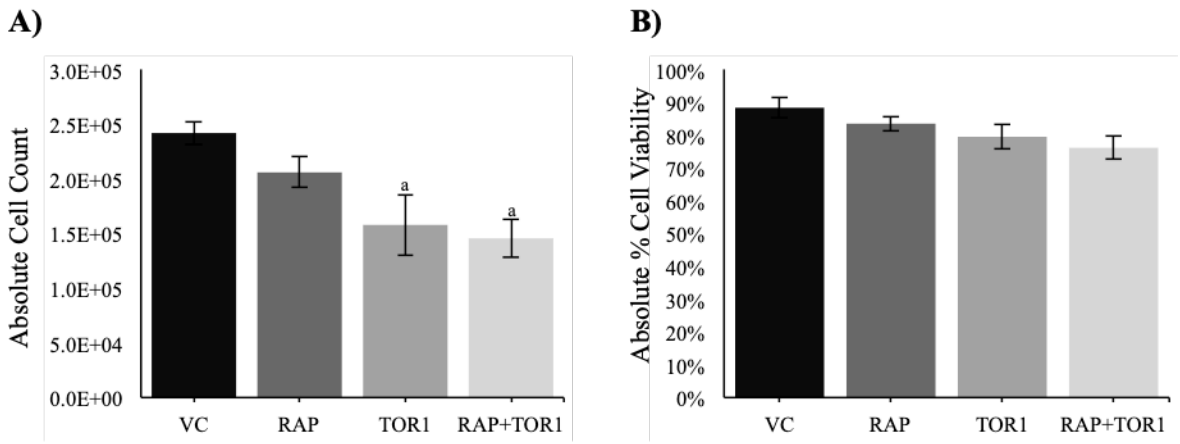

Figure 17. mTOR Pharmacology Inhibitor , Cell Counts

Data shown are absolute cell counts with standard error of the mean. Statistical significance: a= different from VC, b= Rapamycin where p=0.05

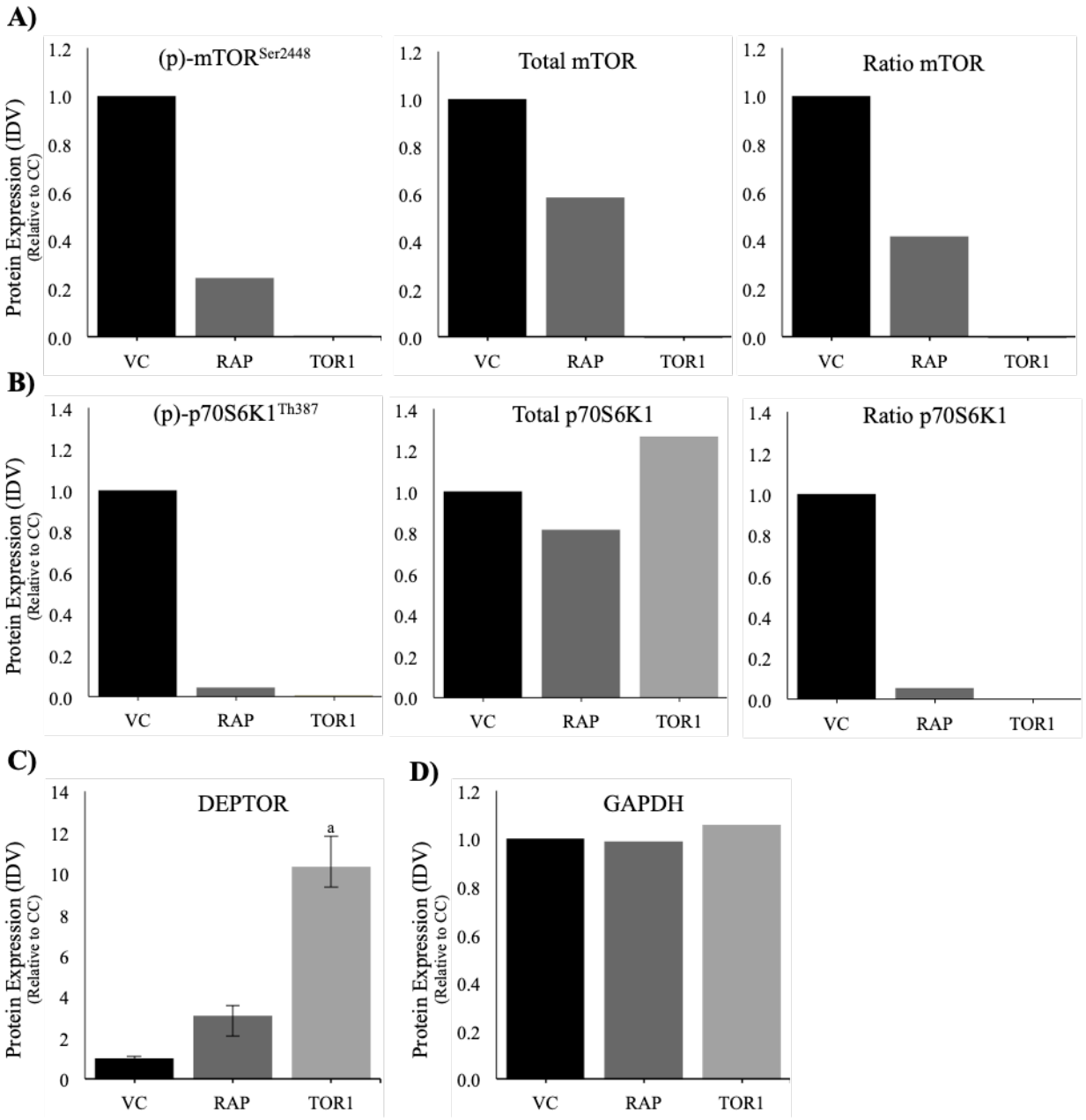

Figure 18. mTOR Pharmacology Inhibitor, Immunoblotting Results

Error bars shown represent standard error of the mean values. Statistical significance: a= different from VC, b= Rapamycin where p=0.05. Nomenclature: VC=Vehicle Control, RAP= Rapamycin, TOR1= Torin1.

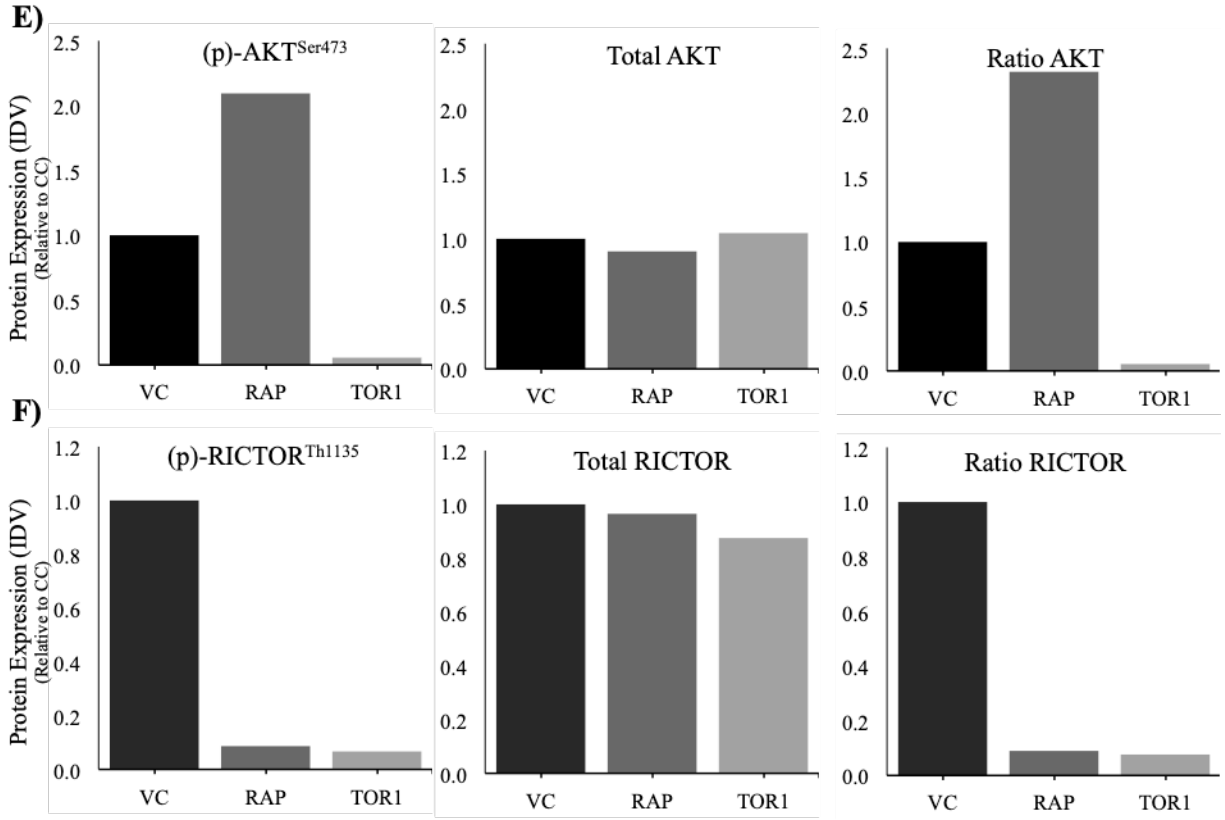

Figure 18. Continued
Nomenclature: VC=Vehicle Control, RAP= Rapamycin, TOR1= Torin1.

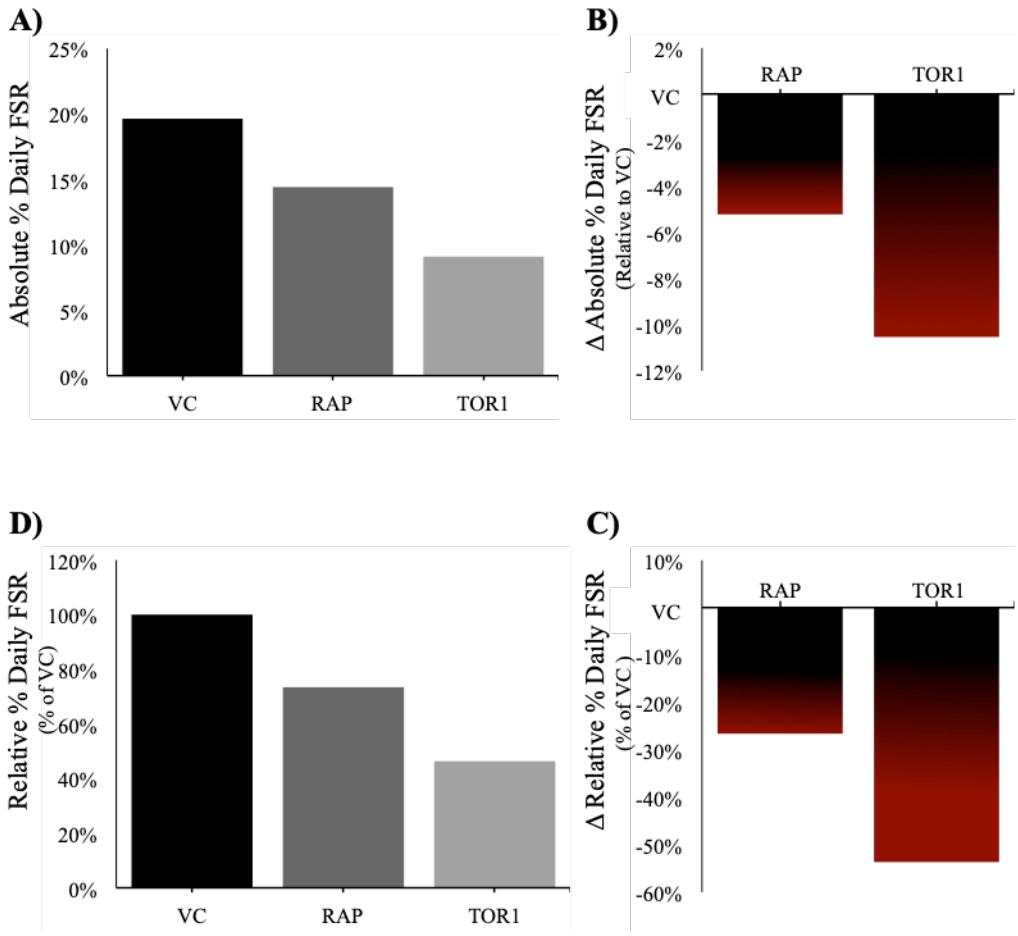

Figure 19. mTOR Pharmacology Inhibitor, 24H Protein Synthesis Results
Nomenclature: VC=Vehicle Control, RAP= Rapamycin, TOR1= Torin1.

Gene Expression Pharmacology Inhibitor Experiments. Relative expression ratios are illustrated in Figure 20. A combined reference of ACTB, RPLP0 and EIF4EBP1 genes was found to be the most stable control for analysis (Table 1). Treatment with ActinomycinD resulted in inhibition of transcription, thus mRNA detected is assumed resultant of transcription activity prior treatment. Thus serves as a baseline of mRNA content prior to pharmacological intervention.

Gene(s) Combinations	± S	Number of Genes
ACTB, RPLP0, EIF4EBP1	0.24	3
ACTB, GAPDH, RPLP0, EIF4EBP1	0.25	4
ACTB, GAPDH, EIF4EBP1	0.29	3
ACTB, EIF4EBP1	0.30	2
ACTB, GAPDH, RPLP0	0.31	3
GAPDH, RPLP0, EIF4EBP1	0.33	3
GAPDH, RPLP0	0.34	2
RPLP0, EIF4EBP1	0.35	2
GAPDH, EIF4EBP1	0.40	2
ACTB, RPLP0	0.40	2
GAPDH	0.42	1
ACTB, GAPDH	0.43	2
RPLP0	0.44	1
EIF4EBP1	0.47	1
ACTB, PUM1, GAPDH, RPLP0, EIF4EBP1	0.56	5
PUM1, GAPDH, RPLP0, EIF4EBP1	0.56	4
ACTB, PUM1, GAPDH, EIF4EBP1	0.68	4
ACTB, PUM1, RPLP0, EIF4EBP1	0.69	4
PUM1, GAPDH, EIF4EBP1	0.70	3
PUM1, RPLP0, EIF4EBP1	0.72	3
ACTB, PUM1, GAPDH, RPLP0	0.75	4
ACTB	0.76	1
PUM1, GAPDH, RPLP0	0.80	3
ACTB, PUM1, EIF4EBP1	0.89	3
ACTB, PUM1, GAPDH	0.97	3
ACTB, PUM1, RPLP0	0.99	3
PUM1, EIF4EBP1	1.01	2
PUM1, GAPDH	1.13	2
PUM1, RPLP0	1.18	2
ACTB, PUM1	1.45	2
PUM1	2.24	1

Table 1. Reference Gene Combinations

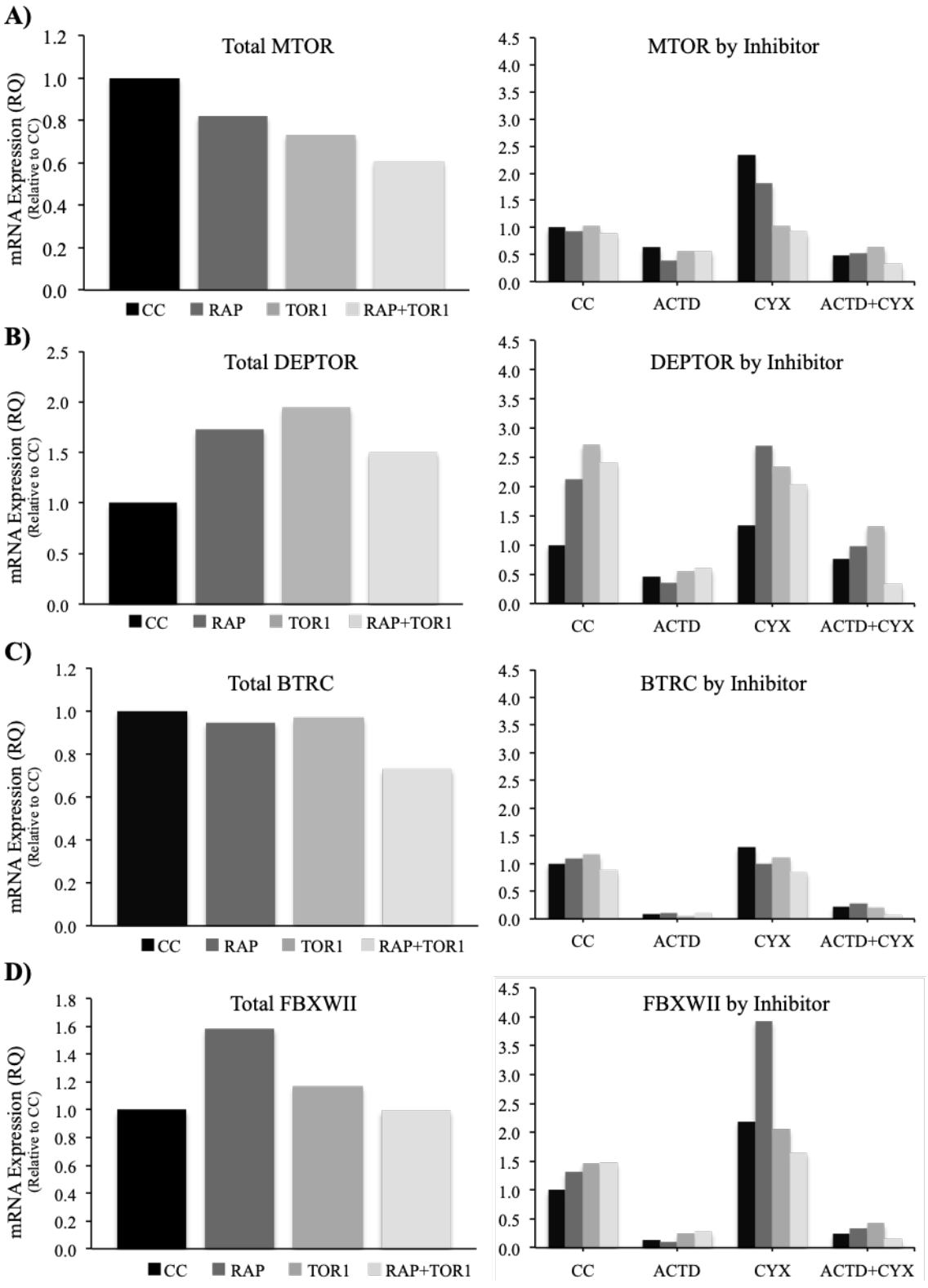

Figure 20. Gene Expression Pharmacology Inhibitors, RT-qPCR Results
VC=Vehicle Control, RAP= Rapamycin, TOR1= Torin1, ACTD=ActinomycinD, CYX=Cycloheximide,

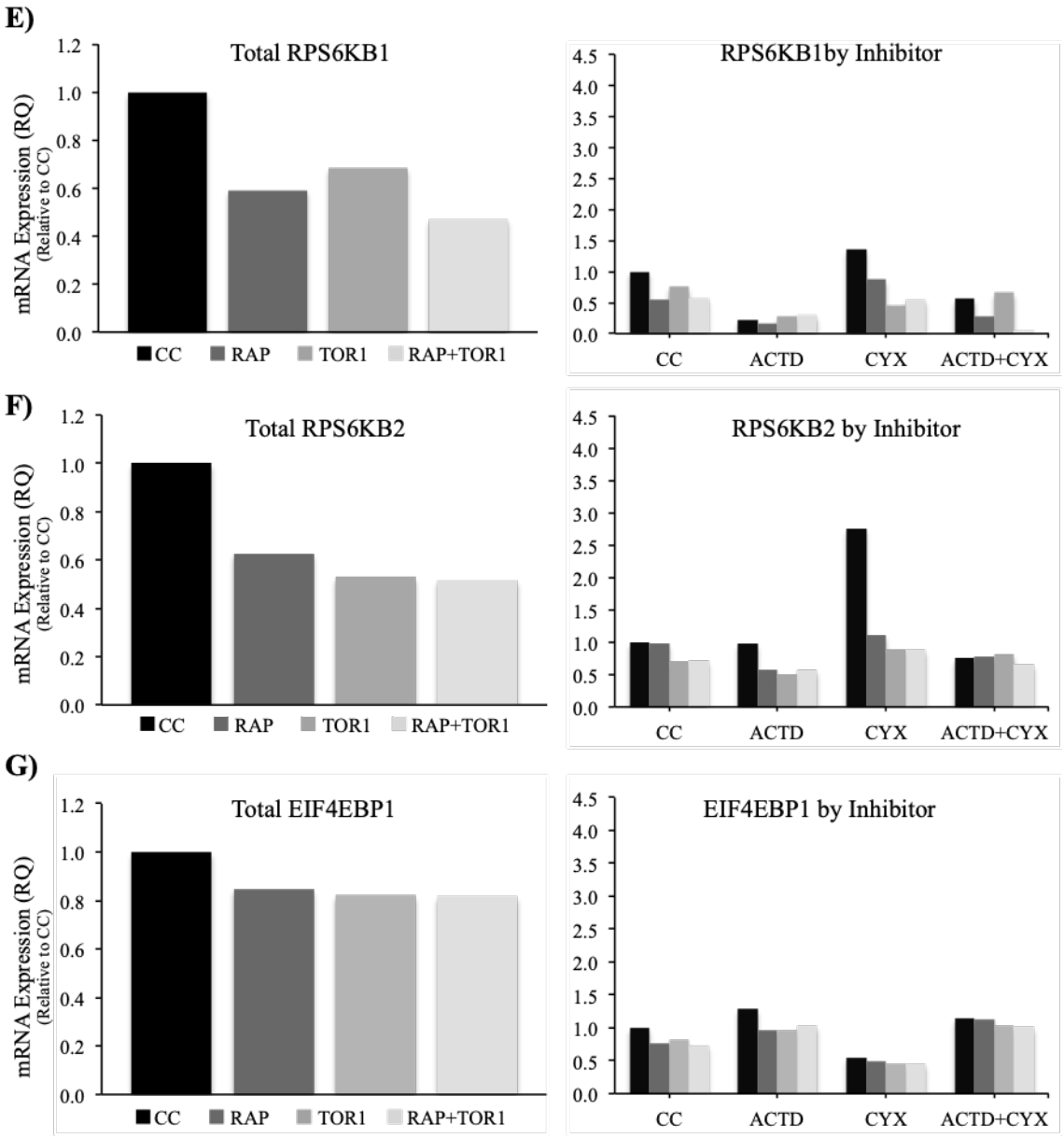

Figure 18. Continued

VC=Vehicle Control, RAP= Rapamycin, TOR1= Torin1, ACTD=ActinomycinD, CYX=Cycloheximide,

2.5. Discussion

The benefit of physical activity to breast cancer patients is well documented [8, 9, 128-133], leading to an evolved appreciation for exercise-combined therapies advocated by clinicians [93-96]. Despite early animal work which demonstrated increased rates of apoptosis in epithelial breast cancer cells exposed to exercise, comparatively little is known regarding the impact of exercise on the anabolic activity of breast cancer and whether specific proliferative pathways are altered leading to suppressed cell growth and/or induction of apoptosis. In this study we aimed to determine how a breast cancer cell's anabolic profile is affected by exercise and to identify a mechanistic basis of exercise facilitated muscle-cancer crosstalk. Understanding and identifying the mechanistic impact of exercise on cellular anabolism, a critical component of tumor growth, will facilitate targeted pharma-, chemo- and exercise-combined therapeutic strategies that may be used to protect at-risk populations. The data presented here provide a molecular basis for how exercise affects the growth and proliferation of breast cancer via the suppression of cellular anabolism.

Several points of evidence support the aforementioned findings. First, exposure of epithelial breast cancer cells to medium collected from contracting skeletal muscles resulted in suppressed cellular protein synthesis, concomitant with diminished mTOR activity and elevated DEPTOR expression. Second, inhibition of mTOR via pharmacology suppresses protein synthesis and consequent anabolic signaling in breast cancer cells, with a rescue of DEPTOR protein. Previous studies have demonstrated that exercise can alter rates of apoptosis, the flip side to mTOR's regulation of anabolism. Using the same surgical preparation, Westerlind et al. demonstrated not only increased apoptosis in cells treated *in vitro* with perfusate of stimulated muscles [25], but increased tumor time to latency and tumor growth retardation with moderate

exercise training *in vivo* [23]. Our data suggests that, the benefit of exercise on breast cancer appears to be directed toward the suppression of mTOR activation by facilitating an acute overexpression of a potent and specific kinase binding protein called DEPTOR, resulting in reduced anabolic responses and increased apoptosis [25]. DEPTOR is an mTOR binding protein which inhibits the mTOR activity, and whose expression is low in most cancers [37, 40, 43, 58, 59] and a characteristic of invasive breast cancer [70]. Our findings are consistent with other studies that indicate the control of mTOR activation, and ultimately cancer proliferation, can be achieved by indirectly restoring DEPTOR protein through direct interference of its degradative pathway [37, 134, 135]. Although future studies need to determine the extent to which DEPTOR expression is altered in our model, either through *de novo* protein synthesis or the rescue from degradation, we nevertheless established diminished DEPTOR protein content and concomitant mTOR hyperactivity are important contributors to MCF7 cellular proliferation, in vitro. Interestingly, the increased expression of DEPTOR was not only concomitant with decreased mTORC1 activity as indicated by downstream effectors S6K and 4EBP1, but also a dramatic decrease in global protein synthesis with treatment. Thus, in MCF7 cancer cells, *diminished DEPTOR protein content and concomitant mTOR hyperactivity are important contributors to MCF7 cellular proliferation, in vitro.*

With respect to pharmacological regulation of mTOR activity, *in vitro* treatment of either rapamycin, Torin1 or combined suppressed anabolic signaling and consequent cellular protein synthesis, with a rescue of DEPTOR protein content, although not as robustly as muscle-perfusate treatment. Our results are consistent with previous findings whereby inhibition of the mTOR signaling pathway blocks the destruction of DEPTOR [40, 103]. Additionally, inhibition of mTOR using Torin1 resulted in an increase in DEPTOR expression, findings consistent with

Gao et al [103] who also found blocked DEPTOR destruction with both mTORC1 and mTORC2 suppression via pharmacology. Inhibition of both mTOR complexes is significant in the context of cancer, for without suppression of mTORC2 activity, the complex's "pro survival function" via feedback signaling is maintained, and mTORC1 can remain transiently active, leading to continued proliferation [57, 83]. Additionally, we found higher phosphorylation of AKT^{Ser473} in TOR1 vs RAP treated cell, concomitant with previously mentioned p70 data, support the association between phosphorylated $p70^{Th389}$ mediated inhibition of the Rictor protein within the mTORC2 complex [53, 54, 136] and that signal crosstalk, between the complexes, increased the complexity of understanding mTOR regulation [100, 107, 137]. The mTOR kinase is a key molecule whose altered activity by exercise can be modeled using pharmacology in culture. However, mTOR suppression, per se, cannot solely account for DEPTOR's acute overexpression exercise, as total DEPTOR accumulation by silencing upstream activation of mTOR was less than half of what is observed with muscle contractions.

With the knowledge that DEPTOR is an unstable protein which rapidly degrades upon mTOR stimulation [40], we favor the concept that its expression is influenced by the activity of its dichotomous interacting partner, mTOR. Moreover, the mTOR signaling network has known transduction intermediate proteins that act both upstream and downstream to influence cellular anabolic activity, thusly inducing proliferative or apoptotic states [45, 48, 53, 103-110]. Our investigations into the expression of these intermediate proteins suggest their dependency on mTOR's activity for their translation. Our data propose that mTOR activity influences the availability of a transcript for translation potential via the transcripts association with translational machinery. It has been shown that peptide-chain initiation involves specific factors, namely eIF4E or eIF3d, that preferentially select transcripts for the translational apparatus,

depending on the nature of its 5'UTR [138]. While the newly characterized eIF3d mechanism is lesser understood [139], it is well known that the eIF4E complex can only participate in this selective translational process only when mTORC1 is active [140-142]. Therefore, when a transcript is cap-dependent, its translation is mTOR-dependent, meaning that it requires mTORC1 activity to allow for eIF4E direction of the transcript to the ribosome. If translation of the transcript arises when mTOR activity is suppressed, the transcript is thought to be cap-independent. Our data demonstrate that an increase in DEPTOR mRNA is concomitant with decreased mTOR activity, suggesting that its translation is cap-independent

Unlike cap-dependent transcripts, there are an abundance of transcripts that have internal ribosome entry sites (IRES), which allow these transcripts to find the translational apparatus independent of eIF4E, and therefore, independent of mTORC1. It has been speculated that the activation of mTORC1 not only selectively directs cap-dependent transcripts to the translational apparatus, but also that cap-dependent translation occurs preferentially at the expense of cap-independent translation. Cap-independent transcripts are thought to include proteins that respond to specific stressors in the cell, impact cell survival, and/or are involved with directing the cell into specific phases of the cell cycle [138]. Our pharmacology and exercise model data show increased DEPTOR protein coincides with changes in the anabolic state of the breast cancer cell. The increased DEPTOR protein content is adjuvant to a suppression of mTOR activity, a markedly decreased anabolic rate, and an elevation of apoptosis. We propose that this translational dependency of DEPTOR on mTOR's suppression, which is strongly suggestive of cap-independent behavior, is key to its characteristically low expression in invasive breast cancers. Furthermore, not only do we demonstrate that hyperactivity of mTORC1 may eliminate the possibility of DEPTOR being translated, but also that mTORC1 may also prevent the

transcription of the DEPTOR gene. The use of ActinomycinD and Cycloheximide in tandem in the present investigation provides a baseline of transcript levels in the cell. As such, at 18h, the increase in DEPTOR transcript with rapamycin and Torin1 treatment is not due to the preservation of a pre-existing transcript, but the increase mRNA transcription, suggesting that mTORC1 suppresses DEPTOR gene transcription, albeit by mechanisms unknown to us at this time. Only when we silence mTORC1 do we see a rescue on the protein and transcript of this important mTOR binding partner. Due to mTOR's hyperactivity in breast cancer, the DEPTOR transcript is reduced, and what is bioavailable is not available for translation in the cap-dependent preferred environment, and thus DEPTOR's endogenous inhibitory role on mTOR eludes the cell.

We should note that there have been numerous attempts to use pharmacology to silence mTOR in an effort to manage cancer proliferation, with limited to marginal success. While we acknowledge that others have demonstrated that the phosphorylation of many mTORC1 substrates are resistant to rapamycin treatment, including translational regulators [111, 115, 143, 144] the combination of Rapamycin and Torin1 in our hands did not elicit any significant difference in the translation of the DEPTOR protein compared to Torin1 treatment alone. Furthermore, with the recognition that pharmacological disruption of the proteolytic mechanism responsible to degrade DEPTOR has resulted in limited successful control of mTOR activation [37, 134, 135], our findings suggest that the impact of exercise on breast cancer is by directly influencing the synthesis of new DEPTOR molecules in a capacity that is far more robust than current pharmacological outcomes (Figure 21), and likely not due to a physiological alteration of DEPTOR degradation.

3. STUDY INTERLUDE

Although the reasons underlying differences in protein expression between exercise and pharmacology remain to be fully elucidated, it is enticing to speculate about the mechanistic contribution of contracting skeletal muscle to exert a therapeutic control on anabolic gene expression.

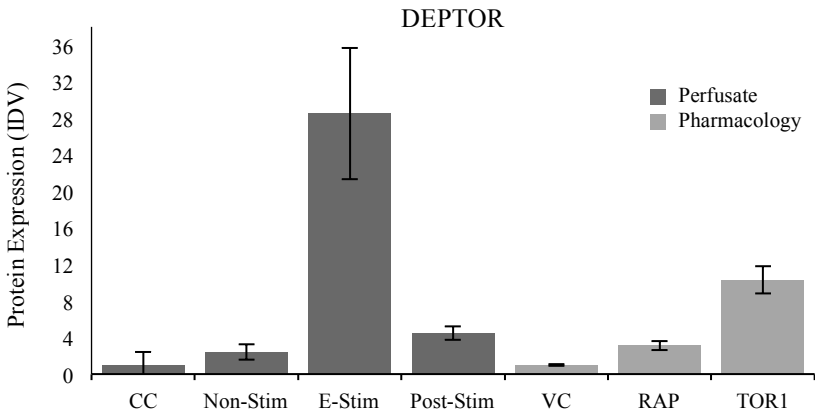

Figure 21. Protein Expression Differences
CC= Cell Control, Non-Stim= without contraction, E-Stim= with Contraction, Post-Stim = after contraction, VC= Vehicle Control, RAP= Rapamycin, TOR1=Torin1

The synthesis of specific proteins is dependent upon tight control over translation of their mRNA [145]. mTORC1 is tightly linked to several steps of protein synthesis including ribosome biogenesis, translation initiation and elongation [100]. Specific mTORC1 active-site inhibitors have been shown to significantly reduce overall rates of protein synthesis in proliferating cells [56, 107, 112]. Furthermore, the regulation of gene expression for both cellular anabolism and apoptosis are sensitive to environmental stimuli resulting in functional aberration of cellular control, a critical event in disease progression [145-147]. Myokines, molecules released from

muscle that impact other cells or cell types in organism, are one such example of how the investigation into the anabolic transcript may elucidate exercise's biological regulation on cancer. MicroRNAs (miRNA) are small non-coding RNAs found in abundance in skeletal muscle that have been proposed as possible myokines. The miRNA have the capacity of regulating gene expression post-transcriptionally by masking either the cap-dependent 5' or IRES start codons and preventing the transcript from entering the translational apparatus. Thus, these miRNAs have the capacity to act as negative gene regulators of gene expression on a variety of proteins, including those involved with the mammalian target of rapamycin (mTOR) pathway. In fact, studies have shown that up to 30% of overall gene expression is affected by the presence of miRNA [27], and this regulation has been implicated in the pathophysiology of muscle growth, atrophy, metabolism and insulin resistance [57, 148-151]. Overall, discrepancies in the translational regulation of altered proliferative factors within the mTOR cascade (Figure 21) highlight the potential for exogenous sources (*i.e.*, not produced in the cancer cell) of anabolic regulation in breast cancer cells, such as microRNA arising from another cell/tissue type. Therefore, a first step in understanding whether microRNA could be a possible regulator in the aforementioned altered anabolic capacity of breast cancer will be to profile the appearance of microRNA from skeletal muscle, termed myomiRs, with and without exercise. The hind limb perfusion preparation studies in the current study provide an excellent experimental paradigm to monitor the appearance of miRNA arising from skeletal muscle and if that appearance is altered by muscle contraction.

4. EXERCISE INDUCED MYOMIRS AS POTENTIAL CHEMOTHERAPEUTIC MYOKINES

4.1. Summary

Underappreciated is skeletal muscle's ability to release molecules into circulation facilitated by muscular contraction. Termed *myokines*, these molecules have the ability to influence many disease states, including breast cancer. MicroRNAs (miRNA) are small non-coding RNAs found in abundance in skeletal muscle that impact cellular function by altering the availability of mRNA for translation. Mechanistically, this translational alteration is accomplished by masking start codons, which prevent the transcript from entering the ribosome, and represents a way that cells can modify gene expression post-transcriptionally. Recent interest in miRNA has been focused on findings demonstrating that these post-transcriptional modifiers can be released from skeletal muscle and delivered to other cell types in the organism, and thus, may be an example of a myokine arising from skeletal muscle and capable of exerting a biological action in another cell of the body. We have unique preliminary findings that myokines released during electrically-stimulated muscle contraction of hemicorpus-prepared rats affects the anabolic activity and capacity of breast cancer cells. When MCF-7 cancer cells were treated with perfusate collected during muscle contraction, a significant inhibition of proliferation was noted alongside diminished mTOR activity and global rates of protein synthesis. **Purpose**: The purpose of this study was to identify microRNA released into circulation during a controlled hind limb perfusion preparation, and how that profile was affected by muscle contractions. **Methods:** Female Wistar rats underwent a hemicorpus hindlimb perfusion preparation with and without electrically-stimulated muscular contractions. RT-PCR analysis of select microRNAs, known to

impact cellular anabolism, was performed on both muscle and perfusate samples collected pre- and post-contraction (Non-Stim=4. E-Stim=4, respectively). **RESULTS**: MicroArray analysis identified 52 microRNA across all samples, with an average of 65 microRNAs detected per sample. Significant differential expression of 8 microRNA E-Stim and Non-Stim samples within an animal ($p<0.05$), and 15 microRNAs between E-Stim and Non-Stim groups ($p<0.05$) were determined. Expression of mir16-5p was 4% higher in hindlimb muscle exposed to E-Stim compared to Non-Stim ($p>0.05$), and was 147% higher in E-Stim perfusate samples compared to Non-Stim ($p<0.05$). **CONCLUSION**: Results suggest that skeletal muscle is a rich endogenous source of cancer microRNA that are released into the circulation, and muscle contraction results in the release of specific miRNAs that are associated with altered mTOR signaling. The observation that muscular contraction comparable to resistance exercise facilitates the release of microRNA into systemic circulation supports the concept that there is an exercise-facilitated crosstalk between muscle and cancer.

4.2. Introduction

The burden of breast cancer affects over 2.8 million women in the United States, with 238,130 *new cases* in the year 2015 alone [99]; however, this burden can be reduced with exercise. Currently there is an evolving appreciation for exercise-combined therapies advocated by clinicians [93-96]. Given breast cancer's prevalence in the United States, and the recognized benefit of exercise in breast cancer patients [93-96], identifying and understanding the mechanistic impact of exercise on cancer will advance research and may lead to new treatments. Our initial experiments have identified a causal role for exercise in cancer management/prevention through an, as yet, unidentified release of (a) myokine(s). A

mechanistic understanding of this causal role would have major translational impact in cancer prevention and patient survivorship, as even a small 1% reduction in incidence of cancer would result in $500 billion in health care savings. Unfortunately, the exercise mechanisms associated with a 20-40%% reduction in breast cancer risk are not well understood [2, 8, 9]

Skeletal Muscle as an Endocrine Organ

Skeletal muscle, the mechanistic machinery of exercise, has established a positive association with regard to enhanced activities of daily living and improved quality of life, and its protective effect on multiple metabolic disease states [37, 57, 87]. Underappreciated however, is that skeletal muscle exhibits an endocrine-like behavior during exercise where exercise-induced 'hormones' are released into circulation in both rodents and humans post muscular contraction [19, 20, 35, 88]. Specifically, contracting muscle releases "myokines" (factors), which are molecules that effect signaling pathways involved with muscle homeostasis, inflammation, and colon cancer, amongst others [19, 22, 89, 90].

The earliest established connection between myokines and cancer, previously termed "fatigue substance", leading to an inhibited tumor progression dates back to the early 1960s. It was found that extract released from rat muscle during passive electrically-stimulated contraction resulted in significant tumor growth inhibition when administered via subcutaneous injections into tumor-bearing rodents [4, 21, 24]. Most recently, our lab has contributed to the field's current findings that i*n situ* produced muscle-contraction medium significantly inhibits cancer cell growth, proliferation *in vitro* [22, 26, 91, 92] potentially through the altered cellular anabolism. Furthermore, expression differences of key anabolic proteins between muscle-

perfusate and pharmacology data (see above) lead to enticing speculation about the mechanistic contribution of contracting skeletal muscle on gene expression.

MicroRNA

MicroRNA are endogenous, small, but mature, single stranded RNAs with gene silencing capabilities. These small, non-coding ~22 nucleotide sequence long molecules are believed to control 30% of overall gene expression [27]; thus they are implicated in the pathophysiology of muscle growth, atrophy, metabolism and insulin resistance [57, 148-151]. These miRNA have post-transcriptional abilities allowing them to act as negative gene regulators of gene expression. This is accomplished through association with the RNA Induced Silencing Complex (RISC), which exposes an unpaired strand of the miRNA, directing it to bind to messenger RNAs (mRNAs) with a partially complementary sequence [152]. Of particular interest to us, are the recent investigations that miRNA can be manufactured in one cell, packaged in exosomes or protein carriers, and released into circulation where they can be taken up by other cells or even cell-types [153]. Upon an miRNAs uptake by a receiving cell, resultant translation repression or mRNA degradation can occur, subsequently modulating protein expression post-transcriptionally [145].

A relevant and key feature of miRNAs is their remarkable stability; they are well preserved both in their tissue of origin as well as in circulation, as they are often packaged in vesicles or on carrier proteins to protect them from RNAse activity in the blood.. In circulation, these non-coding nucleotide sequences may be delivered to a recipient cell to regulate translational activity of targeted genes. In breast cancer research, circulating microRNA, collected in biological fluids, act as minimally invasive biomarkers for disease progression and

risk factors. Recently, Heneghan et al. showed that circulating tumor-associated microRNAs have the potential to serve as a marker of breast cancer in its earliest stages, providing information regarding histologic features, hormone receptor and lymph node status [154]. Basic experiments of breast cancer and miRNA have evaluated microRNA's role in cell growth and proliferation *in vitro*, directed primarily to apoptotic and autophagy pathways [29, 60-69].

Moreover, miRNA expression is observed to be altered in physiological states, such as exercise. Additionally, miRNA have previously been found in abundance in skeletal muscle, thusly making them attractive candidates for membership under the myokine umbrella. MyomiRs, microRNA released by skeletal muscle, have been found to regulate multiple facets of muscle development [149, 155] and myogenesis [156-158]. A myomiR of interest due to it absence or downregulateion in many types of cancers is miR16-5p-1 [159-162]. In breast cancer, which has low basal expression of miR-16-5p-1, the overexpression of this microRNA attenuates cellular growth and proliferation, and promotes apoptosis [159, 163]. Interestingly, it has also been implicated in regulating protein synthesis through the mTOR network [151, 164, 165]. Ramaiah et al [165] demonstrated that overexpression of exogenous sourced miR-15/16 led to inhibition of cell proliferation, with demonstrated direct binding of miR-16 to a subunit of mTORC1's downstream effector p70S6K1. These findings encourage the evaluation of miR-16-5p-1 as a myomiR release during contraction to potentially facilitate altered mTOR-mediated anabolism with muscle-breast cancer crosstalk.

In context of cellular anabolism, changes in mitotic capacity and proliferation can both be attributed to changes in gene expression at the translational level. Thus, many pathways have been implicated in the development and progression of breast cancer. Among them, the mTORC signaling network has garnered much acclaim due to its superlative role as regulator of

coordinated cell growth, proliferation and survival. With the knowledge that mTORC1 is tightly linked to several steps of protein synthesis including ribosome biogenesis, translation initiation and elongation [100], the investigation into differences in anabolic transcript of a cancer cell with muscle-contraction exposure may elucidate exercise's biological regulation on tumor- and carcinogenesis [101, 102] Furthermore, the mTOR kinase's sensitivity to environmental stimuli and cellular metabolic environment further encourage investigation into its response to exercise exposure beyond metabolic substrate and oxygen availability. However, it remains unclear as to whether anabolic repressor microRNA are found in circulation following muscle contraction, and whether they have the ability influence the bioavailability of mTOR proliferative factors.

As such, the purpose of this study was to profile microRNA released from skeletal muscle into circulation, with and without lower limb muscular contractions to determine if the myomiR profile is altered by exercise. Results herein illustrate the secretome profile of both quiescent and maximally contracting skeletal muscle, and we offer speculation on how the contracting profile may affect breast cancer cells.

4.3. Methods

Muscle Perfusate and Skeletal Muscle Collection

Female Wistar rats (N=10) 8-12 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). All procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Animals were house two-rats per cage under standard 12h photoperiod, provided with normal Rat Chow with water *ad libitum.* Following a 2 day acclimation period after arrival, animals underwent a non-survival hemicorpus hind limb

perfusion preparation (HHLP) (Figure 7) as described previously [91, 92, 122-125]. Briefly, midline to caudal end of the animal was surgically prepared so both hind limb limbs could be perfused with an oxygenated Krebs-Heinseliet Buffer during electrically stimulated muscle contraction. Electrical stimulation was administered using a stimulator (Model #) (Grass Instruments, West Warwick, RI), a force transducer (Warner Instruments, Harvard Bioscience Inc., Holliston, MA) at a surgically exposed sciatic nerve on a single hind limb of the animal. Perfusate medium was maintained at 31.7°C using a bipolar temperature controller (Model # CL-100, Warner Instruments, Harvard Bioscience Inc., Holliston, MA) and administered at a flow rate of 12ml/min, by peristaltic pump (MPL 8-Channel) (Watson Marlow, Marlo, United Kingdom).

Perfusion sample collection is depicted previously in Figure 8. During perfusion, medium was collected on ice in 50ml sterile conical tubes (Corning Inc., Corning, NY) before electrical stimulation (Non-Stim, NS), during electrical stimulation (E-Stim, ES), and following electrical stimulation (Post-Stim, PS). Following collection, samples was centrifuged at 2500 rpm at 4°C to remove red blood cells and the supernate was then stored at -80°C until analysis. Skeletal muscles of both hind limbs were anatomically separated into soleus, plantaris, gastrocnemius and snap frozen in liquid nitrogen, and stored at -80°C until analysis.

miRNome Profiling of Muscle Perfusate

Sample Preparation. A total of 8 samples (NS=4, ES=4) were profiled for microRNA Total RNA was extracted by using the miRCURY exosome RNA isolation kit #300102 (Exiqon, South Korea), and then followed by miRNeasy Micro Kit #217084 (Exiqon, South Korea). Three

RNA spike-ins (UniSp2, UniSp4 and UniSp5) were pre-mixed, each at different concentration in 100 fold increments, added to samples, and used as an RNA isolation control.

RT-qPCR. All microRNAs were polyadenalyted and reverse transcribed into cDNA in a single reaction step. A total of 4 µl of RNA was reverse transcribed in 20 µl reactions using the miRCURY LNA Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon, South Korea). To confirm reverse transcription and amplification efficiency in all samples, UniSp2 was used as a spike in (RNA Spike-In Kit, ID#339390, Exqion). cDNA was diluted 50 x and assayed in 10 ul PCR reactions using a pipetting robot; each microRNA was assayed once by qPCR on the miRCURY LNA miRNA Human Cancer Focus PCR Panel CAT# YAHS-102Y(EXIQON, South Korea), using ExiLENT SYBR® Green master mix. A total of 88 primers were assayed. Negative template controls (NTC) were profiled similar to the samples. The amplification was performed in a LightCycler 480 Real-Time PCR System (Roche) in 384-well plates. The amplification curves were analyzed using the Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis.

Data Analysis. The amplification efficiency was calculated using algorithms similar to the LinReg software. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Assays with several melting points, or with melting points deviating from assay specifications were flagged and removed from the data set. Reaction amplification efficiency was set at 1.6 or above. Additionally, assays must be detected with 5 Cqs less than the negative control, and with Cq<37 to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis. Cq was calculated as the 2^{nd} derivative.

Normalization. Data were normalized to correct for potential overall differences between samples using as identified best by NormFinder software (Anderson, C. et al). The formula used to calculate normalized Cq values was as follows:

$$\text{Normalized Cq} = \text{average Cq} - \text{assay Cq (sample)}$$

RT-qPCR Validation in Muscle Perfusate and Muscle Tissue

Sample Preparation. Enriched and Isolated microRNA samples from miRNome profiling were utilized. Total RNA from pooled soleus and gastrocnemius muscle tissue samples were manually extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and 1-Bromo-3-chloropropane (Biomedical Research Centre) protocol, and precipitated in isopropanol. Following precipitation, RNA was treated with a series of ethanol washes to prevent downstream salt inhibition, and then re-suspended in 50ul of nuclease free water. RNA concentration was then quantified by a Nanodrop using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) according to manufacturer's specifications. RNA integrity (RIN) was assessed on a 4200 TapeStation System (Agilent Technologies, Santa Clara, CA), which assigned a RINe value on a scale of 1 to 10. RNA samples with RINe quality values >7.5 were included in RT-qPCR assays.

RT-qPCR. Reverse transcription for cDNA synthesis was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) with 40ng and 200mg of total RNA (perfusate vs muscle samples, respectively) in reactions of 15ul according to manufacturers instructions. Corresponding negative reverse transcriptase reactions (NRT) were also prepared for each sample. miRNA expression of target miRNA was quantified with real time PCR using Applied Biosystems 7900HT Fast Real-Time PCR System (Carlsbad, CA).

TaqMan MicroRNA Assay has-miR-16-5p (ID# 000391) and US snRNA (ID 1973) were handled according to the manufacturer's instructions. Quantitative PCR was performed in triplicates of 20ul reactions using 1X TaqMan Universal Master Mix II, no UNG (Applied Biosystems, Carlsbad, CA) with corresponding NRT and no template controls (NTC). Thermal conditions were as follows: 10min at 95°C, and 15s at 95°C with 1min at 60°C for 40 cycles. Expression of target gene was normalized to US snRNA. All data analysis was conducted with Sequence Detection Software v 2.2.2 (Applied Biosystems, Carlsbad, CA).

Statistical Analyses

MirNome Profiling of Muscle Perfusate. The normal distribution of the data was assessed by a Shapiro-Wilks normality test. If the data were normal, a t-test was performed for microRNAs detected in at least three samples per group to assess differential expression between Non-Stim and E-Stim groups. If the data were not normal ($p>0.05$), a Wilcoxon test was performed on all assays detected in at least three samples per group. A chi-square test was performed on all assays to detect if any assay was under represented in one of the two groups. A low p-value from chi-square test indicated that the presence of the miRNA was different between the E-Stim and Non-Stim groups.

RT-qPCR Validation in Muscle Perfusate and Muscle Tissue. A t-test was performed to assess differential expression of miR16-5p-1 between Non-Stim and E-Stim samples of exercise perfusate and muscle, followed by Fisher LSD post hoc test (SigmaStat 3.5; Systat Software Inc., San Jose, CA, USA).

4.4. Results

miRNome Profiling of Muscle Perfusate

Profiling of perfusate samples was successfully completed for all samples (n=8). Controls (NTC and RNA spike-in) indicated good technical performance of the profiling experiment. No inhibition during qPCR was detected. Unsupervised analysis demonstrated that samples from the E-Stim group are grouped together and samples from the Non-Stim group are grouped together. 52 microRNA were identified across all samples, with an average of 65 microRNAs detected per sample (Figure 22).

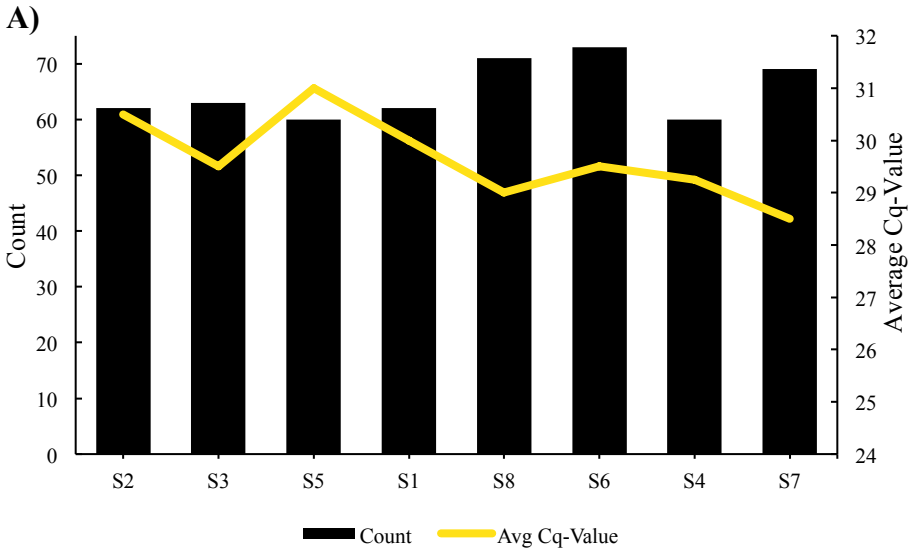

Figure 22. Graphical Illustration of MicroRNA Content.
The bars represent the number of microRNAs detected and the line shows the average Cq value for the commonly expressed microRNAs. On average, 65 microRNA were detected per sample.

Using NormFinder the best normalizer was found to be the average of assays detected in all samples. All data were normalized to the average of assays detected in all samples (average – assay Cq). Several microRNA were found to be differently expressed when using a significance

level of 0.05 (Figure 23). When comparing grouped E-Stim to Non-Stim samples, 8 microRNAs were found to be differentially expressed between E-Stim and Non-Stim samples ($p<0.05$) (Table 2). When comparing paired E-Stim vs Non-Stim samples, 15 microRNAs were found to be differentially expressed ($p<0.05$), with one passing a Benjamin-Hochberg correction ($p<0.05$) (Table 3).

MirName	Fold Δ (±S)	p-value
hsa-miR-143-3p	-2.4±0.15	0.00043
hsa-miR-7-5p	-1.3±0.06	0.0067
hsa-miR-24-3p	-1.2±0.09	0.0074
hsa-miR-145-5p	-3.2±0.53	0.0082
hsa-miR-196a-5p	1.8±0.19	0.015
hsa-miR-125b-5p	-2±0.4	0.017
hsa-let-7d-5p	-1.3±0.16	0.018
hsa-miR-130a-3p	-1.9±0.4	0.019
hsa-miR-99a-5p	-2±0.45	0.023
hsa-miR-15a-5p	-1.8±0.41	0.026
hsa-miR-93-5p	2±0.49	0.029
hsa-miR-10b-5p	-1.9±0.5	0.032
hsa-miR-16-5p	1.4±0.24	0.033
hsa-miR-195-5p	-1.5±0.3	0.034
hsa-miR-126-3p	-1.5±0.34	0.038

Table 2. MicroRNA Profiling, Paired t-Test Results
This table shows the standard deviation (SD) across groups, followed by the average normalized Cq values for each group, and fold change between the two groups. The last column shows the p-value from the paired t-test.

MirName	Avg ΔCq (± SD)		Fold Δ	p-Value
	Non-Stim	E-Stim		
hsa-let-7d-5p	-0.77+ 0.13	-0.4+0.14	-1.3	0.008
hsa-miR-181b-5p	-2.4+0.29	-3.2+0.38	1.7	0.02
hsa-miR-101-3p	0.82±0.24	-1.7+0.46	1.8	0.027
hsa-miR-99a-5p	-2.2+0.5	-1.2+0.51	-2	0.035
hsa-miR-143-3p	-2.3+0.67	-1+0.65	-2.4	0.036
hsa-miR-145-5p	-0.28+1	1.40±0.62	-3.2	0.036
hsa-miR-186-5p	-4.3+0.33	-5.1+0.37	1.8	0.037
hsa-miR-181a-5p	-1.1+0.25	-1.7+0.38	1.5	0.047

Table 3. MicroRNA Profiling, t-Test Results

This table shows the standard deviation (SD) across groups, followed by the average normalized Cq values for each group, and fold change between the two groups. The last column shows the p-value from the t-test

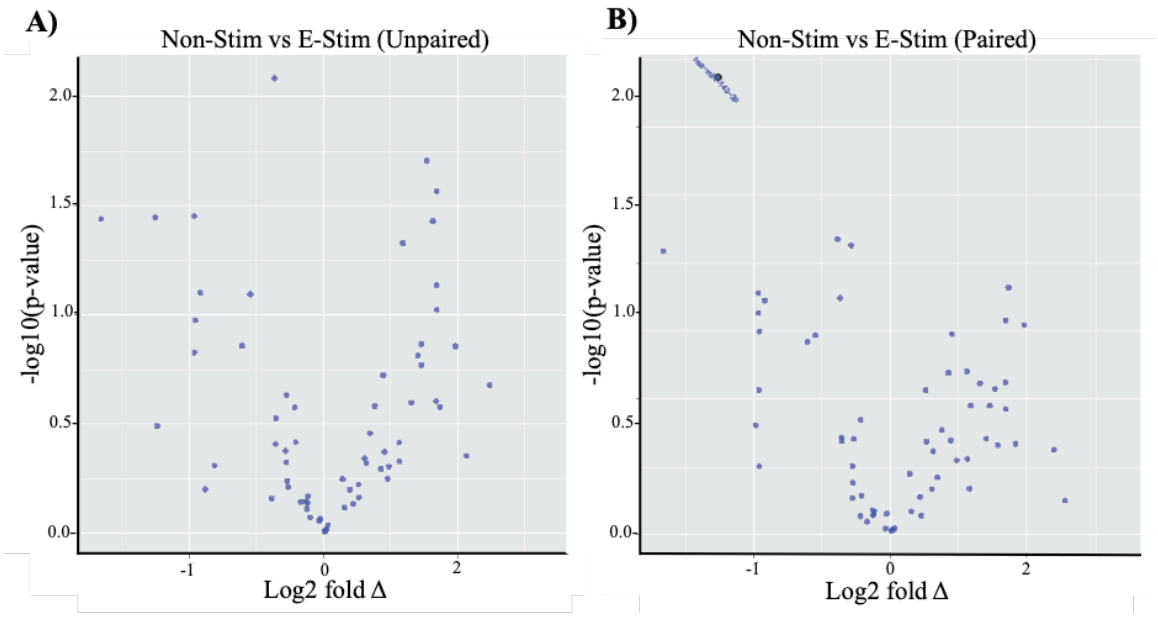

Figure 23. Volcano Plot of Differential MicroRNA Expression

The volcano plots shows the relation between the p-vales and the ΔΔCq. Highlighted are microRNAs with p-values below 0.05 after Benjamin-Hochberg correction for multiple testing.

RT-qPCR Validation in Muscle Perfusate and Muscle Tissue

Expression of mir16-5p-1 was 4% higher in hind limb muscle exposed to E-Stim compared to Non-Stim ($p>0.05$), and was significantly 147% higher in E-Stim perfusate samples compared to Non-Stim ($p<0.05$) (Figure 24).

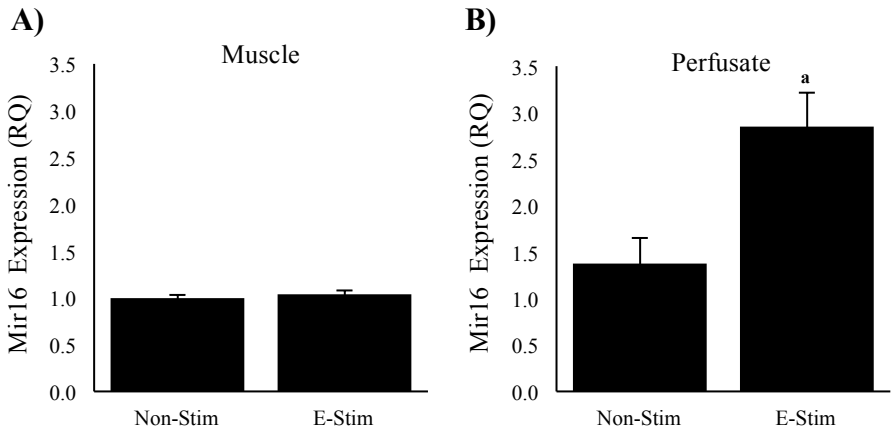

Figure 24. miR16-5p-1 RT-qPCR Results
Data are expressed at relative expression ($RQ=2^{-\Delta\Delta Ct}$). Statistical significance: a= different from Non-Stim at p=0.05.

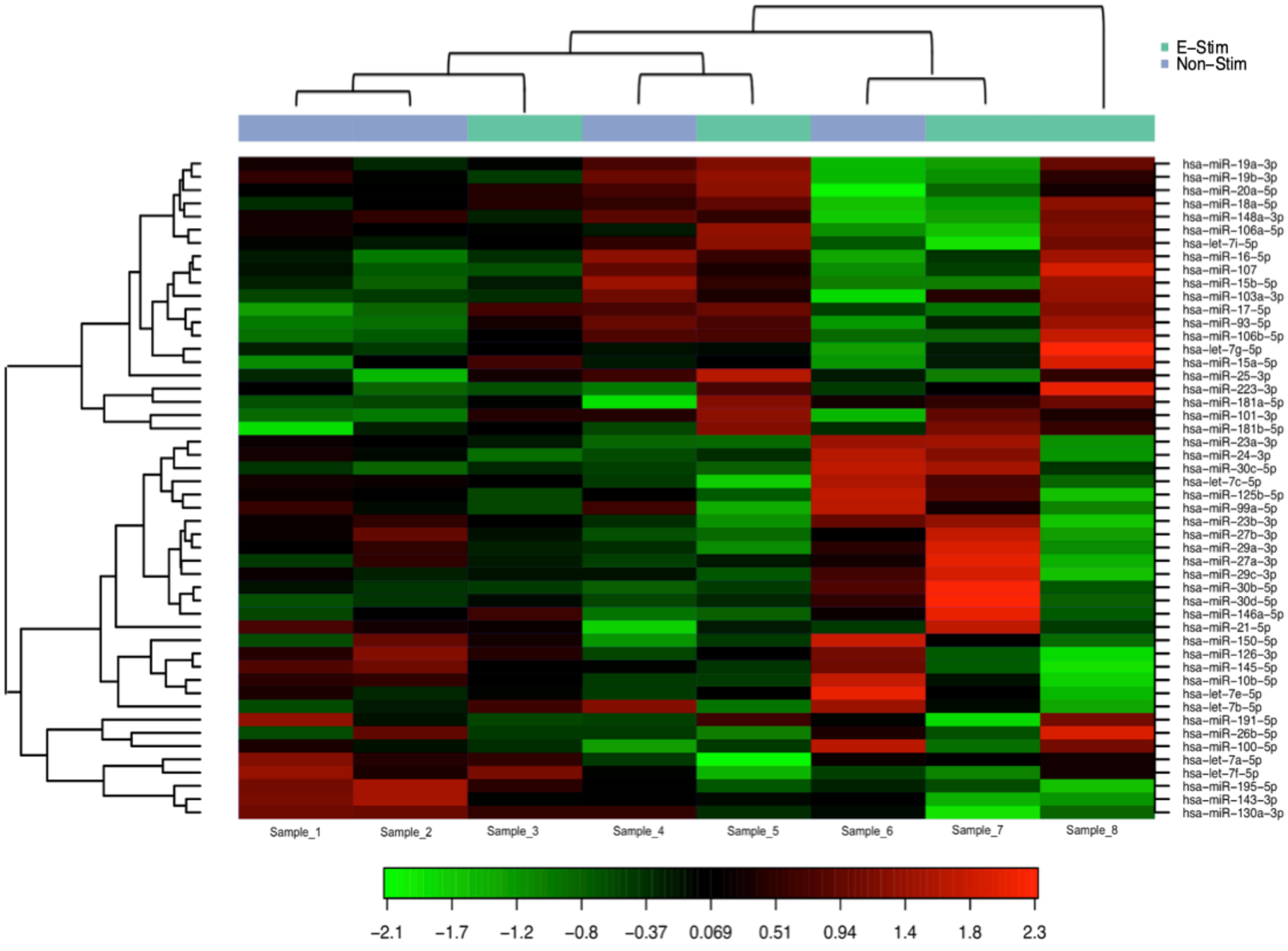

Figure 25. Heat Map and Unsupervised Hierarchical Clustering of top 50 microRNA.

Results of the two-way hierarchical clustering of top 50 microRNAs and samples. Each row represents one microRNA, and each column represents one sample. The microRNA-clustering tree is shown on the left. Relative expression level of a microRNA across all samples are indicated by according to color scale (red= above mean, green=below mean)

4.5. Discussion

In the United States, breast cancer accounts for 30 % of all new cancer diagnoses in women alone [1]. The benefit of physical activity to breast cancer patients is well documented [8, 9, 128-133], leading to an evolved appreciation for exercise-combined therapies advocated by clinicians [93-96]. Skeletal muscle, the mechanistic machinery of exercise, has a unique ability to release molecules into systemic circulation, which have auto- and paracrine capabilities [8, 19-22]. Despite the knowledge that anabolic protein expression dictates function, and that skeletal muscle is a potent reservoir of endogenous gene repressors, microRNA (myomiRs), little is known regarding circulating myomiRs as potential sources of cancer anabolic gene repressors. In this study we aimed to evaluate the effect of contraction on the microRNA 'secretome' of skeletal muscle, and to profile expression of circulating myomiRs that may impact the growth and proliferation of cancer. As such, the present study is an "intermediate" investigation of mTOR gene expression regulation, evaluating the role of exercise as facilitator of muscle and breast cancer crosstalk observed in dissertation work discussed above (study 1 and 2), in addition to previously published work [25, 91].

Several points of evidence support this notion. First, muscular contraction comparable to resistance exercise facilitates the release of microRNA into systemic circulation. The physiological relevance of systemic miRNA is dependent upon a mature miRNA's stability and concentration in biofluids to maintain functional integrity to carry-out post-translational modifications of target genes on recipient cells [28, 166, 167]. Aligning with our results, other studies have shown detectability of circulating microRNA after incubation at room temperature [154, 168] and after multiple freeze-thaw cycles [166, 169]. Differences in mTOR network gene expression between muscle perfusate (study 1) and pharmacology (study2) reported above

suggest that an endogenous molecule arising from skeletal muscle, such as a myomiR, acts a key physiological mechanism contributing to the benefit of exercise above pharmacology alone. Second, the secretome of skeletal muscle differs at rest and during electrically-simulated contraction via the nerve. Previous studies have demonstrated that miRNA can be released both actively and passively into systemic circulation [170-175]. Our data show expression differences in Non-Stim (quiescent) and E-Stim (contracting) medium collected in real-time from the hind limbs of female rats. This collection technique, which eliminates the candidacy of other tissue and organs as sources of microRNA, highlights a direct connection between skeletal muscle contraction and altered cancer anabolism observed in dissertation work discussed above (study 1 and 2), in addition to previously published work [25, 91]. The earliest investigation that demonstrated neoplasia inhibition with exercise in a rodent model was by Rusch and Kline in 1944, utilizing a rotating drum as the exercise mode [176]. The vast majority of exercise and cancer prognoses in both rodent and human models have utilized acute e-stim or aerobic exercise training. Even though the use of treadmill and wheel running is accepted as a surrogate model for physical activity and training effects in humans, isolated muscle contraction and resistance exercise are associated with greater muscle recruitment and systemic myokine secretion [19, 177-179]. As such, the hemi-corpus hind-limb perfusion preparation with an electrically-stimulated for muscle contraction via the sciatic nerve methodology utilized here provided a controlled and directive approach to evaluate muscle involvement of exercise on cancer, eliminating confounding variables such as the voluntary exercise response variability, hormonal and catecholamine involvement, and other systemic factors including alterations of the vasculature [180-182].

With respect to the chemotherapeutic impact of muscle contraction on breast cancer, our data indicate that 'exercise' has the potential to facilitate crosstalk between muscle and breast cancer, and this impact directly affects anabolic gene expression leading to disrupted or attenuated growth. Previously, collaborative research has shown miR16-5p-1 expression is significantly elevated in quiescent skeletal muscle in conjunction with normal anabolic function and elevated expression of the mTOR inhibitory protein, DEPTOR [151]. Interestingly, after anabolic-promoting exercises in skeletal muscle, there is a significant reduction of miR16-5p-1, leading to a loss of DEPTOR and heightened anabolic function. Based on the current studies, we now know that the loss of miR16-5p-1 after exercise may be due to the release of that molecule into the circulation. The expression of miR16-5p-1 is noted to be absent or downregulated in many types of cancer [159-162], and with the dysregulated anabolism in some muscle diseases [151]. In circulation, miR-16-5p-1 is encapsulated in microvessicles, protecting it from high RNAse activity of blood serum and plasma [183]. As such, miR-16 is generally thought to be a key tumor-suppressive miRNA and a likely candidate for myokine designation. In breast cancer, which has low basal expression of miR-16-5p-1, the overexpression of this microRNA attenuates cellular growth and proliferation, and promotes apoptosis [159, 163]. Our investigation showed significant increase in circulation of miR-16-5p-1 in muscle perfusate. Results of the targeted RT-qPCR validation experiments supported the results of the microarray, demonstrating a significant elevation in miR16-5p-1 in the E-Stim muscle perfusate as compared to Non-Stim, without a decline of expression in contracting muscle, suggesting that this microRNA was being manufactured during contraction for reasons unknown. Results from study 1 reported above showed significant elevation in DEPTOR protein expression in MCF7 cells cultured in the same muscle-perfusate used here-in (study 3). Collectively, this suggests that miR-16-5p-1 expression

may contribute to altered anabolic capacity of breast cancer cells, potentially through repressed anabolic gene expression. Future work from our laboratory will systematically assess the impact of miR-16-5p-1 on cancer cells, as well as define the anabolic targets it impacts. Those studies are beyond the scope of the current study.

Moreover, myomiRs that would target anabolic proteins and serve to repress their translation would be likely candidates to be released from skeletal muscle with contraction. This would be a means to protect/elevate the contribution of mTOR anabolic signaling to muscular adaptations like hypertrophy. Thus, upon secretion into systemic circulation, whether packaged in exosomes or microvessicles, myomiRs could be delivered to recipient surrounding cells, including cancer, to impart their post-translational control to suppress growth and cellular proliferation capacity of tumors.

When extrapolating miRNA expression differences found in our study, it is important to reiterate that the contraction model used in this study is most comparable to maximal activation with resistance exercise that is accompanied by complete motor recruitment of the muscle, albeit without any load imposed. Others have not only shown that miRNA expression differs over the exercise and recovery duration, but also by mode of exercise performed [155, 175, 184-192], which may be due to loads imposed on the muscle or the amount of muscle mass recruited to perform the work. Furthermore, fiber type specificity may also play a role, and the participation of specific fiber types is often dictated by the forces necessary to overcome loads imposed upon them. As such, lack of distinction in exercise mode and intensity may explain variation in response to exercise interventions and physical activity levels observed in epidemiological studies.

In conclusion, this investigation collectively provides a mechanism of exercise-facilitated muscle and cancer crosstalk through the secretion of known cancer associated microRNA. Understanding myomiR expression levels and their clinical significance as it pertains to tumor progression, may provide insight into the pathobiology of breast cancer cell growth and proliferation observed with combined exercise-therapeutics. Further elucidation of the transport of these molecules in circulation and their affinity for specific gene targets within the anabolic network may provide potential therapeutics to treat cancer and other mTOR hyperactive diseases.

5. CONCLUSIONS

Collectively, our results provide insight how exercise may act as a modulator for muscle and cancer crosstalk, altering vital anabolic signals within cancer cells through manufacture and release of factors into the circulation. Our data strongly suggest that the mTOR-DEPTOR interaction is an important participant in cancer proliferation and that the rescue of DEPTOR, either through silenced mTORC1/2 signaling or altered protein expression of DEPTOR, may serve a critical role in the reduced proliferation/elevated apoptosis in response to the medium perfused through contracting skeletal muscle. A critical question for the future is whether regulated DEPTOR expression, endogenous or exogenous (i.e., via genetic engineering), can contribute to the suppressed anabolism in a cancer cell, long-term. As such, efforts focused on the rescue of DEPTOR expression as opposed to altering its degradative-pathways, may be therapeutically helpful to counteract the limited success of rapalogs and first generation inhibitors in the clinic. Furthermore, the discovery of miRNA differences in skeletal muscle during and after contraction support the naming of skeletal muscle as an endocrine organ. These data suggest that endogenous muscle miRNA, myomiR, with their potential regulation of messenger RNAs that encode proteins, are suitable candidates of myokine influence. The data presented in these studies indicate that (a) factor(s) arising from skeletal muscle affects the anabolic function and survival of cancer cells, which appears to be directly focused on the key anabolic pathway involving mTOR. Subsequent studies using pharmacology to affect mTOR activation were largely successful in reproducing that work, albeit on a smaller scale. Collectively, our data indicate that the mechanistic target of muscle and breast cancer crosstalk with exercise is centrally focused on the capacity of the mTOR pathway to promote anabolism,

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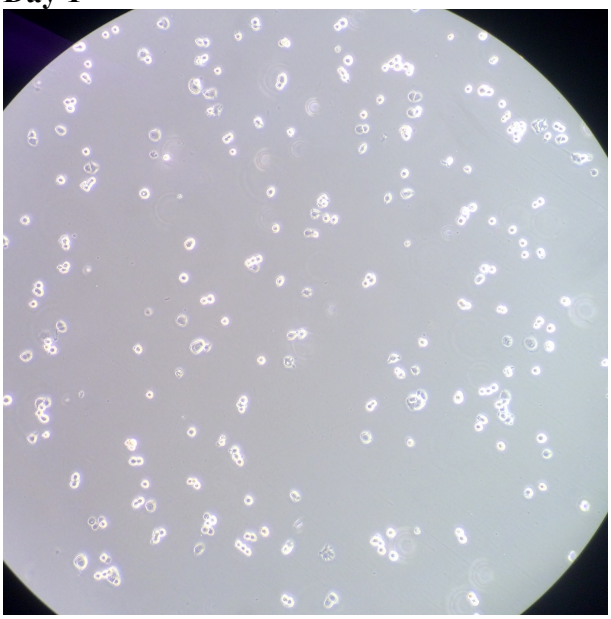
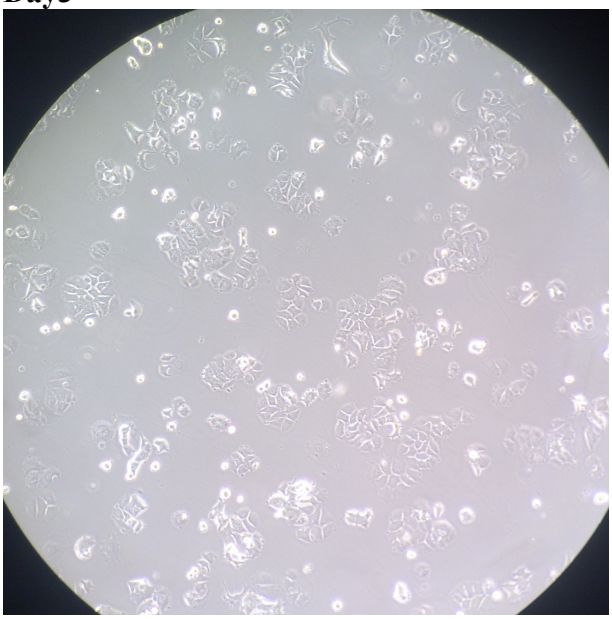
APPENDIX A

ADDITIONAL TABLES AND FIGURES

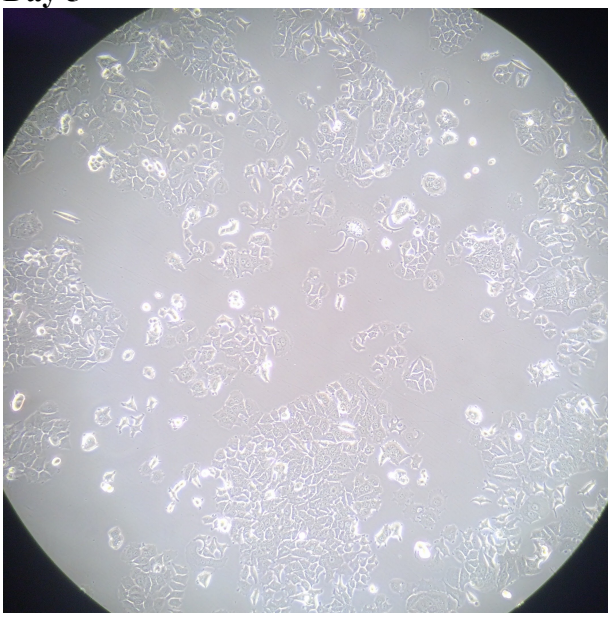
Day 1



Day3



Day 5



Day 7

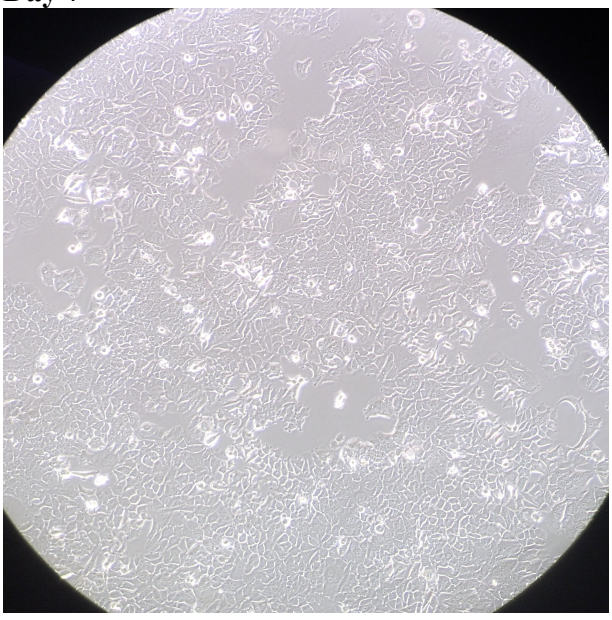


Figure 26. MCF7 Growth Profile

Sample*	NanoDrop (ng/ul)	Qubit (ng/ul)	TapeStation (ng/ul)	TapeStation (RINe)
CC	676.38	600	979	10
CC + ActD	216.52	203	187	9.9
CC + ActD +CYX	188.56	175	137	9.5
CC + CYX	445.69	446	378	9.6
VC	585.46	570	449	9.7
VC + ActD	195.57	165	159	9.8
VC + ActD +CYX	216.48	198	187	9.7
VC + CYX	359.21	329	238	9.6
RAP	441.94	405	275	9.8
RAP + ActD	215.34	197	170	9.7
RAP + ActD +CYX	131.34	113	97.1	9.8
RAP + CYX	287.42	277	215	9.7
RAP+TOR	300.61	269	284	9.4
RAP+TOR + ActD	145.93	113	92.4	9.6
RAP+TOR + ActD +CYX	113.93	101	85.3	9.5
RAP+TOR + CYX	195.88	177	158	9.5
TOR	366.74	340	195	9.8
TOR + ActD	134.87	118	97.2	9.8
TOR + ActD +CYX	116.14	98.6	80.4	9.4
TOR + CYX	230.09	205	139	9.7

Table 4. RNA Sample Purity and Quality

Gene Symbol	Gene ID	Ref ID	Sequence (bp)	Exon Boundary	Amplicon (bp)	Assay ID
MTOR	2475	NM_004958.3 XM_005263438.2 XM_011541166.2 XM_017000900.1 XM_017000901.1 XM_017000902.1	8733	20 - 21 20 - 21 20 - 21 17 - 18 18 - 19	103	Hs00234508_m1
DEPTOR	64798	NM_001283012.1 NM_022783.3 XM_017013735.1	2333-2636	3 - 4 5 - 6 3 - 4	67	Hs00961900_m1
RPS6KB1	6198	NM_001272042.1 NM_001272043.1 NM_001272044.1 NM_001272060.1 NM_003161.3 XM_011525101.2 XM_011525102.2 XM_011525103.2 XM_017024929.1 XM_017024930.1 XM_017024931.1 XM_017024932.1 XM_017024933.1	4332-5497	6 - 7 7 - 8 8 - 9 7 - 8 7 - 8 7 - 8 7 - 8 9 - 10 8 - 9 8 - 9 9 - 10 8 - 9 8 - 9	97	Hs00177357_m1
RPS6KB2	6199	NM_003952.2 XM_005274164.1 XM_006718655.3 XM_006718656.3 XM_006718657.1 XM_017018108.1	1782	4-5 3-4 4-5 3-4 3-4 4-5	74	Hs00177689_m1
BTRC	8945	NM_001256856.1 NM_003939.4 NM_033637.3 XM_006718054.2 XM_011540320.2 XM_017016870.1 XM_017016871.1 XM_017016872.1 XM_017016873.1 XM_017016874.1	6072-6180	12 - 13 12 - 13 13 - 14 13 - 14 10 - 11 12 - 13 13 - 14 13 - 14 13 - 14 11 - 12	116	Hs00182707_m1
FBXWII	23291	NM_012300.2 XM_005265855.4	4575	1-2 1-2	63	Hs00362667_m1
EIF4EBP1	1978	NM_004095.3	877	2-3	69	Hs00607050_m1

Table 5. qPCR Target Gene Assay Details
All assays were FAM-MGB detector-quenchers

Gene Symbol	Gene ID	Ref ID	Sequence (bp)	Exon Boundary	Amplicon (bp)	Assay ID
EIF4EBP1	1978	NM_004095.3	877	2-3	69	Hs00607050_m1
GAPDH	2597	NM_001289746.1 NM_002046.5	1407 1285	2 3	122	Hs_99999905_m1
RPLP0	6175	NM_001002.3 NM_053275.3	1105 1289	3 3	105	Hs_99999902_m1
ACTB	60	NM_001101.2	1793	1	171	Hs_99999903_m1
PUM1	9698	NM_001020658.1 NM_014676.2	5416 5410	20-21 20-21	77	Hs00982775_m1

Table 6. qPCR Reference Gene Assay Details
All assays were FAM-MGB detector-quenchers

APPENDIX B

HEMICORPUS HIND LIMB PERFUSION PREPARATION

8.1. Surgical Preparation

Equipment Calibration:

Warner Instrument Box (Perfusate Temperature Regulator)

1. Attach water hose to tap and use a light flow
2. Set temperature using the "set temperature" dial
3. Connect the thermometer to the "monitor temperature" input
   Note: The catheter dissipates heat, so set temperature just above 36.8°C (~37°C) because the end temperature of the catheter (essentially the perfusate entering circulation) needs to *be* 36.8°C.

Peristaltic Pump:

- When not in use, unclamp the tubes on the box
- When in use, have the clip at the same slot
- To lock hold "start" and "stop" at the same time
- Pump rate: 12ml/min

Saline Drip:

- Invert the saline pump/tube/drip to allow it to fill to protect from bubbles emptying into the system (do this before attaching the catheter to the animal)
- Monitor this level to see if there is a change in the systemic pressure in the animal

Procedure And Equipment Set-Up:

1. Turn on / hook up $O_{2(g)}$
2. Turn on pump pro and set at correlated rpm
3. Pour perfusate into glass vial
4. Divert O_2 tank to flow into the glass (note: you only want slow low bubbles to oxygenate the perfusate)
5. Turn on glass turner so that the vial of the perfusate is constantly moving
6. Set-up overflow vial in draw and place surgery overflow tube in there
7. Turn on water flow, turn on temperature box
8. Check that electrodes are connected.
9. Set up 50ml conical collection vial

Surgical Procedure:

1. Inject 0.75ml of Ketamine into intraperitoneal cavity
2. Check animal for negative hind limb reflex
3. 1st Cut: cross abdominal cut with angled proximal ends (blunt dissectors)
4. 2nd Cut: cut a deep mid sagittal line proximal to sternum-xiphoid process, through the abdominal and thoracic musculature. Be careful not to pierce gastrointestinal organs, kidneys or lobes of liver.
5. 3rd Cut: widen the cross abdominal cut to expose the base of the bladder and distal reproductive organs

6. Use gauze pads to separate gastrointestinal tissue, reproductive tissue from fat and fascia. Make sure to push fecal matter away from the pelvic floor to all room for clean ties
7. 1st Tie: place surgical silk under the long intestine and fallopian tubes, including the bladder and below the fallopian bifurcation. Make sure the double tie is as close to the pelvic floor before cutting off excess thread.
8. 2nd Tie: place surgical silk under the proximal intestine up by the liver, under the lobes of the liver, make sure not to include the lobes of the kidney. Cut tissue and discard.
9. Wipe body cavity up to the kidneys, main descending artery and vein. Keep the cavity and tissue moist by soaking with saline to prevent tearing vascular tissue.
10. *OPTIONAL: Step 10 is optional. Tie off the kidney bifurcation with two separate ties (left tie, right tie).*
11. 3rd and 4th Tie: Find kidney artery bifurcation, lift up with hook forceps, grab with forceps, and pull surgical silk through. Make left and right ties.
12. Re-wipe body cavity and moisten with saline.
13. 5th & 6th Tie: tie off two other distal bifurcations to eliminate perfusion flow to other distal tissue.
14. *Prepare for Cannulation:* The artery lays to the left of the large veins. Pull on the side tissue to show the interconnecting space between the artery and vein. Use forceps and blunt dissectors to tweeze away the tissue to separate the artery and vein. *Note: Use the nose of the dissectors and/ forceps) to tweeze apart the space. Pinch the forceps closed and place on space, then slowly allow the forceps to open to the length of the vein and artery in a stroking motion with light downward pressure*.
15. 7th Tie: leave forceps under the separated artery. Feed the tie under the forceps and make a loose tie.
16. 8th Tie: repeat 7th tie but distal to 7th tie , on the artery, above the bifurcation.
17. 9th & 10th Tie: use forceps to tweeze under the vein and expose on the other side. Be careful not to knick the vein. Pull two sutures under the veins and place them parallel to ties 7 and 8. Create loose loop ties.

Canulate Artery:

18. Moisten vascular tissue and body cavity with saline.
19. Cannulate as proximal as possible to the distal bifurcation, below the 7th tie.
20. Hold the catheter needle, bevel up.
21. Hold the catheter with cannulation forceps on outside of the artery tissue, as the needle is gently push/inserted into the artery, threading it deep toward the caudal bifurcation, through the loose loop tie.
22. Take the distal 8th tie and tie around the catheter, over the artery tissue.
23. Tie off 7th tie which is already through the proximal artery tie. Again, ties around the cannula and the artery tissue.
24. Attach the artery perfusion line. Be careful not to over twist as it will twist the cannula and tear the vascular tissue.

Canulate Vein:

25. Moisten vascular tissue and body cavity with saline.
26. Cannulate the vein as proximal as possible to the distal bifurcation, below the 9th tie.

27. Hold the catheter needle, bevel up.
28. Hold the catheter with cannulation forceps on outside of the vein tissue, as the needle is gently push/inserted into the vein, threading it deep toward the caudal bifurcation, through the loose loop tie.
29. Take the distal 10^{th} tie and tie around the catheter, over the artery tissue.
30. Tie off 9^{th} tie which is already through the proximal vein tie. Again, ties around the cannula and the vein tissue.
31. Attach the perfusate collection line. Be careful not to over twist as it will twist the cannula and tear the vascular tissue.
32. Switch on peristaltic pump. Set timer to 0m and begin 8min of flush.

Electrode Placement:

33. Locate the hip socket. Using sharp dissection scissors, cut away skin at the hip to exposure muscle.
34. Take sharp dissection scissors and create a proximal-distal opening cut on the fascia line.
35. Using blunt dissection scissors, widen the cut in the muscle with its natural separation space.
36. Push on lower hip to expose a white stipe (sciatic nerve)
37. Hook both positive and negative electrodes around the sciatic nerve. Moisten with saline to ensure the nerve does not dry out.

In-situ Perfusate Collection

38. Carefully move the animal to the perfusion cage
39. Cover body cavity with 4x4 gauze pad soaked in saline. Continue to keep the gauze pad moist during perfusion.
40. After 8m flush, reset timer to 0m. Begin electrical stimulation at 20V increments, increasing to ensure continuous muscle contraction.
41. Following e-stim collection, cease electrical stimulation, set time to 0m and begin post-stim flush.

8.2. Krebs-Heinseliet Buffer Recipe

The following methods are appropriate perfusion procedures in the hemicorpus hind limb perfusion preparation:

Stock Solution Recipe:

1. Measure out the following volumes in a 6L volumetric flask.

NaCl-692.3g	Q.S. to 4L with di H_20 (25x)
KCL-35.34g	Q.S. to 1L with di H_20 (100x)
$CaCl_2$. $2H_20$ -37.35g	Q.S. to 1L with di H_20 (100x)
KH_2PO_4 – 16.19g	Q.S. to 1L with di H_20 (100x)
$MgSO_4$. 7 –H_20 -29.33g	Q.S. to 1L with di H_20 (100x)

Fresh Solution Procedure:

1. Weigh out 2.1g of $NaHCO_3$- per liter (L) of fresh solution, and mix into 500ml of Di H_20 to dissolve.
2. Take $NaHCO_3$- 500ml solution and gas for 30min with 95% ^{02}; 5% CO_2.
3. Prepare Working Buffer Solution while $NaHCO_3$- solution is being gassed.

Working Buffer Solution:

1. Measure out the following volumes in a 1L volumetric flask.
   118.5 mM NaCl-40ml
   4.7 mM KCL-10ml
   3.4 mM $CaCl_2$. $2H_20$-10ml
   1.2 mM KH_2PO_4 – 10 ml
   1.2 mM $MgSO_4$. 7 –H_20 – 10 ml

2. Take the mix with $NaHCO_3$ solution and Q.S. to 1 L (using di H_20)
3. Once working solution is completely mixed, add Fraction V Bovine Serum Albumin (BSA) to obtain 3.5% solution (3.5g/100ml). BSA should be present both in the transport/wash and incubation buffers.
4. Add dextrose to obtain 90-100 mg%
5. Leave solution to mix with stir bar until BSA is completely dissolved (~1h).
6. To store, seal the volumetric flask keep in cold room or fridge at -4C until ready to use

   Note: Shelf life of working buffer is2-3 days at -4C. Transport buffer should be cool but wash buffer should be maintained at 37C for perfusion experiments.

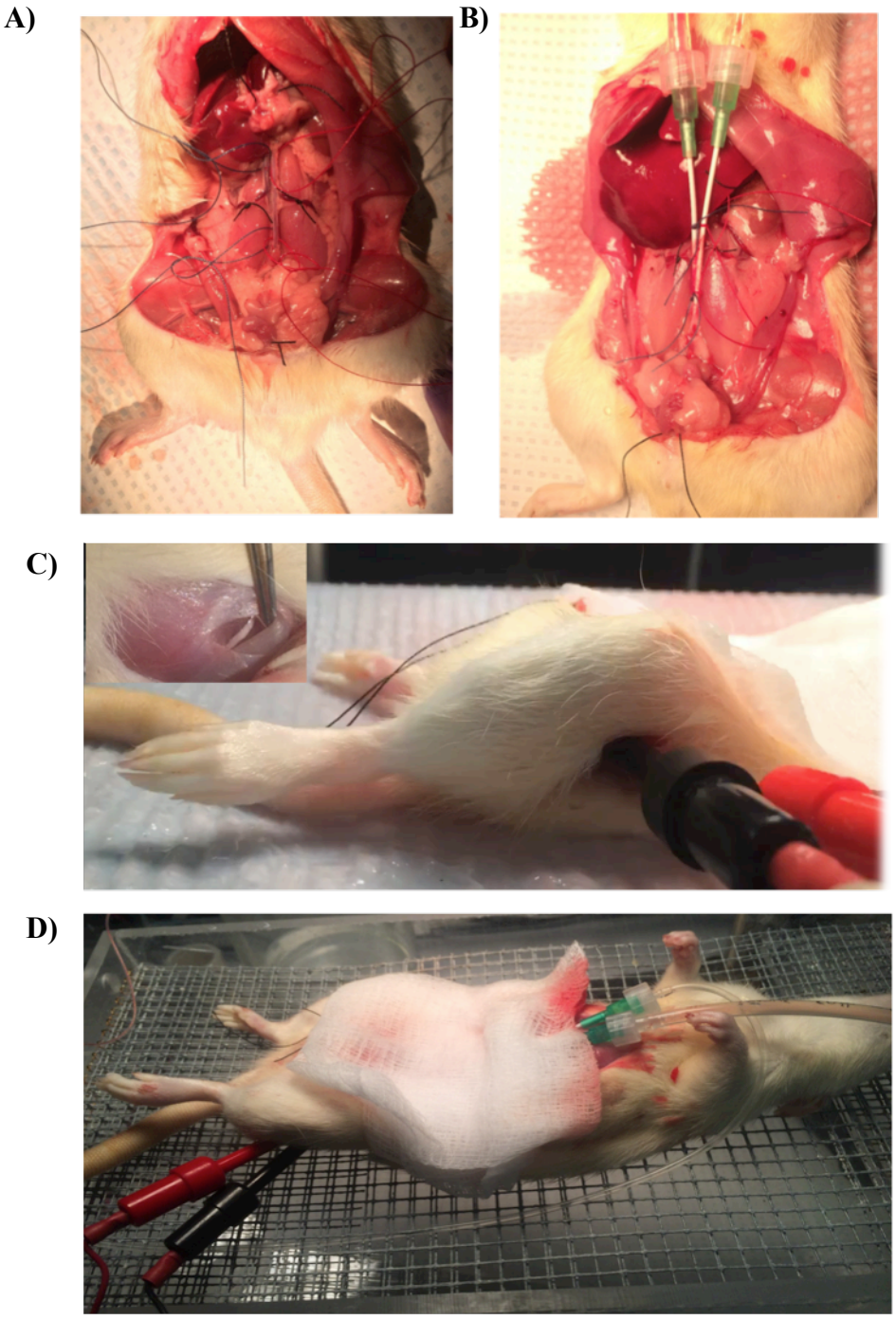


Figure 27. Hemicorpus Perfusion Preparation, Surgical Images
A) Placement of surgical ties, B) Artery and vein cannula placement, C) Sciatic nerve exposure and electrode placement, D) Final perfusion preparation