Muscle Inflammatory Signaling Regulates Eccentric Contraction-Induced Protein Synthesis during Cancer Cachexia

Justin Perry Hardee

University of South Carolina - Columbia

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MUSCLE INFLAMMATORY SIGNALING REGULATES ECCENTRIC CONTRACTION-INDUCED PROTEIN SYNTHESIS DURING CANCER CACHEXIA

by

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2017

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DEDICATION

This dissertation is dedicated to family, friends, and colleagues who have lost a loved one to cancer. May our work provide insight for the improvement of future treatment and management strategies to enhance survival and quality of those diagnosed with cancer.
ACKNOWLEDGEMENTS

I first would like to thank my parents Donnie and Elizabeth Hardee for their unconditional love and support throughout all aspects of life. I would like to extend a special thank you to my sister Ashley and her family (Jamie, Afton, Harper) for the constant inspiration to be better than the day before. I would like to thank Dr. James Carson for his outstanding mentorship and dedication to excellence; these qualities have led me to achieve more than I could have imagined. I would like to thank my dissertation committee members Dr. Ho-Jin Koh, Dr. Xuewen Wang, and Dr. Edie Goldsmith who have given their time and expertise to improve my development as a scientist. I thank them for their patience and guidance. I would like to thank both past and present members of the Integrative Muscle Biology Laboratory who has assisted me throughout my time: Shu Sato, Melissa Puppa, Kandy Velazquez, Aditi Narsale, Song Gao, Kimbell Hetzler, Johannes Aartun, Josh Mangum, Dennis Fix, Brandon VanderVeen, Brittany Counts, Ryan Montalvo. I will cherish the long hours and fun times we have spent together. Funding for this dissertation was supported by National Institute of Health grants R01 CA121249 and P20 RR-017698 (JAC), SPARC Graduate Research Grant from the Office of the Vice President for Research at the University of South Carolina (JPH), and an ACSM Foundation Research Grant from the American College of Sports Medicine (JPH).
ABSTRACT

Cancer-related cachexia is a complex metabolic syndrome that involves the unintentional loss of skeletal muscle mass that cannot be reversed by standard nutritional intervention. Skeletal muscle mass depletion directly impacts patient survival and life quality, and therefore treatments that preserve muscle mass and function may have significant implications for cancer patient treatment and survival. Systemic and muscle inflammation has been implicated in the regulation of skeletal muscle homeostasis, and the cytokine interleukin-6 (IL-6) and muscle gp130 receptor signaling have established roles in cancer-induced muscle wasting. Resistance exercise is a nonpharmacological treatment that can improve physical function and metabolic health in many disease conditions, and has proven beneficial outcomes during cancer patient treatment and survival. Unfortunately, no studies to date have examined the effects of exercise in the cachectic cancer patient, and significant gaps remain in our understanding of the interaction between exercise and the systemic cachectic environment during cancer. Therefore, the purpose of this dissertation was to determine how cancer-induced inflammation affects wasting muscles ability to respond to eccentric contractions (ECC). We hypothesized that chronic muscle inflammatory signaling would attenuate the anabolic response to acute ECC. In experiment 1, we determined if muscle inflammatory signaling regulated the cachectic muscle’s acute anabolic response to ECC. We found that muscle inflammatory signaling regulated basal and ECC-induced protein synthesis. In experiment 2, we determined if systemic IL-6 and muscle gp130 signaling regulated basal and ECC-induced protein
synthesis and mTORC1 signaling. We found that systemic IL-6 signaling regulated basal and ECC-induced protein synthesis. In addition, muscle gp130 could regulate ECC-induced protein synthesis. In experiment 3, we determined if altering muscle inflammatory signaling by training improved basal and ECC-induced protein synthesis and mTORC1 signaling. We found that training could improve basal and ECC-induced protein synthesis. These findings provide initial evidence that cachectic muscle is responsive to acute responses and training adaptations induced by exercise. Additionally, we provide evidence for a potential interaction between muscle inflammation, protein synthesis, and oxidative metabolism, which can be influenced by exercise during cancer cachexia.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AH-130</td>
<td>Yoshida ascites hepatoma AH 130</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>Apc</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CNT</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>COXIV</td>
<td>Cytochrome c oxidase subunit IV</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRP-1</td>
<td>Dynamin-related protein-1</td>
</tr>
<tr>
<td>ECC</td>
<td>Eccentric contraction</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FIS1</td>
<td>Mitochondrial fission protein 1</td>
</tr>
<tr>
<td>FNIII</td>
<td>Fibronectin type III</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead transcription factor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
</tbody>
</table>
GLUT4 ................................................................. Glucose transporter 4
GP130 ................................................................. Glycoprotein 130 receptor
Hz ................................................................. Hertz
IL-1 ................................................................. Interleukin-1
IL-6 ................................................................. Interleukin-6
IL-6 KO ............................................................... Interleukin-6 knockout
IL-6R ............................................................... Interleukin-6 receptor
INFγ ................................................................. Interferon gamma
IOD ................................................................. Integrated optical density
JAK ................................................................. Janus kinase
kDa ............................................................... Kilodalton
LIF ................................................................. Leukemia inhibitory factor
LLC ................................................................. Lewis lung carcinoma
LFES ............................................................. Low-frequency electrical stimulation
MAC16 ............................................................. Murine adenocarcinoma 16
MAPK ............................................................. Mitogen-activated protein kinase
MFN ................................................................. Mitofusin
MIN ................................................................. MIN
mRNA .............................................................. messenger RNA
ms ................................................................. Millisecond
mtDNA .......................................................... Mitochondrial DNA
mTOR ............................................................. mechanistic target of rapamycin
MuRF-1 ........................................................... Muscle RING-finger protein-1
NF-κB .............................................................. Nuclear Factor-kappa B
NRF ................................................................. Nuclear respiratory factor
OPA1 .......................................................................................... Optic Atrophy 1
OSM ........................................................................................... Oncostatin M
PBS .......................................................................................... Phosphate buffered saline
PDTC ........................................................................................ Pyrrolidine dithiocarbamate
PGC-1 .............................................................. peroxisome proliferator-activated receptor coactivator-1
PI3K ........................................................................................ Phosphoinositide 3-kinase
PPAR ....................................................................................... peroxisome proliferator-activated receptors
PVDF ...................................................................................... Polyvinylidene fluoride
RNA ........................................................................................ Ribonucleic acid
SDH ........................................................................................ Succinate dehydrogenase
SDS ........................................................................................ Sodium dodecyl sulfate
STAT3 ................................................................. Signal transducer and activator of transcription-3
TA ............................................................................................. Tibialis Anterior
TBST .................................................................................. Tris buffered saline with tween
TFAM ................................................................. Mitochondrial transcription factor A
TNF-α ................................................................. Tumor necrosis factor-alpha
V ............................................................................................... volts
CHAPTER 1
INTRODUCTION
1.1 INTRODUCTION

Cachexia, a complex metabolic syndrome that involves the unintentional loss of bodyweight that cannot be reversed by nutritional intervention, directly impacts patient survival and life quality (K. Fearon et al., 2011). Cachexia development does not occur with all cancers, but is most prevalent in pancreatic, lung, colorectal, and gastrointestinal cancers (Dodson et al., 2011). The progression of cachexia is directly associated with cancer patient morbidity and mortality. Cancer cachexia accounts for approximately 20% of all cancer related deaths and about 40% of deaths related to colon cancer (Bruera, 1997; Tisdale, 2002). The prevention and treatment of cancer cachexia will have a major impact on cancer patient survival and life quality. While cachexia results in the loss of both skeletal muscle and adipose tissue, maintenance of skeletal muscle mass has proven to be of importance. Given that the maintenance of skeletal muscle mass and metabolic function are critical for health (Wolfe, 2006b), the loss of skeletal muscle mass loss is directly associated with increased mortality.

Skeletal muscle mass depletion associated with cancer cachexia contributes to increased patient morbidity and mortality (K. C. Fearon, 1992; Tisdale, 2009). This muscle wasting is accompanied by the suppression of muscle protein synthesis and the activation of muscle protein breakdown (Samuels et al., 2001; Smith & Tisdale, 1993; White, Baynes, et al., 2011). Skeletal muscle size can be highly plastic and influenced by the dynamic balance between the rates of protein synthesis and breakdown (Murton & Greenhaff, 2010). While our understanding of basal protein breakdown and synthesis has dramatically increased (Bonaldo & Sandri, 2013; Sandri, 2013; Schiaffino & Mammucari, 2011), we have a limited understanding of how the cachectic environment affects wasting muscles.
ability to respond to anabolic stimuli, which is clinically important in the treatment of the cachectic cancer patient.

Protein synthesis control involves the regulation of both translational efficiency and capacity, which can be directed by the mechanistic target of rapamycin complex 1 (mTORC1). The activation of ribosomal protein 6 protein kinase 1 (S6K1), a direct mTORC1 downstream target, participates in cap-dependent translation and ribosomal biogenesis (Bentzinger et al., 2013; Bentzinger et al., 2008; Kimball, Farrell, & Jefferson, 2002; Laplante & Sabatini, 2009). We have previously reported that muscle protein synthesis and mTORC1 signaling is suppressed during the initiation of cachexia, and further decreased during severe cachexia (Bentzinger et al., 2013; Bentzinger et al., 2008; Kimball et al., 2002; Laplante & Sabatini, 2009; White, Baynes, et al., 2011). Muscle 5’-adenosine monophosphate-activated protein kinase (AMPK), a negative regulator of mTORC1, is activated in pre-clinical models of cancer cachexia (Hardee et al., 2016; Puppa, Murphy, Fayad, Hand, & Carson, 2014; White, Baynes, et al., 2011; White, Puppa, Gao, et al., 2013). Additionally, activation of AMPK and signal transducer and activator of transcription 3 (STAT3) by IL-6 overexpression coincides with suppressed muscle protein synthesis and mTORC1 signaling in tumor bearing mice (White, Puppa, Gao, et al., 2013). While these studies highlight a potential role of muscle AMPK and IL-6/STAT3 signaling in the dysregulation of muscle mTORC1 signaling during cachexia, it is currently unknown if suppressed muscle protein synthesis and mTORC1 signaling can be activated in the present of the cachectic environment.

Resistance exercise, consisting of concentric and eccentric contractions, is a potent stimulator of mTORC1 signaling and protein synthesis, and repeated bouts can lead to
muscle hypertrophy in healthy adults (Charette et al., 1991; Chesley, MacDougall, Tarnopolsky, Atkinson, & Smith, 1992; Eliasson et al., 2006). Moreover, resistance exercise has also been demonstrated to attenuate skeletal muscle mass loss associated with various muscle wasting conditions (Alberga et al., 2012; Hardee, Porter, Sidossis, et al., 2014; Sharif et al., 2011). Eccentric contractions (ECC) induced by high-frequency electrical stimulation (HFES) have been used to examine signaling associated with muscle hypertrophy (Baar & Esser, 1999; Y. W. Chen et al., 2002; Nader & Esser, 2001; Witkowski, Lovering, & Spangenburg, 2010). A single bout of ECC can increase mTORC1-dependent signaling related to translational efficiency and ribosomal biogenesis (Jacobs et al., 2013; Nader & Esser, 2001; O’Neil, Duffy, Frey, & Hornberger, 2009; West et al., 2016), and multiple bouts can induce muscle and myofiber growth (Baar & Esser, 1999; Hardee et al., 2016). Related to cancer cachexia, initial evidence suggests skeletal muscle can initiate growth in response to increased loading by synergist ablation or ECC in tumor-bearing mice (al-Majid & McCarthy, 2001; Hardee et al., 2016; Norton, Lowry, & Brennan, 1979; Otis, Lees, & Williams, 2007). Repeated ECC bouts started at the time of C26 tumor implantation can prevent mouse extensor digitorum longus (EDL) muscle protein loss (al-Majid & McCarthy, 2001). While these initial findings suggest cachectic skeletal muscle is responsive to exercise training, further is work is needed to determine if single and repeated ECC bouts can modulate suppressed mTORC1 activity in cachectic skeletal muscle.

Despite the clinical significance of maintaining muscle mass during cancer and the known benefits of exercise, there is currently limited information related to exercise training in the cachectic cancer patient. We have previously found that tumor derived
cachectic factors blocked the mechanical activation of protein synthesis in vitro (Gao & Carson, 2016), but whether this occurs in vivo has not been examined. Further, the interaction between inflammatory signaling and ECC on muscle mass regulation could have important ramifications in future treatments. Therefore, the mechanistic basis of acute exercise responses and training adaptations are being examined in preclinical models of cancer cachexia. The ApcMin/+ (MIN) mouse is an established pre-clinical model of colorectal cancer that develops a slow progression of cachexia, which allows treatments to be performed after significant muscle wasting has occurred (Baltgalvis et al., 2010; Hardee et al., 2016; Narsale et al., 2016; White, Baynes, et al., 2011). We previously found that repeated bouts of ECC after the initiation of cachexia induced myofiber growth despite the presence of a systemic cachectic environment (Hardee et al., 2016). Interestingly, improvements were accompanied by the suppression of muscle inflammatory signaling and chronically activated AMPK. However, the activation of mTORC1 signaling and protein synthesis by ECC was not investigated.

**Overall Premise:** Cancer cachexia is a severe wasting condition associated with chronic inflammation and muscle atrophy. Muscle wasting is accompanied by altered proteostasis and oxidative metabolism, which have been associated with enhanced muscle inflammatory signaling (STAT3, NFkB). Protein breakdown activation through FOXO signaling and protein synthesis suppression by Akt/mTORC1 are thought to be critical for this regulation. While attenuating protein breakdown can attenuate muscle mass loss during cachexia progression, the ability of anabolic stimuli to activate cachectic muscle protein synthesis is not well understood. ECC can produce growth through protein synthesis activation. However, the effect of the cancer-induced cachectic environment on
the anabolic response to muscle contractions has not been well described. Furthermore, cachectic skeletal muscle’s capacity to stimulate protein synthesis and Akt/mTORC1 signaling has not been established.

The **overall purpose** of this study is to determine how cancer-induced inflammation affects wasting muscles ability to respond to ECC. The **central hypothesis** is that chronic muscle inflammatory signaling will attenuate the anabolic response to acute ECC. Our working model is that muscle gp130 receptor signaling will be required for the suppression of ECC-induced protein synthesis through altered mTORC1 activation. First, this study will examine if systemic IL-6 and muscle inflammatory signaling regulate cachetic muscle’s acute anabolic response to ECC. Next, this study will examine if systemic IL-6 and muscle gp130 signaling independent of the cachectic environment can regulate basal and ECC-induced protein synthesis through mTORC1 signaling. Lastly, this study will examine if altering muscle inflammatory signaling by training improves the acute anabolic response to ECC. The MIN mouse, an established preclinical model of colorectal cancer that develops cachexia, will be used to examine the anabolic response to acute ECC during cancer cachexia. Plasma cytokine levels and muscle inflammatory signaling will be manipulated using non-pharmacological (exercise), pharmacological (systemic inhibitors), molecular (plasmid electroporation), and genetic (knock-out) approaches.

**Specific Aim 1 will determine if cachetic muscle inflammatory signaling disrupts the anabolic response to a single ECC bout in MIN mice.** Aim 1.1 will determine cachetic muscle regulation of ECC-induced mechano-sensitive pathways in MIN mice. Aim 1.2 will determine cachetic skeletal muscle regulation of ECC-induced
protein synthesis and mTORC1 signaling in MIN mice. Aim 1.3 will determine muscle STAT3/NFkB regulation of ECC-induced mTORC1 signaling in MIN mice.

**Specific Aim 2 will determine if IL-6 signaling through muscle gp130 independent of the cachectic environment can regulate basal and ECC-induced protein synthesis and mTORC1 signaling.** Aim 2.1 will determine if IL-6 regulates basal and ECC-induced mTORC1 signaling and protein synthesis in B6 mice. Aim 2.2 will determine if IL-6 regulates basal and ECC-induced mTORC1 signaling and protein synthesis in MIN mice. Aim 2.3 will determine if IL-6 signaling through muscle gp130 regulates basal and ECC-induced protein synthesis regulation in tumor-free mice.

**Specific Aim 3 will determine if training regulates ECC-induced protein synthesis in MIN mice.** Aim 3.1 will determine if ECC training alters cachectic skeletal muscle regulation of protein synthesis and mTORC1 signaling in MIN mice. Aim 3.2 will determine if ECC training alters cachectic skeletal muscle regulation of oxidative capacity in MIN mice. Aim 3.3 will determine if training alters cachectic skeletal muscle regulation of ECC-induced protein synthesis and mTORC1 signaling in MIN mice.
Figure 1.1. Overall Working Model. The overall purpose of this study is to determine how cancer-induced inflammation affects wasting muscles ability to respond to ECC. The central hypothesis is that chronic muscle inflammatory signaling will attenuate the anabolic response to acute ECC. Our working model is that muscle gp130 receptor signaling will be required for the suppression of ECC-induced protein synthesis through altered mTORC1 activation. First, this study will examine if muscle inflammatory signaling regulates cachectic muscle’s acute anabolic response to ECC. Next, this study will examine if systemic IL-6 and muscle gp130 signaling can regulate basal and ECC-induced protein synthesis and mTORC1 signaling. Lastly, this study will examine if altering muscle inflammatory signaling by training improves the acute anabolic response to ECC. The MIN mouse, an established preclinical model of colorectal cancer that develops cachexia, will be used to examine the anabolic response to acute ECC during cancer cachexia. Plasma cytokine levels and muscle inflammatory signaling will be manipulated using non-pharmacological (exercise), pharmacological (systemic inhibitors), molecular (plasmid electroporation), and genetic (knock-out) approaches.
CHAPTER 2
REVIEW OF LITERATURE

2.1 CANCER CACHEXIA

Cancer cachexia is defined as a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment (K. Fearon et al., 2011). Cachexia development does not occur with all cancers, but is most prevalent in pancreatic, lung, colorectal, and gastrointestinal cancers (Dodson et al., 2011). The progression of cachexia is directly associated with cancer patient morbidity and mortality. Additionally, cachexia is associated with reduced physical function and tolerance to anticancer therapy. Given that cancer cachexia accounts for approximately 20% of all cancer related deaths and about 40% of deaths related to colon cancer (Bruera, 1997; Tisdale, 2002). The prevention and treatment of cancer cachexia will have a major impact on cancer patient survival and life quality.

Systemic disruption induced by cachexia include systemic inflammation, anorexia or reduced food intake, increased energy expenditure, insulin resistance, anemia, and hypogonadism (K. Fearon et al., 2011; Tisdale, 2010). Physical activity levels are also reduced in cancer patients, and may contribute to systemic wasting processes (Eheman et al., 2012; Irwin et al., 2008; Michaud et al., 2001). These systemic disruptions can promote whole-body wasting through altered metabolism and physical function. While treatments that impede cachexia are important to reduce morbidity and mortality in cancer patients, there are currently no FDA approved treatments for cancer cachexia. This is in part due to the complex nature of the disease and the severity of cachectic symptoms varying between patients (Baracos, 2013). Significant progress has been made in our understanding of cellular pathways regulating wasting during cancer. However, many studies have
examined cachexia prevention in preclinical models, and far fewer studies have been
designed to treat the cachectic condition by initiating treatments after the development of
cachexia, which has clinical significance since many cancer patients are cachectic at the
time of diagnosis (Tisdale, 2009; Wigmore, Plester, Richardson, & Fearon, 1997). Thus,
it remains to be determined if cachexia can be prevented or reversed after significant
wasting has occurred.

Loss of skeletal muscle is a hallmark of cancer cachexia, and muscle mass loss is
directly related to increased morbidity and mortality. Given that the maintenance of
skeletal muscle mass and metabolic function are critical for health (Wolfe, 2006b),
understanding the regulation of muscle wasting is critical for the development of
preventative and therapeutic strategies for the proper treatment of cancer cachexia. Muscle
mass is regulated through an intricate balance between the rates of protein synthesis and
breakdown, termed protein turnover (Kimball et al., 2002; Schiaffino & Mammucari,
2011). While significant progress has been made in our understanding of protein
breakdown during wasting, much less is known related to protein synthesis suppression
during cancer cachexia. It is currently unknown if the cachectic phenotype related to
disrupted protein turnover and oxidative metabolism can be modulated while the cachectic
environment is present.

2.2 MUSCLE MASS REGULATION

In healthy adults, skeletal muscle about 40% of total body weight and represents
the largest protein reservoir (Wolfe, 2006b). The maintenance of skeletal muscle mass is
critical for metabolic health and physical function (Wolfe, 2006b), and the loss of skeletal
muscle mass is associated with increased morbidity and mortality (Levy & Welch, 2015). Skeletal muscle is a highly plastic tissue in which its size can be influenced by the balance between the rates of protein synthesis and degradation, termed protein turnover (Maddocks, Murton, & Wilcock, 2011; Murton & Greenhaff, 2010). The rates of protein synthesis and degradation can be regulated by nutrient status/energy balance, mechanical loading, and growth factors (Kimball et al., 2002). In general, skeletal muscle mass is maintained due to equal rates of muscle protein synthesis and degradation. While circadian fluctuations in the rates of protein synthesis and degradation occur, sustained alterations to either component can lead to alterations in muscle mass (Horstman, Olde Damink, Schols, & van Loon, 2016). Given the importance of skeletal muscle to overall health, understanding the mechanisms regulating protein synthesis and breakdown are critically important for improving cancer cachexia treatments.

2.3 MUSCLE PROTEIN SYNTHESIS REGULATION

2.3.1 Protein synthesis and Akt/mTORC1 signaling

Skeletal muscle protein synthesis is regulated by several factors such as nutrient status, activity level, and inflammation. Protein kinase B (PKB, also known as Akt) and the mechanistic target of rapamycin complex 1 (mTORC1) have established roles for the integration of anabolic signaling initiated by growth factors, nutrients, and mechanical loading to regulate protein synthesis (Kimball et al., 2002; Laplante & Sabatini, 2009; Schiaffino & Mammucari, 2011). In mammals, the mTORC1 complex is composed of four known subunits: raptor (regulatory associated protein of mTOR), PRAS40, mLST8, and mTOR. Raptor acts as a scaffold to recruit downstream substrates such as 4EBP1 and
ribosomal S6 kinase (p70S6K1), to the mTORC1 complex (Nojima et al., 2003; Schalm, Fingar, Sabatini, & Blenis, 2003). Growth factors such as insulin and insulin-like growth factor 1 (IGF1) stimulate mTORC1 signaling through the activation of Akt (Kimball et al., 2002; Schiaffino & Mammucari, 2011). Binding of insulin/IGF1 to its respective cell surface receptor initiates tyrosine kinase activity and phospho-inositol 3-kinase (PI3K)-dependent activation of Akt T308 through phosphoinositide-dependent kinase 1 (PDK1).

The rapamycin-insensitive mTOR complex 2 (mTORC2) has also been implicated in growth factor signaling through the phosphorylation of Akt S473 (Fayard, Tintignac, Baudry, & Hemmings, 2005). Subsequent downstream targets of activated Akt which can control muscle protein synthesis include tuberous sclerosis 2 (TSC2), glycogen synthase kinase-3B (GSK3B), and proline-rich Akt substrate 40 kDa (PRAS40) (Laplante & Sabatini, 2009; Schiaffino & Mammucari, 2011). Akt phosphorylates and inhibits GSK3B, which leads to the activation of eukaryotic translation initiation factor 2B (eIF2B) and protein synthesis initiation (P. Cohen & Frame, 2001; Rommel et al., 2001; Schiaffino & Mammucari, 2011). Inactivating eIF2B stops the initiation of protein synthesis (Proud & Denton, 1997). In addition, Akt can indirectly activate mTORC1 through the phosphorylation of TSC2, which relieves the inhibitory effects of TSC1/2 complex on the Ras homologue enriched in brain (Rheb) activation of mTORC1 (Kimball et al., 2002; Zoncu, Efeyan, & Sabatini, 2011). Mechanical signaling and amino acids can also stimulate mTORC1 through PI3K-independent, but Rheb-dependent mechanisms. Upstream signaling pathways implicated in the mechanical activation of mTORC1 include phospholipase D and extracellular signal-regulated kinases 1/2 (ERK1/2) (Hornberger et al., 2006; Miyazaki, McCarthy, Fedele, & Esser, 2011). While it has long been recognized
that amino acids stimulate protein synthesis in skeletal muscle (Preedy & Garlick, 1986), the precise signaling molecules or interactions responsible for this activation remained elusive. Recently, the localization of mTORC1 to the lysosome has emerged as a critical regulatory point in the activation of mTORC1 by amino acids (Bar-Peled & Sabatini, 2014). While mTORC1 can be found throughout the cytoplasm during amino acid deprivation, the Ragulator-Rag complex can target mTORC1 to the lysosomal surface where it can interact with and be activated by the small GTPase Rheb upon amino stimulation (Sancak et al., 2010). Regardless of the upstream activator, mTORC1 promotes protein synthesis by phosphorylating the eukaryotic initiation factor 4E (4E-BP1) and the p70 ribosomal S6 kinase (S6K1). The hyperphosphorylation of 4E-BP1 prevents binding to eukaryotic initiation factor 4E (eIF4E) and the formation of 4E-BP1-eIF4E complex, resulting in the assembly of the eIF4F complex and translation initiation (Kimball et al., 2002). In addition, S6K1 activation by mTORC1 has been implicated in cap-dependent translation, translation elongation, and ribosomal biogenesis (Bentzinger et al., 2013; Bentzinger et al., 2008; Laplante & Sabatini, 2009). Collectively, these studies highlight the unique role of Akt/mTORC1 signaling in the regulation of muscle protein synthesis.

2.3.2 Protein Synthesis and Cancer Cachexia

While it is well established that muscle protein breakdown is activated during wasting, whether suppressed protein synthesis regulation contributes to muscle mass loss during cachexia is not well known (Combaret, Ralliere, Taillandier, Tanaka, & Attaix, 1999; Llovera et al., 1998). To date, only a few studies have examined muscle protein synthesis and breakdown rates in cancer patients (Deutz et al., 2011; Dillon et al., 2012;
Despite a lack of human studies, it has been concluded that changes in both basal and postprandial muscle protein synthesis and breakdown rates contribute to the muscle wasting observed in cachectic cancer patients (Horstman et al., 2016). While cancer patients demonstrate a muscle protein synthetic response to protein ingestion (Deutz et al., 2011; Dillon et al., 2012; Dillon et al., 2007; Horstman & Sheffield-Moore, 2015; Williams et al., 2012), there is evidence to support that the responsiveness to protein administration is strongly reduced during cancer cachexia (Deutz et al., 2011; Horstman & Sheffield-Moore, 2015; Williams et al., 2012). Thus, this anabolic resistance to feeding attenuates the postprandial rise in muscle protein synthesis and may contribute to the loss of muscle mass observed in cancer cachexia. To circumvent the issues with performing tracer studies in cachectic cancer patients, the majority of data related to muscle protein synthesis has been collected in rodent models of cancer cachexia. Indeed, basal protein synthesis rates are reduced in preclinical models of cancer (Smith & Tisdale, 1993). Our laboratory has routinely demonstrated suppressed muscle protein synthesis throughout the progression of cachexia in the MIN mouse (Narsale et al., 2016; White, Baynes, et al., 2011). During the natural progression of cachexia, muscle protein synthesis is reduced during the initial stages of weight loss (<5% loss) and is further suppressed with the severity of cachexia progression (White, Baynes, et al., 2011). The suppression of muscle protein synthesis corresponds to a reduction in muscle IGF1 expression and mTORC1 signaling, however these alterations in mTORC1 signaling are independent of Akt (White, Baynes, et al., 2011). Interestingly, muscle AMPK activity was not changed during the initiation of cachexia, but was increased during late-stages of cachexia progression (White, Baynes, et
al., 2011). While the administration of the IL-6 receptor antibody was sufficient to attenuate muscle mass loss and AMPK activity, it did not alter the suppressed muscle protein synthesis, mTORC1 signaling, or IGF-1 mRNA expression (White, Baynes, et al., 2011). In contrast to specific IL-6 receptor antibody, short-term pyrrolidine dithiocarbamate (PDTC) treatment improved disrupted protein turnover regulation through the activation of protein synthesis and suppression of protein breakdown (Narsale et al., 2016). To further examine the role of systemic IL-6 on muscle protein synthesis during cachexia, we have also examined protein synthesis regulation by IL-6 in prior to the initiation of cachexia in pre-cachectic MIN mice. Systemic IL-6 overexpression is sufficient to suppress muscle protein synthesis and mTORC1 signaling in pre-cachectic MIN mice (White, Puppa, Gao, et al., 2013). Interestingly, treadmill exercise training was sufficient to block IL-6 suppression of mTORC1 signaling (White, Puppa, Gao, et al., 2013). It is currently unknown whether systemic IL-6 overexpression can reduce Akt/mTORC1 signaling in healthy skeletal muscle independent of cancer. Long-term IL-6 exposure can decrease myotube diameter and mTORC1 signaling in C2C12 cells, and inhibition of AMPK can also relieve IL-6 inhibition of protein synthesis (White, Puppa, Gao, et al., 2013). In the Lewis lung carcinoma (LLC) tumor model, cachexia suppressed the phosphorylation of the mTORC1 substrates 4EBP-1 and S6RP, and this suppression was not affected by skeletal muscle gp130 receptor loss (Puppa, Gao, Narsale, & Carson, 2014). We have also examined C2C12 myotube protein synthesis suppression by LLC tumor-derived factors. We have found that LLC-derived media decreased basal mTORC1 activity and protein synthesis, which coincided with the activation of STAT3 and AMPK (Gao & Carson, 2016; Puppa, Gao, et al., 2014). Interestingly, inhibition of AMPK, but
not gp130 signaling, could restore basal protein synthesis suppression by LLC derived factors (Gao & Carson, 2016). Collectively, these studies highlight that while IL-6/STAT3 signaling is associated with suppressed muscle protein synthesis and mTORC1 signaling, singular inhibition inflammatory signaling pathways is not sufficient to restore basal protein synthesis during the progression of cancer cachexia.

2.4 MUSCLE PROTEIN BREAKDOWN REGULATION

Muscle atrophy involves the reduction in size due to the coordinated loss of proteins, cellular organelles, and cytoplasmic volume (Bonaldo & Sandri, 2013; Sandri, 2013). The ubiquitin-proteasome system and the autophagy-lysosome pathway are the two main proteolytic systems involved in the muscle atrophy process (Sandri, 2013). Other proteolytic systemic implicated in muscle proteostasis include calpains and caspase-mediated proteolysis. Calpains are Ca\(^{2+}\)-dependent cysteine proteases which can cleave cytoskeletal proteins for further breakdown at the proteasome (Huang & Zhu, 2016). In contrast, caspases (cysteine-aspartic proteases) are a family of proteolytic enzymes that are most commonly known for their role in initiating apoptosis (Bell, Al-Khalaf, & Megeney, 2016). The following section will highlight the role of the ubiquitin-proteasome and autophagy-lysosomal systems in protein breakdown.

2.4.1 Ubiquitin-proteasome pathway

The bulk of intracellular proteins are degraded by the ubiquitin-proteasome pathway (Lecker, Solomon, Mitch, & Goldberg, 1999). The ubiquitin–proteasome system includes 3 enzymes that conjugate ubiquitin to intracellular proteins that are then recognized and degraded in the proteasome (Lecker et al., 1999; Sandri, 2013). The
enzymatic components that link ubiquitin chains onto proteins include the E1 (Ub-activating enzyme) and E2 proteins (Ub-carrier or conjugating proteins), which prepare ubiquitin for conjugation. E1 enzymes activate ubiquitin proteins after the cleavage of ATP. The ubiquitin is then moved from E1 to members of the E2 enzyme class. The third enzyme, E3 Ub-protein ligase, is a key factor in terms of the specificity of proteolysis because specific E3 enzymes will recognize a specific protein substrate. E3 enzymes catalyze the transfer of activated ubiquitin to the substrate until a chain of 4–5 Ub's are attached. E3 binds to E2 and the protein substrate, inducing the transfer of ubiquitin from E2 to the substrate. The conjugation reactions form Ub-conjugated proteins which can be recognized by the 26S proteasome. The proteasome removes ubiquitin and degrades the substrate protein into smaller peptides (Bonaldo & Sandri, 2013; Rajan & Mitch, 2008), which can then be used to resynthesize new proteins.

In wasting conditions, two muscle-specific E3 Ub-conjugating enzymes, Atrogin-1 (also known as MAFbx) and MuRF-1, are thought to be critical for the breakdown of muscle proteins (Bodine, Latres, et al., 2001). Indeed, in cultured muscle cells Atrogin-1 mRNA expression correlates closely with rates of protein breakdown (Sacheck, Ohtsuka, McLary, & Goldberg, 2004; Sandri et al., 2004; Stitt et al., 2004). We have also found that IL-6 can modulate Atrogin-1 expression in C2C12 cells (White, Puppa, Gao, et al., 2013). In several models of muscle wasting, Atrogin-1 and MuRF-1 gene expression are dramatically increased and corresponds to periods of rapid muscle atrophy (Bodine, Latres, et al., 2001). Thus, it commonly thought that these E3 mRNAs might prove useful as biomarkers of excessive proteolysis in muscle. Two upstream transcription factors that regulate Atrogin-1 and MuRF-1 expression have been identified, which include FOXO and
NFkB (Sandri et al., 2004; C. L. Wu, Cornwell, Jackman, & Kandarian, 2014). These transcription factors stimulate their expression, and evidence from both human cancer patients and preclinical models support their involvement in atrogene transcriptional regulation associated with muscle wasting (Cai et al., 2004; W. A. He et al., 2013; Judge et al., 2014).

2.4.2 Lysosomal/autophagy

Autophagy plays a crucial role in the turnover of cell components during normal homeostasis and in response to various stimuli such as cellular stress, nutrient deprivation, and muscle contraction (Mizushima, Levine, Cuervo, & Klionsky, 2008). Indeed, there is emerging evidence that the lysosomal-autophagy system plays a critical role during muscle wasting conditions (Asp, Tian, Wendel, & Belury, 2010; McClung, Judge, Powers, & Yan, 2010). Autophagy, specifically macroautophagy in skeletal muscle, is primarily considered to be a non-selective degradation pathway, however more selective removal of specific organelles such as mitochondria by mitophagy is becoming increasingly evident (Bonaldo & Sandri, 2013). Unlike the ubiquitin proteasome, this is an ATP-independent process. Several genes have been identified as autophagy-related which include LC3B, Gabarpl1, Atg12l, PI3kIII, Ulk2, Atg4b and Beclin-1 (Sandri, 2013). Disruption in the expression of these genes can cause deleterious effects, as deletion of Atg7, a critical gene involved in autophagy, results in skeletal muscle atrophy, abnormal mitochondria and disorganization of sarcomeres (Masiero et al., 2009). There is evidence to suggest that the lysosomal/autophagy system is disrupted during cancer cachexia (Asp et al., 2010; Lecker et al., 2004). Moreover, while the molecular components of autophagy/lysosomal pathways have been described, their regulation is not well known.
2.4.3 Upstream signaling regulating protein breakdown

In addition to protein synthesis regulation, Akt can also regulate protein breakdown processes through the phosphorylation and inhibition of the forkhead box O (FOXO) transcription factor. Skeletal muscle expresses three FOXO family members, including FOXO1, FOXO3 and FOXO4, with both FOXO1 and FOXO3a significantly upregulated in cachectic muscles from LLC and C26 tumor-bearing mice (Cornwell, Mirbod, Wu, Kandarian, & Jackman, 2014; Reed, Sandesara, Senf, & Judge, 2012). Moreover, FOXO1 is also upregulated in skeletal muscle of human cancer patients, and was recently identified as a cachexia-associated gene (Skorokhod, Bachmann, Giese, Martignoni, & Krakowski-Roosen, 2012). Akt can phosphorylate FOXO at Thr24, Ser256, and Ser319, which prevents nuclear entry and activation of gene expression (Latres et al., 2005). FOXO has been shown to regulate gene expression related to the ubiquitin proteasome and lysosomal-autophagy systems (Milan et al., 2015; Sandri et al., 2004). AMPK can also stimulate protein breakdown through the regulation of FOXO signaling. AICAR has been shown to increase the mRNA expression of Atrogin-1 and MuRF-1, and the nuclear localization of FOXO in C2C12 myotubes (Tong, Yan, Zhu, & Du, 2009). Additionally, AICAR treatment increased FOXO1 and FOXO3 mRNA expression, and stimulated protein degradation in vitro (Nakashima & Yakabe, 2007). Nonetheless, it is still unclear how AMPK activation can lead to FOXO activation and ubiquitin proteasome protein degradation.

mTORC1 has also been implicated in the regulation of autophagy. Activation of mTORC1 inhibits autophagy through the phosphorylation of multiple autophagy-related proteins that promote autophagy initiation and autophagosome nucleation. mTORC1
inhibits the autophagy-initiating UNC-5 like autophagy activating kinase (ULK) complex by phosphorylating complex components including autophagy related gene 13 (ATG13) and ULK1/2 (C. H. Jung et al., 2009; C. H. Jung, Ro, Cao, Otto, & Kim, 2010). Additionally, mTORC1 can regulate lysosomal and autophagy gene expression by modulating localization of the transcription factor EB (TFEB), as phosphorylation of S142 and S211 results in cytoplasmic sequestration of TFEB, thereby inhibiting transcriptional activity (Rocznia-Ferguson et al., 2012). Thus, it appears modulating suppressed Akt/mTORC1 activity can have significant implications on protein turnover regulation during cancer cachexia.

2.4.4 Protein Breakdown and Cancer Cachexia

It is well established that protein breakdown is elevated during cancer cachexia, which results from both the activation of the ubiquitin proteasome and lysosomal-autophagy systems. We have found strong associations between systemic IL-6, myofiber cross-sectional area, and muscle Atrogin-1 mRNA expression in tumor bearing mice (Baltgalvis et al., 2009). More specifically, systemic IL-6 overexpression is sufficient to decrease myofiber cross-sectional area, which is associated with the induction of Atrogin-1 gene expression in MIN mice (Baltgalvis et al., 2009). Other preclinical models have also observed increased Atrogin-1 expression during severe muscle wasting (Toledo et al., 2016; Toledo, Penna, Busquets, Lopez-Soriano, & Argiles, 2014). Related to the natural progression of cancer cachexia, total protein degradation was increased in mice with initial weight loss, and was further increased throughout cachexia progression (White, Baynes, et al., 2011). The initial activation of protein degradation corresponds to increased ATP dependent degradation, while an increase in both ATP-dependent and –independent protein
degradation activity occurs during severe cachexia (White, Baynes, et al., 2011). During the initiation of cachexia muscle there was an increase in total muscle ubiquitination, E3 ligase expression, and proteasomal subunit expression, which was further increased as cachexia severity increased. In contrast to the ubiquitin proteasome system, autophagy related protein expression (Beclin-1, Atg7, LC3B) was not increased until late-stage cachexia in MIN mice (>5% loss) (White, Baynes, et al., 2011). These findings highlight differential expression related to the ubiquitin and lysosomal-autophagy systems during the progression of cachexia. Interestingly, both of these proteolytic systems could be attenuated with IL-6 receptor antibody treatment (Fujita et al., 1996; White, Baynes, et al., 2011), further establishing a role for IL-6 in the regulation of muscle protein breakdown. In addition to the MIN mouse, we and others have demonstrated enhanced ubiquitin proteasome and lysosomal-autophagy systems in cachectic muscle of C26 and LLC tumor bearing mice (Pin et al., 2015; Toledo et al., 2016; Toledo et al., 2014). Indeed, the expression of a dominant negative FOXO inhibited the cachexia induction of atrogin-1, MuRF1, cathepsin L, and/or Bnip3 gene expression, and inhibited muscle fiber atrophy in LLC tumor bearing mice (Reed et al., 2012). Collectively, these results demonstrate that while the proteolytic systems may be activated during different degrees of cachexia severity in MIN mice, both systems are activated in cachectic skeletal muscle regardless of tumor model.

2.5 MUSCLE OXIDATIVE METABOLISM

Mitochondria oxidative capacity and function are important for the maintenance of muscle mass. The maintenance of healthy mitochondria, which can directly affect muscle oxidative capacity, involves the coordinated processes related to biogenesis, fission, fusion,
and mitophagy (Yan, Lira, & Greene, 2012), and the disturbance to any of these processes can disrupt muscle metabolism and function. Mitochondrial biogenesis is required for the maintenance of newly synthesized mitochondria. The peroxisome-proliferator gamma-activated receptor (PGC-1) family of co-activators has been described as the ‘master regulators’ of muscle oxidative metabolism. PGC-1α regulates mitochondrial biogenesis by nuclear translocation and activation of oxidative gene transcription (Z. Wu et al., 1999), and is a critical regulatory step of mitochondrial biogenesis through the transcriptional control involves mitochondrial proteins, mitochondrial transcription factor A (TFAM), and nuclear respiratory factor-1 (NRF-1) and NRF-2 (Hood, Irrcher, Ljubicic, & Joseph, 2006; Z. Wu et al., 1999). Emerging evidence suggest the fission and fusion of mitochondria, a process known as mitochondrial dynamics, plays a critical role in mitochondrial function (Ding et al., 2010). Mitochondrial fusion proteins Mitofusin-1 and -2 (Mfn1/2) promote mitochondrial elongation and activity, whereas the mitochondrial membrane protein fission-1 (Fis1) promotes mitochondrial fragmentation (James, Parone, Mattenberger, & Martinou, 2003). Maintaining mitochondrial quality requires the removal of damaged mitochondria (Yan et al., 2012), and the selective removal of damaged or dysfunctional mitochondria is known as mitophagy. AMPK/FOXO and mTORC1 signaling have been implicated in the control of several autophagy-lysosomal pathway components (Bonaldo & Sandri, 2013; Sandri, 2013). For example, the transcription factor FOXO can control the expression of autophagy-lysosomal proteins LC3 and Bnip (Bonaldo & Sandri, 2013). Additionally, activation of AMPK induces stimulatory phosphorylation of ULK1 for induction of autophagy, whereas mTORC1 inhibits autophagy through ULK1 phosphorylation (D. Egan, Kim, Shaw, & Guan, 2011). Thus, perturbations to any of these
aforementioned mitochondrial quality control points could lead to the accumulation of damaged mitochondrial, which would ultimately impair muscle oxidative capacity and function.

2.5.1 Muscle Oxidative Metabolism During Cancer Cachexia

Skeletal muscle oxidative capacity is disrupted during the progression of cancer cachexia (White, Baltgalvis, et al., 2011; White et al., 2012). Indeed, several aspects of mitochondrial quality control related to biogenesis, content, fission/fusion, and dynamics are altered during the progression of cachexia (White et al., 2012). We have found a strong relationship between the degree of muscle wasting and the loss of muscle oxidative capacity (White, Baltgalvis, et al., 2011). The suppression of PGC-1α precedes the loss of mitochondrial content (mtDNA) and protein expression (Cytochrome C and COXIV) during the progression of cachexia (White, Baltgalvis, et al., 2011; White et al., 2012). The loss of muscle oxidative capacity parallels skeletal muscle mass atrophy and the disruption of protein turnover (Puppa, Murphy, et al., 2014; White et al., 2012). However, systemic IL-6 receptor inhibition in MIN mice can increase mitochondrial content, PGC-1α expression, and mitochondrial protein expression while attenuating the progression of cancer cachexia (White et al., 2012). Similar effects have also been observed in response to exercise training during systemic IL-6 overexpression in MIN mice (White et al., 2012). Reductions in PGC-1α and mitochondrial fusion proteins Mfn1 and 2 precede the loss of mitochondria during the initial stages of cachexia, while the induction of Fis1 occurs in late cachexia (White, Baltgalvis, et al., 2011; White et al., 2012). In contrast, mitochondrial fission is not induced until severe cachexia (White, Baltgalvis, et al., 2011). Nonetheless, evidence suggest that both mitochondrial fusion and fission are IL-6 sensitive processes.
Systemic IL-6 over-expression decreases Mfn1/2 protein expression and increases Fis1 protein expression in MIN mice (White et al., 2012). Additionally, long-term IL-6 exposure to C2C12 myotubes induces Fis1 protein expression (White et al., 2012). During the natural progression of cachexia, IL-6 receptor (IL-6r) antibody administration after the initiation of cancer cachexia can improve disrupted fusing and fission protein expression in MIN mice (White et al., 2012). Although we have made dramatic progress in the understanding of the regulation of muscle oxidative metabolism during the progression of cachexia, whether skeletal muscle retains the plasticity to improve mitochondrial quality control, and ultimately function, after the initiation of cachexia is still unclear. Given the ability of PGC-1α and mTORC1 signaling axes to regulate muscle oxidative capacity and protein turnover, treatment strategies that can modulate these pathways, such as exercise training, are of potential interest for the attenuation or reversal of skeletal muscle atrophy occurring with cancer.

2.5.2 Muscle Oxidative Metabolism and Exercise During Cancer Cachexia

Exercise training is a potential non-pharmacological treatment to attenuate muscle wasting with cancer, and the metabolic responses to endurance and resistance exercise are distinct. Endurance training is associated with increased mitochondrial density, capillary supply, and key metabolic enzymes in muscle (Holloszy & Booth, 1976). After an acute bout of exercise PGC-1α is rapidly upregulated leading to mitochondrial-associated gene transcription and mitochondrial biogenesis (Baar et al., 2002; Pilegaard, Saltin, & Neufer, 2003). Repeated bouts of exercise increase several mitochondrial proteins such as PGC-1α, mitochondrial transcription factor A (TFAM), and nuclear respiratory factor (NRF), leading to improved muscle oxidative capacity (J. W. Gordon, Rungi, Inagaki, & Hood,
Additionally, endurance exercise can increase Mfn1/2 and Fis1 mRNA, which positively regulates mitochondrial function (Ding et al., 2010). The two primary protein kinases involved in the regulation of PGC-1α in skeletal muscle are AMPK and p38γ mitogen-activated protein kinase (p38γ MAPK) (Akimoto et al., 2005; Yan et al., 2012). Paradoxically, AMPK activity is increased during late-stage cachexia which coincides with the loss of PGC-1α expression and mitochondrial content (White, Baltgalvis, et al., 2011; White, Baynes, et al., 2011). Treadmill exercise training could increase basal PGC-1α and mitochondrial protein expression, suppress the activation of AMPK and FOXO, and reduce autophagy related proteins (Puppa et al., 2012; White et al., 2012). Additionally, we recently found that a single bout of concentric contractions could stimulate PGC-1α expression in cachectic skeletal muscle (Puppa, Murphy, et al., 2014). However, despite the contraction-induced increase of PGC-1α, there was not a corresponding induction in downstream PGC-1α targets NRF1 and TFAM in cachectic muscle (Puppa, Murphy, et al., 2014). These data suggest cachexia disrupts the muscle metabolic response a single bout of concentric contractions. Whether repeated bouts of concentric contractions increase basal PGC-1α expression and improve the suppression of mitochondrial oxidative metabolism has yet to be examined.

Resistance exercise is associated with increased muscle mass, fiber hypertrophy, and strength (B. Egan & Zierath, 2013). Resistance exercise consisting of ECC can induce muscle protein synthesis through the activation of mammalian target of rapamycin complex 1 (mTORC1) and downstream substrates p70S6K and 4EBP-1 (Hornberger & Esser, 2004). In rodents, a single bout of ECC elicits a rapid and sustained activation of p70S6K (Baar & Esser, 1999; Nader & Esser, 2001), and is sufficient to induce muscle hypertrophy.
when repeated ECC bouts are performed (Baar & Esser, 1999). In addition to its role in mTORC1 signaling and protein synthesis, recent evidence suggests that mTORC1 can work in concert with YY1 and PGC-1α to stimulate mitochondrial gene expression and improve oxidative function (Cunningham et al., 2007). PGC-1α protein expression is induced following an acute bout of resistance exercise (Ydfsor et al., 2013), and improved mitochondrial respiratory capacity and mitochondrial complex protein expression have been observed in hypertrophic muscle following 12 wks. of resistance exercise training in humans (Porter, Reidy, Bhattarai, Sidossis, & Rasmussen, 2015). We also have demonstrated that repeated ECC bouts can induce myofiber growth that is associated with improved oxidative capacity during cachexia progression (Hardee et al., 2016), however the effects of ECC on the regulation of mitochondrial quality control and mTORC1 signaling in cachectic muscle has not been investigated.

2.6 MUSCLE INFLAMMATORY SIGNALING

Muscle signaling pathways related to inflammation are disrupted with cachexia progression and appear to have regulatory roles in the wasting process (K. Fearon et al., 2011; Narsale & Carson, 2014). We and others have demonstrated the importance of systemic IL-6 and muscle gp130/STAT3 signaling pathways in the regulation of protein turnover during cancer cachexia (Bonetto et al., 2012; Bonetto et al., 2011; Puppa, Gao, et al., 2014; Seto, Kandarian, & Jackman, 2015). Interestingly, many of these same pathways are regulated by both acute and repeated bouts of exercise (B. Egan & Zierath, 2013). The interaction between the systemic cachectic environment, muscle inflammatory signaling, and muscle contraction has not been well established.
2.6.1 Interleukin-6 / glycoprotein 130 receptor signaling

Interleukin 6 (IL-6) is a pleiotropic cytokine expressed throughout the body. While IL-6 is a 26 kDa protein mainly secreted from T cells and macrophages to produce an immune response, it can be secreted by a variety of cell types including muscle. Depending on the context and duration of exposure, IL-6 has been shown to have both pro-inflammatory and anti-inflammatory properties. IL-6 binds to its specific receptor (interleukin-6 receptor, IL-6R), which then heterodimerizes with the membrane gp130 receptor, forming an active complex to initiate intracellular signaling (Schwantner, Dingley, Ozbek, Rose-John, & Grotzinger, 2004). The glycoprotein 130 (gp130) receptor is a transmembrane receptor for the IL-6 family of cytokines. The interleukin-6 signal transducer (IL6ST) gene, also known as the gp130 receptor, is found on chromosome 5q11, and gp130 protein is ubiquitously expressed in all tissues throughout the body (Rodriguez, Grosgeorge, Nguyen, Gaudray, & Theillet, 1995). Systemic deletion of the receptor is embryonic lethal (Saito, Yoshida, Hibi, Taga, & Kishimoto, 1992; Yoshida et al., 1996). Several different cytokines signal through the gp130 receptor forming either a heterodimer or homodimer with the cytokine, its receptor and gp130. Cytokines within the IL-6 family of cytokines that signal through gp130 receptor include IL-6, IL-11, IL-27, IL-30, IL-31, oncostatin M (OSM), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC) and ciliary neurotrophic factor (CNTF) (Heinrich et al., 2003; Kishimoto, Akira, Narazaki, & Taga, 1995). The gp130 receptor is composed of an Ig-like binding domain and five fibronectin type III (FNIII) repeats on the extracellular portion of the receptor. The first two FNIII repeats form the cytokine binding module. The transmembrane domain is followed by the box1 and box2 regions and the leucine motif...
where JAK/STAT3 activation occurs on tyrosine residues (Heinrich et al., 2003). Mutations in the intracellular region of the gp130 receptor leads to inactivation of the JAK/STAT pathway and IL-6 receptor signaling (Haan, Heinrich, & Behrmann, 2002; Stahl et al., 1994). Downstream signaling pathways activated by IL-6/IL-6R/gp130 include JAK/STAT, RAS/ERK, and PI3K/Akt (Sims & Walsh, 2010). In response to ligand binding and receptor dimerization, constitutively bound Janus family kinases, JAK1, JAK2 or Tyk2, phosphorylate tyrosine residues in the distal cytoplasmic domain of gp130. Phosphorylated gp130 then acts as docking sites for the SH2 domains of the Signal Transducer and Activator of Transcription (STAT) family of transcription factors (typically STATs 1, 3 and 5). Subsequent phosphorylation of STATs then induces STAT dimerization and translocation to the nucleus where they activate gene transcription (Hirano, Ishihara, & Hibi, 2000). Recent evidence also suggests that STAT3 can also enter the mitochondria and regulate oxidative phosphorylation through protein-protein interactions (Gough et al., 2009). In addition to STAT signaling, dimerization of gp130 receptor cytokine receptors can also lead to the activation of MAPK signaling cascades which include ERK1/2, JNK1/2, and p38. The activation of MAPKs by gp130 receptor is mediated through the recruitment of the protein tyrosine phosphatase SHP2 to the gp130 Y759 phosphorylation site, which its recruitment and phosphorylation by JAK. Phosphorylated SHP-2 then interacts with growth-factor receptor bound protein 2 (Grb2), which leads to the activation of the Ras-Raf-ERK1/2 cascade (Heinrich et al., 2003). While IL-6 family of cytokines through gp130 can also induce the phosphorylation and activation stress-activated MAPKs (P38 and JNK), their activation remains poorly understood. Cytokine signaling through gp130 can also lead to the activation of PI3K/Akt. While not
completely understood in skeletal muscle, activation of PI3K signaling has been reported to be involved in the regulation of gp130-dependent hypertrophic signaling in cardiac myocytes (Oh et al., 1998). Interestingly, LIF cytokines stimulation induced JAK1 binds to PI3K, which resulted in enhanced p70 S6 kinase activation and protein synthesis, which would be inhibited by wortmannin and rapamycin (PI3K and mTORC1 inhibitor, respectively). It is currently unknown whether gp130 cytokines can induce PI3K/Akt signaling activation in skeletal muscle cells.

2.6.2 IL-6/gp130/STAT3 Signaling During Cancer Cachexia

Plasma IL-6 levels are elevated in many cachectic conditions associated with wasting. In the MIN mouse model of colorectal cancer, the initiation and progression of cachexia is directly related to tumor burden and circulating IL-6 levels (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Baltgalvis et al., 2009; White, Baltgalvis, et al., 2011). Our laboratory has shown that cachexia severity is directly related to IL-6 and that the inhibition of IL-6 signaling through use of an IL-6 receptor antibody or IL6 knockout mice attenuates/prevents the development of cachexia (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; White, Baynes, et al., 2011). IL-6 can suppress muscle protein synthesis and activate degradation in vivo and in vitro (White, Puppa, Gao, et al., 2013). Plasma IL-6 levels are elevated during the natural progression of cachexia, and systemic IL-6 overexpression can induce cachexia in pre-cachectic MIN mice (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Puppa et al., 2012; White, Puppa, Gao, et al., 2013). Moreover, long-term IL-6 exposure is sufficient to induce atrophy in C2C12 myotubes (White, Puppa, Gao, et al., 2013). The activation muscle STAT3 has been implicated in many preclinical models of cachexia. For example, IL-6 and subsequent muscle signal transducer and
activator of transcription 3 (STAT3) signaling through the gp130 receptor are activated during the progression of cachexia (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Bonetto et al., 2012; Bonetto et al., 2011; White, Baltgalvis, et al., 2011), and either STAT3 inhibition (Bonetto et al., 2012) or gp130 loss can attenuate muscle wasting in some mouse models of cachexia (Puppa, Gao, et al., 2014). During the natural progression of cachexia, inhibition muscle STAT3 signaling can attenuate muscle mass loss in animal models of cancer cachexia (Bonetto et al., 2012; Bonetto et al., 2011). Muscle STAT3 signaling induced by IL-6 overexpression can induce muscle wasting (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Baltgalvis et al., 2009; Bonetto et al., 2012), and the inhibition of muscle protein synthesis signaling (White, Puppa, Gao, et al., 2013). STAT3 inhibition can improve myofiber cross-sectional area in C26 tumor bearing mice (Bonetto et al., 2012; Seto et al., 2015). Furthermore, STAT3 inhibition can block IL-6 induced myotube atrophy in vitro (Bonetto et al., 2012). Additionally, reducing muscle signaling pathways associated with IL-6 can improve protein turnover regulation (Narsale et al., 2016; Puppa, Gao, et al., 2014; Puppa, Murphy, et al., 2014). These studies indicate a need for further investigation of IL-6/STAT3 signaling on protein synthesis during cancer cachexia.

### 2.6.3 STAT3 and Muscle Contraction

While muscle inflammatory signaling and its link to protein degradation regulation have established roles in cancer cachexia-induced muscle wasting (S. Cohen, Nathan, & Goldberg, 2015), these same pathways can also be regulated by muscle contraction or exercise, and have a regulatory role in overload-induced hypertrophy (B. Egan & Zierath, 2013; Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). While muscle IL-6/STAT3 signaling has an important role in the regulation of muscle mass loss with some types of
cancer cachexia (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Baltgalvis et al., 2009; Bonetto et al., 2012; Bonetto et al., 2011; White, Baynes, et al., 2011; White et al., 2012), this pathway has also been implicated in functional overload-induced muscle growth (Serrano, Baeza-Raja, Perdiguero, Jardi, & Munoz-Canoves, 2008; White et al., 2009). However, global IL-6 knockout impedes both myofiber growth and extracellular remodeling in functionally overloaded plantaris muscle (White et al., 2009). Additionally, muscle contraction and functional overload induce IL-6 mRNA expression which may be important for both metabolic and growth processes (Keller et al., 2003; White et al., 2009). However, the IL-6-independent regulation of STAT3 signaling in muscle growth regulation has not yet been clearly established. Functional overload and resistance exercise can increase muscle STAT3 phosphorylation (Begue et al., 2013). Muscle IL-6 mRNA expression is transiently induced by acute exercise (Keller et al., 2003), and treadmill exercise disrupts IL-6-induced regulation of muscle mass loss in MIN mice (Puppa et al., 2012; White et al., 2012). We have previously found that repeated ECC bouts attenuated the cachexia-induced increase in muscle STAT3 phosphorylation in severely cachectic muscle (Hardee et al., 2016), which extends our previous findings that treadmill exercise training can improve muscle mass in the presence of chronically elevated STAT3 signaling (Puppa et al., 2012). Since STAT3 signaling pathways can be induced by both catabolic cachectic stimuli and anabolic muscle contraction, further work is needed to identify the role of contraction-induced regulation of STAT3 signaling during the progression of cachexia.
2.6.4 Adenosine Monophosphate-activated Protein Kinase (AMPK)

5’-AMP-activated protein kinase (AMPK) regulates energy homeostasis (Hardie, 2007), which can be influenced by inflammatory cytokines, energy stress, and muscle contraction. AMPK is a highly conserved heterotrimeric kinase complex composed of a catalytic \( \alpha \)-subunit (\( \alpha \) 1 and \( \alpha \) 2) and two regulatory (\( \beta \)- and \( \gamma \)-) subunits (Ruderman et al., 2010). Further, the \( \beta \) subunit contains a glycogen-binding domain (GBD) and the \( \gamma \) subunit contains four repeat sequences (CBS) that form a pair of Bateman domains responsible for nucleotide binding (Kemp, Oakhill, & Scott, 2007). AMPK containing \( \alpha \) 1 subunit is exclusively cytoplasmic; however, AMPK containing \( \alpha \) 2 subunit is also found in the nucleus (Ruderman et al., 2010). Several upstream kinases that regulate AMPK activity have been identified: serine-threonine liver kinase B1 (LKB1) and calcium/calmodulin kinase kinase-\( \beta \) (CaMKK\( \beta \)) (Ruderman et al., 2010). LKB1 is necessary for AMPK activation by exercise in skeletal muscle (Sakamoto, Goransson, Hardie, & Alessi, 2004). AMPK is activated under conditions of energy stress, when intracellular ATP levels decline and intracellular AMP increase (high intracellular AMP/ATP ratio). Increased binding of AMP to specific domains of the \( \gamma \)-subunit trigger a conformational change that allows upstream kinase to phosphorylate AMPK at threonine residue (Thr172) of the \( \alpha \)-subunit to promote ATP producing catabolic process (Hardie, 2007). Thus, AMPK is an energy-sensing protein that promotes processes that replenish ATP while concurrently inhibiting pathways that consume ATP (Hardie & Sakamoto, 2006). In line with its role in muscle protein turnover and metabolism, AMPK activation can regulate muscle protein synthesis through mTOR inhibition (Bolster, Crozier, Kimball, & Jefferson, 2002), increased muscle-specific ubiquitin ligase expression (Tong et al., 2009), autophagy processes (Kim,
Kundu, Viollet, & Guan, 2011), and mitochondrial biogenesis (Jager, Handschin, St-Pierre, & Spiegelman, 2007). AMPK inhibits mTORC1 through the phosphorylation of TSC2 and Raptor (Gwinn et al., 2008; Inoki, Zhu, & Guan, 2003; Shaw, 2009). The phosphorylation of TSC2 by AMPK inhibits mTORC1 and S6K1 activity (Inoki, Li, Zhu, Wu, & Guan, 2002; Inoki et al., 2003). Moreover, phosphorylation of the mTORC1 binding partner raptor by AMPK at two highly conserved serine residues (S722 and S792) leads to a suppression of mTORC1 kinase activity (Gwinn et al., 2008). Furthermore, treatment with the AMPK activator 5-aminoimidazole-4-carboxamide-1–4-ribofuranoside (AICAR) inhibits mTORC1-mediated signaling and protein synthesis in resting skeletal muscle (Bolster et al., 2002), and protein translation in cultured myotubes (Williamson, Bolster, Kimball, & Jefferson, 2006). AICAR activates AMPK as it is converted to the AMP analog ZMP, thus mimicking an increase in intracellular AMP without disturbing the actual energy status of the cell (Corton, Gillespie, Hawley, & Hardie, 1995). In addition to mTORC1 signaling, AMPK can also activate atrogene expression in skeletal muscle (Tong et al., 2009). The FOXO family of transcription factors regulate the expression of genes related to autophagy and the ubiquitin-proteasome system (Romanello et al., 2010; Sandri, 2013), and AMPK can directly activate FOXO3 independent from Akt (Greer et al., 2007). Further, AMPK has been shown to directly phosphorylate PGC-1α to induce PGC1-dependent gene expression related to glucose metabolism and mitochondrial content (Jager et al., 2007). Collectively, these studies demonstrate that AMPK plays a critical role in the regulation of energy metabolism.

While AMPK is a potent regulator of skeletal muscle metabolism (Jager et al., 2007), its activation is disrupted during cancer cachexia progression. In skeletal muscle,
the metabolic changes induced by AMPK activation are thought to either acute, through direct phosphorylation of metabolic enzymes, or chronic, through control of gene expression (Hardie & Sakamoto, 2006). However, several mouse models of cancer cachexia demonstrate chronically elevated muscle AMPK activity in skeletal muscle (Puppa, Gao, et al., 2014; Puppa, Murphy, et al., 2014; White, Baynes, et al., 2011; White, Puppa, Gao, et al., 2013), which could negatively impact protein and metabolic homeostasis. We have previously found that AMPK is activated during severe cachexia (Hardee et al., 2016; White, Baynes, et al., 2011) or can be induced at earlier time-points with IL-6 overexpression in MIN mice (White, Puppa, Gao, et al., 2013). This activation coincides with the suppression of mTORC1 signaling and activation of ubiquitin proteasome and lysosomal proteolytic systems (White, Baynes, et al., 2011). Interestingly, AMPK inhibition, but not STAT3 inhibition, can rescue IL-6-induced suppression of mTORC1 signaling in myotubes (White, Puppa, Gao, et al., 2013). Unlike healthy skeletal muscle, AMPK activation by cachexia or IL-6 overexpression was not coupled to mitochondrial biogenesis, where muscle oxidative metabolism was suppressed in cachectic muscle (White, Baltgalvis, et al., 2011; White, Baynes, et al., 2011). In healthy muscle, exercise can stimulate AMPK activity and subsequent induction of autophagy and mitochondrial biogenesis (C. He et al., 2012; Hood, 2001). We have found that treadmill exercise at the initiation of cachexia can attenuate AMPK phosphorylation independent of reductions in elevated muscle STAT3 signaling (Puppa et al., 2012). Moreover, repeated ECC bouts suppressed chronically activated AMPK and improved muscle oxidative capacity, independent to STAT3 inhibition (Hardee et al., 2016). Further work is needed to determine the relationship between AMPK and STAT3 signaling for the suppression of
anabolic signaling and muscle oxidative metabolism in cachectic skeletal muscle. Given
the potential role of AMPK in muscle protein and metabolic homeostasis, further work is
required to determine if restoring AMPK activity may be a potential therapeutic target for
muscle wasting syndrome.

2.7 RODENT MODELS OF CANCER CACHEXIA

Approximately 50% of cancer patients will experience progressive wasting of
adipose or skeletal muscle tissue (Tisdale, 2002, 2010). However, there are currently
limited studies that have defined the cachectic response in cancer patients. This is in part
due to the difficulty related to obtaining patient samples, controlling for the type of tumor,
rate and duration of cachexia, and diverse treatment regimens used following diagnosis.
Therefore, several preclinical models have been developed to mechanistically examine the
initiation and progression of cancer cachexia. Genetic and tumor implantation rodent
models have been used to study muscle mass regulation with cancer. In vitro cell culture
models have also been developed to mechanistically define specific cachectic factors and
signaling pathways associated with wasting. While it is acknowledged that many different
preclinical models are available, the following section will highlight mouse and rat models
that have been commonly used by our laboratory and others for the examination of cancer
induced muscle wasting.

2.7.1 Injectable Models of Cancer Cachexia

Injection of cancer cells into mice has become one of the most frequently used
models to study cancer cachexia. The two most commonly used cancer cell lines to
produce cachexia include the colon tumor 26 (C26) and Lewis lung carcinoma (LLC). The
development and utility of these tumor models are briefly discussed. The C26 cell line originated as a chemically induced tumor, while the LLC model was observed naturally occurring. First described in 1975, colon tumors were chemically induced by exposing mice to carcinogens (Corbett, Griswold, Roberts, Peckham, & Schabel, 1975). Of the colon tumors that developed across 82 mice, 4 survived the 1st passage and were serially transplanted. The C26 tumor that developed in a female BALB/c mouse was classified as an undifferentiated grade 4 carcinoma, and demonstrated high metastases to the lungs and other tissues when transplanted. In contrast, the spontaneous LLC tumor cell line was first isolated in 1951 by Dr. Margaret R. Lewis from B6 mouse lung. This model also displays metastasis to the liver and lung tissues. In both models, tumor cells can be grown in culture and then injected into the flank of a recipient mouse. While not commonly employed in cachexia research, donor mice can also be used induce tumor growth and then tumor fragments can be transplanted into the recipient mouse (Aulino et al., 2010). Regardless of delivery method, significant wasting can be achieved within ~14-30 days depending on the cell type (C26 vs LLC), cell culture conditions, and cell phenotype (moderate vs severe) (Judge et al., 2014; Puppa, Gao, et al., 2014; Seto et al., 2015; Toledo et al., 2014). Key features of cachexia observed in humans, which include anorexia, weight loss, adipose and skeletal muscle wasting and increased energy expenditure (K. Fearon et al., 2011), can be recapitulated throughout various time points following implantation. However, the cytokines or cachectic factors associated with cachexia initiation and progression can differ between tumor cell lines. The cytokines IL-6 and LIF are elevated in C26 conditioned culture media or mouse plasma (Pin et al., 2015), whereas increased TNFα and IL-6 levels have been observed in LLC conditioned media or mouse plasma (J. A. Chen et al., 2015).
It should be noted that culture conditions during tumor cell growth (i.e., serum vs serum-free) or tumor-host interactions can alter the cytokine profile in vitro or in vivo, respectively. One common advantage of the LLC model is the utility of genetically modified mice (e.g., knock-out) since B6 mice can be used (Pin et al., 2015; Puppa, Gao, Narsale, & Carson, 2014). However, while BALB/c and CDF1 mice are commonly used in the C26 model, this can be surpassed using molecular approaches such as plasmid electroporation to overexpress or knock-down candidate proteins (Cornwell, Mirbod, Wu, Kandarian, & Jackman, 2014; Judge et al., 2014; Seto, Kandarian, & Jackman, 2015). Both tumor models have been utilized in exercise studies to examine potential effects of endurance and resistance type exercise on muscle wasting processes (al-Majid & McCarthy, 2001; Penna et al., 2011; Pin et al., 2015). Collectively, these two injectable tumor models have greatly enhanced our current understanding of the potential regulators of wasting during cachexia progression.

2.7.3 Genetically engineered mouse models

The MIN mouse is an established model of colorectal cancer and that develops cachexia. This mouse is on a B6 (B6) background, and has a naturally occurring nonsense germline mutation at codon 850 in the Adenomatous polyposis coli (Apc) gene which predisposes mice to multiple intestinal neoplasia (Moser, Pitot, & Dove, 1990). Mutations to the APC gene occurs in approximately 70% of familial adenomatous polyposis (FAP), a dominant inherited syndrome that predisposes individuals to intestinal adenomas. Therefore, this mouse model is commonly used in cancer biology to study the initiation and progression of colon cancer. While the MIN mouse does not fully recapitulate the observed cancer phenotype in humans (i.e., small intestinal polyps vs colon tumors), our
laboratory and others have found this mouse develops a cachectic phenotype (i.e., muscle wasting, high IL-6, hypogonadism) similar to that observed in the human cancer patient (White, Baltgalvis, et al., 2011; White, Baynes, et al., 2011; White, Puppa, Narsale, & Carson, 2013). Furthermore, this model can be advantageous due to the slow onset and progression of cancer cachexia (discussed in detail below).

The MIN mouse develops intestinal polyps beginning as early as 4 weeks of age, and total polyp formation is reached by approximately 12 weeks of age (McClellan et al., 2012; Puppa et al., 2011). The majority of polyps are observed in all intestinal segments (polyp segments), while only a few are observed in the colon. The initiation and progression of cachexia in this mouse is directly related to the intestinal tumor burden and IL-6 levels. Indeed, our laboratory has demonstrated an IL-6 dependent cachexia phenotype between 3 and 6 months of age (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Baltgalvis et al., 2009; White, Baynes, et al., 2011). During the natural progression of cachexia, initial body weight loss occurs at approximately 14 weeks of age, and progressive loss can continue over several weeks (Baltgalvis et al., 2010). This overall wasting corresponds to both adipose and muscle tissue loss. This slow cachexia progression allows treatments to be initiated prior to or after significant wasting has occurred (prevention versus treatment). Indeed, our laboratory has examined both exercise in the prevention of wasting (Baltgalvis, Berger, Pena, Davis, & Carson, 2008; Puppa et al., 2012; White, Puppa, Gao, et al., 2013; White et al., 2012), and the use of pharmaceutical, nutritional, and exercise treatments in the attenuation of cachexia progression (Hardee et al., 2016; Narsale et al., 2016; Velazquez et al., 2014; White, Baynes, et al., 2011).
We have observed strong relationships between tumor burden, circulating IL-6 levels, and cachexia severity during the progression of cachexia (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Baltgalvis et al., 2009; White, Baltgalvis, et al., 2011). Our laboratory has completed several studies demonstrating the importance of plasma IL-6 in the development of cachexia in the male MIN mouse. In our initial studies, we found that while MIN mice lacking IL-6 (MIN; IL-6-/-) did not develop cachexia, wasting could be achieved in these mice when systemic IL-6 was reintroduced by muscle plasmid overexpression (Baltgalvis et al., 2009). We have also found that systemic IL-6 inhibition by a receptor antibody after the initiation of cachexia could attenuate muscle wasting (White, Baynes, et al., 2011). Lastly, elevating systemic plasma IL-6 levels by muscle plasmid electroporation can accelerate cachexia progression in young weight stable MIN mice, which does not occur in polyp free wild-type littermates (i.e., B6). These studies provided strong evidence for the use of the male MIN mouse as an IL-6-dependent model of cancer cachexia. Interestingly, we have found sex differences in cachexia progression. While female mice will eventually develop severe cachexia, the time course and IL-6 dependence on cachexia development differs from the male (Hetzler et al., 2015). For example, IL-6 is not correlated to wasting during cachexia progression, and elevating systemic IL-6 through muscle electroporation does not accelerate cachexia development. We are currently examining the potential sex differences regulating IL-6 sensitivity and cachexia development.

2.7.3 Rat models of cancer cachexia

Similar to mice, several rat models of cancer induced wasting have been developed. Injectable tumor models include methylcholangthrene (MCA) sarcoma cells (Ekman et al.,
1982; Ramos et al., 2004; Ramos et al., 2005), Yoshida AH-130 hepatoma (Busquets et al., 2000; Busquets et al., 2004; Carbo et al., 1997; Muscaritoli et al., 2003; Tessitore, Bonelli, & Baccino, 1987), and Walker 256 carcinosarcomas (Clark & Goodlad, 1975; Goodlad & Clark, 1972, 1973; Goodlad, Tee, & Clark, 1981). These cell lines also recapitulate the cachectic phenotype observed in humans (discussed above), however cachexia initiation and progress is very rapid in these models. Body weight and muscle mass loss can be observed after only seven days of tumor implantation depending on tumor model.

2.7.4 In vitro Models

In vitro models have also been developed to examine alterations in protein turnover and oxidative metabolism in response to various cytokines and tumor-derived cachectic factors. This model employs exposing cultured myoblast and myotubes to individual cytokines or media from cultured tumor cells (Gao & Carson, 2016; Seto et al., 2015). We have found that long-term IL-6 exposure to C2C12 myotubes can recapitulate the cachectic phenotype observed in MIN mice. For example, long-term IL-6 exposure can suppress mTORC1 signaling, activate FOXO signaling, and reduce mitochondrial protein expression (White, Puppa, Gao, et al., 2013). Long-term exposure of leukemia inhibitory factor (LIF), another IL-6 family of cytokine, also induce myotube atrophy through STAT activation in vitro (Seto et al., 2015). Additionally, we have found that C2C12 myotubes preincubated in LLC-derived media promoted atrophy through the suppression of mTORC1 signaling and protein synthesis, which coincided with the activation of muscle inflammatory (STAT3, ERK1/2, P38, NFkB) and AMPK signaling (Gao & Carson, 2016). Similar findings have also been observed using culture media from C26 cells.
Interestingly, C2C12 myotubes treated with C26 conditioned medium induced myotube atrophy that was associated with enhanced STAT transcriptional activation (Seto et al., 2015). These important findings in vitro could be replicated in vivo, as STAT3 inhibition in skeletal muscle has been shown attenuate myofiber atrophy in vivo (Bonetto et al., 2012; Seto et al., 2015). Collectively, these studies highlight that key cytokine and intracellular signaling pathways implicated in cancer induced wasting can be mechanistically determined using this model system.

### 2.8 Rodent Models of Physical Activity, Exercise and Muscle

#### Contraction

**2.8.1 Voluntary cage activity**

It is well established that physical activity levels can have a significant role in the etiology of cancer. While physical inactivity has been well documented with cancer cachexia, it is often characterized as an outcome of wasting and not a contributor to the process. The two most commonly employed methods to examine physical activity levels in rodents include general cage monitoring and running wheels. Several customized models of cage monitoring systems are available to examine daily spontaneous physical activity. In general, rodent cages are placed in fitted racks that contain infrared photobeams. Spontaneous physical activity can be detected and measured by infrared arrays in the x-, y-, and z-axis. Physical activity across x- and y-axis allow the determination of ambulatory (locomotor activity), while the z-axis allows for determination of vertical movement (rearing or standing). The running wheel can also be used to assess levels of physical activity. In general, healthy rodents with access to a wheel
will engage in physical activity. Therefore, the running wheel has been used to study spontaneous activity, but also to examine the effects of exercise training. It should be noted that access to running wheel can alter physical activity and behavior patterns, which could influence the study outcome and has been discussed elsewhere (Novak, Burghardt, & Levine, 2012). Nonetheless, when the rodent is engaging in physical activity the number and velocity of revolutions of the wheel can be simultaneously quantified through computer or other devices. Regardless of method, physical activity patterns can be monitored during specific times of day (light vs dark cycle) or across time (pre- and post-treatment). Therefore, careful monitoring of spontaneous cage activity over time could help to reconcile the potential contribution of physical inactivity or muscle disuse as contributing to the cancer-induced wasting.

2.8.2 Voluntary Cage Activity during Cancer Cachexia

As previously highlighted, the potential interaction between muscle use and the systemic environment could have significant implications on muscle mass regulation during cancer. Related to our previous discussion, access to a wheel could alter cachexia progression through serving as a training stimulus compared to an animal without wheel access. Indeed, several indices of cachexia progression are attenuated in rodents given access to wheel. While voluntary wheel running increased over time in C26 tumor bearing mice, it was reduced compared to non-tumor bearing controls (Coletti et al., 2013). Nonetheless, wheel running has been shown to attenuate muscle mass loss and improve myofiber cross sectional area across multiple studies in C26 mice (Coletti et al., 2016; Pigna et al., 2016). Moreover, these studies have demonstrated improvements in ex vivo force (Pigna et al., 2016) and reduced muscle inflammatory signaling related to NFkB
activation (Coletti et al., 2016). More clinically relevant, there was a positive correlation between running distance and survival days (Pigna et al., 2016). Thus, it appears that participating in voluntary exercise can have significant clinical and functional ramifications in tumor bearing animals.

2.8.3 Treadmill Exercise

Treadmill exercise is one of the most commonly used methods to increase physical activity levels and induce exercise training adaptations in rodents. The overall concept of treadmill exercise training in rodents is similar to humans in that varying speeds and inclines during the exercise bout can be achieved. An advantage to this model is that a specific, quantifiable dose can be delivered to the rodent. However, a disadvantage is that the exercise is forced and can be stressful to the animal. Nonetheless, instrumental information related to the mechanisms associated with the acute responses and chronic adaptations to endurance exercise have been identified using this exercise model. In addition to the acute response to exercise, treadmill run to fatigue test is also commonly utilized to examine exercise capacity. Treadmills can also be coupled to respiratory chambers that allow for the determination of oxygen consumption during exercise. These systems have been used to examine resting, submaximal, and maximal oxygen consumption. Similar to spontaneous physical activity, this can provide valuable information related to phenotype alterations throughout lifespan or treatment.

2.8.4 Treadmill Exercise During Cancer Cachexia

Treadmill exercise training is a potent stimulus to improve whole body metabolism. Our laboratory has examined treadmill exercise training before (<12 wks. of age) or after
(~12wks of age) intestinal polyp formation in MIN mice. We have found that treadmill exercise training (6 days/wk., 9wks) decreased crypt depth: villus height ratio (CVR) in 13 wk. male and female MIN mice prior to body weight loss (Mehl, Davis, Clements, et al., 2005). Additionally, training decreased total poly number and large size polyps (>1 mm) in male MIN mice (Mehl, Davis, Clements, et al., 2005). The potential mechanisms for reduced poly formation by exercise training include reductions in immune cell infiltration, apoptosis, and beta-catenin signaling (Baltgalvis, Berger, Pena, Davis, & Carson, 2008). In addition to polyp formation, exercise training prior to and during IL-6 overexpression could prevent body weight and muscle mass loss, improved systemic glucose and fatty acid metabolism, increased muscle oxidative metabolism, and restored mTORC1 signaling in MIN mice (Puppa et al., 2012; White, Puppa, Gao, et al., 2013). Other laboratories have also reported treadmill exercise prior to tumor injection and during treatment improved survival rate and prevented/delayed muscle mass loss in metastatic C26 tumor model (Jee, Chang, & Yang, 2016). However, not all studies have reported attenuated muscle mass loss by exercise training in tumor implantation models (Pin et al., 2015), however training was sufficient to improve muscle strength and oxidative capacity depending on the tumor model (Pin et al., 2015). Collectively, there is sufficient evidence to suggest that exercise training prior to and during cachexia progression can attenuate muscle mass and strength loss, which may be related to improved oxidative metabolism and protein synthesis.

2.8.5 Synergist Ablation/Compensatory Hypertrophy

Compensatory overload of synergist muscles is commonly used to study muscle growth and the mechanical signals activating transcription and translation. Two commonly used models of compensatory overload include tenotomy of synergist and synergist
ablation. Regardless of the model, the synergist muscle is inactivated by tenotomy or removal and the remaining muscle compensate for the increased load, which leads to a robust hypertrophic growth response. First described by Goldberg, tenotomy (i.e., severing of tendon) of the gastrocnemius muscle redistributes load to the synergist soleus and plantaris muscles and promotes rapid muscle growth (Goldberg, 1967, 1968). Disadvantages to this model include rapid edema and inflammatory induction during the initial phase of loading resumption, and tendon reattachment during long-term loading. In contrast, the synergist ablation model places functional overload to the muscle by surgical removal of the synergist muscle. The distal one-third of the gastrocnemius and soleus muscles are removed leaving the plantaris to bear load upon normalization of ambulatory activity (McCarthy et al., 2011). The soleus can also be left intact to examine the loading responses in oxidative versus glycolytic muscle (Carson, Nettleton, & Reecy, 2002; Kilikevicius, Bunger, & Lionikas, 2016; W. J. Lee, McClung, Hand, & Carson, 2003). Similar to tenotomy, this model is associated with edema and inflammation infiltration during reambulation, however significant muscle hypertrophy occurs following this initial phase. The improvements in muscle mass are accompanied by increased myofiber cross-sectional area and the accretion of protein and RNA content (Miyazaki et al., 2011). While the compensatory overload model allows the mechanistic investigation of a robust hypertrophic response, whether these findings directly reflect what occurs in humans with resistance training should be considered.

2.8.6 Synergist Ablation/Compensatory Hypertrophy during Cancer Cachexia

While compensatory hypertrophy is an excellent model to examine muscle growth, only a few studies have utilized this in tumor bearing animals. Of these studies, only two
have examined them in cachectic conditions. One of the first pioneering studies found that regardless of overall tumor burden, a comparable growth response was observed tumor- and non-tumor bearing animals (Norton et al., 1979). Interestingly, this study utilized both prevention and treatment perspectives (i.e., overload initiated at or 1-2 wks. post-tumor inoculation), demonstrating that muscle growth could be achieved when loading was initiated at varying tumor burdens. While the next study did not observe cachexia due to the tumor utilized, the authors also found that increased muscle loading could promote muscle growth in tumor-bearing animals (Jaweed, Herbison, Miller, & Ditunno, 1983). These two studies demonstrated for the first time that muscle could respond to increased workload despite the presence of a tumor. However, both studies only examined the initial growth response following 1wk of tenotomy. Therefore, a more recent study examined if long-term loading could prevent muscle wasting (Otis et al., 2007). The authors found that muscle retained the plasticity to adapt to mechanical loading despite significant wasting that occurred with cancer. Collectively, these studies demonstrated that tumor-bearing rodents were responsive to increased loading, and that an initial growth response to mechanical loading was maintained in a cachectic environment.

2.8.7 ECC-induced by High-Frequency Electrical Stimulation

When examining mechanisms related to severe cachexia, exercise feasibility becomes an issue as severely cachectic mice are not capable of performing vigorous exercise training. While not entirely exercise, skeletal muscle’s metabolic and growth response to contraction has been examined using electrical muscle stimulation (Baar & Esser, 1999; Nader & Esser, 2001; Puppa, Murphy, et al., 2014; Witkowski et al., 2010). Electrical stimulation is performed in an unconscious animal, in which electrodes are used
to stimulate a nerve at a specific frequency, thus resulting in contraction of the innervated muscle. Low-frequency electrical muscle stimulation (LFES) has been used to examine the acute responses and training adaptations to endurance like muscle contractions (Nader & Esser, 2001; Patel, Cuizon, Mathieu-Costello, Friden, & Lieber, 1998; Puppa, Murphy, et al., 2014). Related to growth promoting stimuli, high-frequency electrical muscle stimulation (HFES) has examined muscle signaling associated with hypertrophy (Baar & Esser, 1999; Y. W. Chen et al., 2002; B. S. Gordon, Steiner, Lang, Jefferson, & Kimball, 2014; Jacobs et al., 2013; Nader & Esser, 2001; Steiner, Fukuda, Rossetti, Hoffman, & Gordon, 2017; Witkowski et al., 2010). Initial experiments performed by Wong & Booth demonstrated that HFES could induce muscle hypertrophy of the eccentrically contracted tibialis anterior (TA) muscle, while this growth response was not observed in the concentrically contracted gastrocnemius muscle (Wong & Booth, 1988). In this contraction model, the maximal tension generated by the plantar flexors is greater than that of the dorsiflexors, resulting in net plantar flexion of the ankle. This stimulation protocol results in an initial maximal ECC of the dorsiflexor muscles (TA and EDL) due to the greater overall force production of the simultaneously contracting plantar flexors (gastrocnemius, plantaris, and soleus muscles). This is followed by a period of maximal isometric contraction in a lengthened position at full plantar flexion. To date, several research groups have utilized this model to examine the growth response to a single bout of high-force contractions, and have used the same stimulation parameters (Baar & Esser, 1999; Y. W. Chen et al., 2002; B. S. Gordon et al., 2014; Jacobs et al., 2013; Nader & Esser, 2001; Steiner et al., 2017; Witkowski et al., 2010). With these parameters the sciatic nerve is stimulated at 100 Hz in 3-s pulses with a 10-s rest period between repetitions. An
additional 50-s rest is given after the 6th repetition. This cycle of stimulation is continued for 10 sets of 6 stimulations, resulting in 60 contractions over a 22-min period. This protocol has been shown to induce a robust protein synthesis and mTORC1 signaling response in rodent skeletal muscle (Baar & Esser, 1999; Y. W. Chen et al., 2002; B. S. Gordon et al., 2014; Jacobs et al., 2013; Nader & Esser, 2001; Steiner et al., 2017; Witkowski et al., 2010). Moreover, the initial findings revealed that electrical stimulation could produce muscle hypertrophy without external loading (i.e., weighted resistance), since the TA muscle contracted eccentrically against the tension generated by the concentrically contracted gastrocnemius muscle (Baar & Esser, 1999; Wong & Booth, 1988). The major advantage of eliciting muscle contractions via electrical stimulation is related to control of training parameters. For example, variables such as stimulation frequency and duration, the percent of maximal muscle strength generated per contraction, the number of contractions, and rest periods between contractions could be manipulated based on the desired outcome. Other advantages to this model of stimulated muscle contractions include: (a) all motor units are recruited during contraction, (b) contraction is isolated to the muscles innervated by the nerve, (c) contraction is performed involuntary in an unconscious animal (e.g., not dependent on animal motivation), and (d) the contralateral, non-stimulated leg can serve as an intra-animal control. Thus, this model has several advantages in conditions of severe cachexia or chronic disease when voluntary exercise cannot be easily performed. Several disadvantages to this model include: (a) repeated anesthesia, (b) size principal of recruitment pattern not followed, (c) isolated muscle contractions versus whole-body exercise (e.g., concentric and eccentric muscle actions), and (d) repeated bout effects are difficult to determine. Nonetheless, given that an identical
systemic cachectic environment is present between the stimulated and non-stimulated muscles, this can greatly enhance the interpretation of the isolated muscle contraction response in the diseased state.

2.8.8 ECC-induced by HFES during Cancer Cachexia

While the initial synergist ablation studies provided insight into our understanding of muscle loading with cancer, the cachectic muscle response to increased use had yet to be fully resolved. Given that severely cachectic demonstrate reduced activity and are unable to perform exercise training, our laboratory and others have examined this response using electrical stimulation in the unconscious animal (al-Majid & McCarthy, 2001; Hardee et al., 2016; Puppa, Murphy, et al., 2014). We have previously found that cachectic muscle’s anabolic and metabolic response to a single bout of concentric muscle contractions was disrupted in MIN mice (Puppa, Murphy, et al., 2014). Interestingly, inhibition of systemic inflammation related to NF-κB and STAT3 signaling was sufficient to rescue contraction induced anabolic, but not metabolic signaling in cachectic muscle (Puppa, Murphy, et al., 2014). It is currently not known if multiple bouts of exercise or contraction improve the metabolic response of cachectic muscle to an acute bout of contraction. Related to muscle growth by ECC, the first study performed utilized a preventative design and demonstrated that ECC could maintain mass and protein content in the contracted EDL muscle of C26 tumor bearing mice. This was the first study to demonstrate that isolated muscle contractions could preserve mass in the presence of a systemic cachectic environment, evident by body weight loss and wasting of the non-stimulated muscle mass (al-Majid & McCarthy, 2001). Given the importance of treating the cachectic patient, our laboratory performed a similar experiment but initiated training
after the mice had developed cachexia to determine if the muscle was still responsive. Despite significant wasting that occurred with cachexia progression (body weight loss, high circulating IL-6, muscle wasting), these mice responded to training by increasing myofiber cross-sectional area and improving oxidative capacity (Hardee et al., 2016). These changes were accompanied by reduced AMPK activation and inflammatory signaling (Hardee et al., 2016). It is currently unknown whether these benefits reflect changes to improved growth or the attenuation of wasting. Moreover, whether these contractions were sufficient to increase mTORC1 signaling and improve basal protein synthesis has not yet been explored. Nonetheless, these results further establish that tumor-bearing rodents are responsive to increased use by ECC, and that the cachectic muscle can respond to increased loading despite the presence of a systemic cachectic environment.

2.8.9 In-vitro stretch

While increased mechanical loading can induce a potent anabolic response in vivo, muscle’s sensitivity to chronic or intermittent stretch has also been examined in cultured myotubes (Carson & Wei, 2000). Several in vitro stretch models have been developed to examine the muscle’s growth response to mechanical stimuli, and the signaling response to each type of mechanical strain are distinct (Hornberger, Armstrong, Koh, Burkholder, & Esser, 2005). Intermittent, cyclic stretch and chronic, static stretch are two models to study the response to repeated contractions or loading, respectively. Many of the biochemical and physiological responses to these paradigms are similar to that observed in vivo, which has vastly improved our understanding of the interactions between mechanical signaling and growth induction.
2.8.10 In-vitro stretch and Cachectic Factors

While significant understanding of key signaling pathways regulating stretch-induced growth have been gained using cultured myotubes, much less is known about the interaction between stretch and cachectic tumor derived factors. Therefore, recent studies have been performed in which individual cytokines or medium containing tumor derived factors were cultured with stretched myotubes. Our laboratory has found that C2C12 myotubes preincubated in LLC-derived media demonstrated an attenuated stretch induction of protein synthesis (Gao & Carson, 2016). The suppressed induction of protein synthesis by stretch was accompanied by the LLC activation of inflammatory (STAT3, ERK1/2, P38, NFkB) and metabolic (AMPK) signaling pathways. Interestingly, the inhibition of ERK1/2 and p38 rescued the stretch induction of protein synthesis, while AMPK inhibition increased basal mTORC1 signaling activity and protein synthesis in LLC-treated myotubes (Gao & Carson, 2016). We are currently examining the potential role of individual cytokines on the basal and stretch induction of myotube protein synthesis and mTORC1 signaling.
CHAPTER 3

INFLAMMATORY SIGNALING REGULATES ECCENTRIC

CONTRACTION-INDUCED PROTEIN SYNTHESIS IN CACHECTIC

SKELETAL MUSCLE

3.1 ABSTRACT

Skeletal muscle responds to eccentric contractions (ECCs) with an anabolic response that involves the induction of protein synthesis through mTORC1. While we have reported that repeated ECC bouts after cachexia initiation can attenuate muscle mass loss and inflammatory signaling, cachectic muscle’s capacity to induce protein synthesis in response to ECC has not been determined. Therefore, we examined cachectic muscle’s ability to induce mechano-sensitive pathways and protein synthesis in response to an anabolic stimulus involving ECC, and determined the role of muscle STAT3/NFκB signaling on ECC-induced anabolic signaling. Mechano-sensitive pathways and anabolic signaling were examined immediately post or 3h after a single ECC bout in cachectic male MIN mice (N=17; 16 ± 1% body weight loss). Muscle STAT3/NFκB regulation of basal and ECC-induced anabolic signaling was also examined in an additional cohort of MIN mice (N=10; 16 ± 1% body weight loss) that received pyrrolidine dithiocarbamate 24h prior to a single ECC bout. In all experiments, the stimulated tibialis anterior (TA) performed ECC while the non-stimulated TA served as intra-animal control. Data were analyzed by Students t-test or two-way repeated measures ANOVA with Student-Newman-Keuls post hoc when appropriate. The accepted level of significance was set at p<0.05 for all analysis. MIN mice exhibited a cachectic muscle signature demonstrated by perturbed proteostasis (RPS6, P70S6K, Atrogin-1, MuRF1), metabolic (AMPK, PGC-1α, COXIV), and inflammatory (STAT3, NFκB, ERK1/2, P38) signaling pathway regulation. Nonetheless, mechano-sensitive signaling pathways (P38, ERK1/2, AKT) were activated immediately post-ECC irrespective of cachexia. While cachexia did not attenuate ECC-induced P70S6K activation, the protein synthesis induction remained suppressed compared
to healthy controls. However, muscle STAT3/NFκB inhibition increased basal and ECC-induced protein synthesis in cachectic MIN mice. **Conclusions:** These studies demonstrate that mechano-sensitive signaling is maintained in cachectic skeletal muscle, but chronic STAT3/NFκB signaling serves to attenuate basal and ECC-induced protein synthesis.

Keywords: MIN; Cancer Cachexia; Eccentric contractions, Interleukin-6; Muscle Protein Synthesis

### 3.2 INTRODUCTION

Skeletal muscle mass depletion associated with cancer cachexia contributes to increased patient morbidity and mortality (K. C. Fearon, 1992; Tisdale, 2009). Skeletal muscle size is influenced by the dynamic balance between the rates of protein synthesis and breakdown (Maddocks et al., 2011; Murton & Greenhaff, 2010), and disrupted protein turnover accompanies cancer cachexia (Samuels et al., 2001; Smith & Tisdale, 1993; White, Baynes, et al., 2011). While our understanding of suppressed basal protein synthesis and activated breakdown during cachexia has increased dramatically (Bonaldo & Sandri, 2013; Sandri, 2013; White, Baynes, et al., 2011), we have a more limited understanding of how the cachectic environment affects skeletal muscle responsiveness to anabolic stimuli, which is clinically relevant for the treatment of the cachectic cancer patient. Resistance exercise is a potent anabolic stimulus that stimulates muscle hypertrophy through the activation of mechanistic target of rapamycin complex 1 (mTORC1) in healthy adults (Charette et al., 1991; Chesley et al., 1992; Eliasson et al., 2006), and can attenuate skeletal muscle mass loss in several muscle wasting conditions.
(Alberga et al., 2012; Hardee, Porter, Sidossis, et al., 2014; Sharif et al., 2011). Despite the clinical significance of maintaining or improving muscle mass during cancer, limited information currently exists on the cachectic muscle’s anabolic response to resistance exercise.

ECC-induced by high-frequency electrical stimulation have been used to examine signaling associated with muscle hypertrophy in rodents (Baar & Esser, 1999; Y. W. Chen et al., 2002; Nader & Esser, 2001; Witkowski et al., 2010), and have demonstrated great utility for improving our mechanistic understanding of contraction-induced protein synthesis and mTORC1 signaling (Jacobs et al., 2013; Nader & Esser, 2001; O'Neil et al., 2009; West et al., 2016). Related to cancer cachexia, evidence suggests that increased loading by synergist ablation or ECC can maintain muscle mass in tumor-bearing mice (al-Majid & McCarthy, 2001; Hardee et al., 2016; Norton et al., 1979; Otis et al., 2007). Indeed, we have reported that repeated ECC bouts after the initiation of cachexia can attenuate muscle mass loss through reduced inflammatory signaling (Hardee et al., 2016); however, cachectic muscle’s capacity to induce protein synthesis in response to ECC has not been determined and warrants further investigation. We have found that mechano-activation of protein synthesis in stretched myotubes can be disrupted by conditioned media from Lewis lung carcinoma (LLC) cells (Gao & Carson, 2016), suggesting that tumor-derived cachectic factors can interfere with mechanical signaling inducing protein synthesis in vitro. While these studies demonstrate that skeletal muscle from tumor-bearing animals may be responsive to exercise training or loading, the regulation of protein synthesis by muscle contraction in the presence of a systemic cachectic environment requires further investigation.
Suppressed muscle protein synthesis and mTORC1 signaling are associated with interleukin-6 (IL-6) induction of signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and 5’-adenosine monophosphate-activated protein kinase (AMPK) in tumor-bearing mice (Narsale et al., 2016; White, Puppa, Gao, et al., 2013). Indeed, we have previously demonstrated an inverse relationship between plasma IL-6 and muscle protein synthesis during cachexia progression, and systemic IL-6 overexpression can suppress mTORC1 signaling in tumor-bearing mice (White, Puppa, Gao, et al., 2013). In contrast, blocking muscle IL-6 signaling through STAT3 inhibition or glycoprotein 130 (gp130) receptor loss attenuated wasting in tumor-bearing mice (Bonetto et al., 2012; Puppa, Gao, et al., 2014). Additionally, acute and chronic muscle STAT3/NFκB inhibition improved mTORC1 signaling in cachectic mice (Narsale et al., 2016; Puppa, Murphy, et al., 2014). Lastly, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and P38 mitogen activated protein kinase (MAPK) inhibition restored myotube stretch-induced protein synthesis in the presence of LLC derived cachectic factors (Gao & Carson, 2016). Thus, there is a clear rationale that muscle inflammatory signaling involving STAT3/NFκB can disrupt basal and mechanical-induced regulation of protein synthesis during cancer cachexia. However, it is currently unknown if suppressed muscle protein synthesis and mTORC1 signaling can be activated in the cachectic environment. Therefore, we examined cachectic muscle’s ability to induce protein synthesis in response to an anabolic stimulus involving ECC, and determined the role of muscle STAT3/NFκB signaling on ECC-induced anabolic signaling. Interestingly, we report that mechano-sensitive signaling is maintained in cachectic
skeletal muscle, but STAT3/NFκB signaling attenuates basal and ECC-induced protein synthesis.

3.3 METHODS

Animals

Male MIN mice on a B6 background were originally purchased from Jackson Laboratories and bred at the University of South Carolina’s Animal Resource Facility. Mice used in the current study were obtained from the investigators breeding colony in the Center for Colon Cancer Research Mouse Core. Mice were individually housed, kept on a 12:12-h light-dark cycle, and had access to standard rodent chow (cat#8604 Rodent Diet; Harlan Teklad) and water ad libitum. Body weight and food measurements were taken weekly, and the percentage body weight loss from peak body weight was calculated. Mice lacking the Apc mutation (B6) served as controls for all experiments. The University of South Carolina’s Institutional Animal Care and Use Committee approved all animal experimentation in this study.

Experimental Designs

Male B6 (N=15) and MIN (N=27) mice (20 weeks of age) were used to determine cachectic muscle’s ability to induce mechano-sensitive pathways and protein synthesis in response to a single ECC bout. In the first experiment, B6 (N=6) and MIN (N=7) mice were sacrificed immediately following a single ECC bout. In the second experiment, B6 (N=9) and MIN (N=10) mice were sacrificed 3h after a single ECC bout. In the third experiment, an additional cohort of cachectic MIN mice (N=10; 16 ± 1% body weight loss) received a single pyrrolidine dithiocarbamate (PDTC) treatment (10 mg/kg body weight;
cat#: P8765; Sigma Aldrich) 24h prior to a single ECC bout. We have previously found that treatment paradigm can sufficiently lower muscle inflammatory signaling prior to muscle contraction (Puppa, Murphy, et al., 2014). In the current study, a single PDTC treatment did not alter total tumor number (86 ± 4; p=0.91) or plasma IL-6 levels (44 ± 5; P=0.71), as we have previously observed following short-term treatment after the initiation of cachexia (Narsale et al., 2016). In all experiments mice were fasted 2h prior to contraction and remained fasted until sacrifice (immediately or 3h post). Fasting was initiated at ~0800h in each experiment. There were no differences in cachexia indices (e.g., body weight, muscle mass and fat loss) between MIN mice in all experiments, therefore general animal characteristics from each cohort are summarized in Table 3.1. Additionally, protein expression in the non-stimulated TA from MIN mice in experiments 1 and 2 were used to determine the cachectic muscle phenotype (Figure 3.1).

**Eccentric contractions**

ECC of the tibialis anterior (TA) muscle was induced by high-frequency electrical stimulation of the sciatic nerve as previously described with slight modifications (Baar & Esser, 1999; Hardee et al., 2016). Mice were anesthetized via isoflurane (2% in O2 with 1.5% maintenance), the stimulated leg was shaved at the hip region, and two needle electrodes were placed subcutaneously to stimulate the sciatic nerve. Tetanic muscle contractions of one hind limb were generated using a Grass Stimulator (Grass Instruments) for 10 sets of 6 repetitions (100 Hz, 6-12V, 1ms duration). Ten seconds of rest was given between stimulations and 50 seconds of rest was given between sets. The stimulation protocol recruits all motor units and results in net plantar flexion of the ankle (Wong & Booth, 1988, 1990). The dorsiflexors (TA and EDL) undergo ECC while the plantar
flexors (gastrocnemius, soleus, and plantaris) perform concentric muscle contractions. In all experiments, the stimulated TA performed ECC while the non-stimulated TA served as intra-animal control. Our laboratory and others have demonstrated repeated ECC bouts, but not concentric contractions, can induce muscle and myofiber growth in rodents (al-Majid & McCarthy, 2001; Baar & Esser, 1999; Hardee et al., 2016; Wong & Booth, 1988). Therefore, the TA was examined in all experiments. Mice were given an intraperitoneal injection of warm saline following the stimulation procedure and returned to cages upon complete recovery.

Tissue Collection

Mice received an intraperitoneal injection of puromycin (0.04 umol/kg body weight) 30 minutes prior to sacrifice (Goodman, Mabrey, et al., 2011; Narsale et al., 2016). Mice were anesthetized with a subcutaneous injection of ketamine/xylazine/acepromazine cocktail (1.4 ml/kg body weight) at the time of sacrifice. Muscles and organs were rapidly excised, cleared of excess connective tissue, rinsed in phosphate-buffered saline (PBS), dried on blotting paper, weighed, and snap frozen in liquid nitrogen. Immediately prior to dissection blood was collected via retro-orbital sinus with heparinized capillary tubes, placed on ice, and centrifuged (10,000 x g for 10 min at 4°C). The supernatant was removed and stored for plasma IL-6 analysis. Plasma and tissue samples were stored at -80°C until analysis.

Western Blotting

Western blot analysis was performed as previously described (Hardee, Puppa, et al., 2014). Briefly, frozen TA muscle was homogenized in Mueller buffer and protein
concentration was determined by the Bradford method. Crude TA muscle homogenates were fractionated on 7-15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau red to verify equal loading and transfer. Membranes were then blocked at room temperature (RT) for 1-2h in 5% non-fat milk Tris-buffered saline with 0.1% Tween-20 (TBST). Primary antibodies for puromycin (Millipore, cat#MABE343, 1:2000), phospho P70S6K (T389) (cat#9205, 1:1000), total P70S6K (cat#2708, 1:1000), RPS6 (S240/244) (cat#2215, 1:500), total RPS6 (cat#2708, 1:1000), phospho Akt (S473) (cat#4060, 1:1000), total Akt (cat#9272, 1:2000), phospho NFκB (S563) (cat#3033, 1:500), total NFκB (cat#4764, 1:2000), phospho STAT3 (Y750) (cat# 9145, 1:1000), total STAT3 (cat#4904, 1:2000), phospho AMPK (T172) (cat#, 1:2000), total AMPK (cat#2603, 1:1000), phospho ACC (S59) (cat#3661, 1:1000), total ACC (cat#3662), phospho ERK1/2 (T202/Y204) (cat#4370,1:1000), total ERK1/2 (cat#4695, 1:1000), PGC-1α (Abcam, cat#ab54481, 1:1000), COXIV (cat#4844, 1:1000), GAPDH (cat#2118, 1:10000), MuRF1 (ECM Biosciences, cat#MP3401, 1:2000), Atrogin-1 (ECM Biosciences, cat#AP2041, 1:5000), and ubiquitin (cat#3933, 1:2000) were incubated overnight in 5% TBST milk. We have previously validated the specificity of this PGC-1α antibody in tibialis anterior skeletal muscle through somatic gene transfer of empty or PGC-1α overexpression plasmid (data not shown). Membranes were then incubated in 5% milk-TBST containing anti-rabbit (cat#7074, 1:5000) or anti-mouse (cat#7076, 1:5000) IgG horseradish-peroxidase conjugated secondary antibodies for 1h at RT. Exceptions to the aforementioned procedures were that for puromycin incorporation 1% BSA-TBST was used for primary antibody and horseradish-peroxidase conjugated rabbit anti-mouse IgG2a
antibody (LifeTechnologies, cat#610220, 1:5000) in 5% milk-TBST was used for secondary antibody. All antibodies were from Cell Signaling Technology unless otherwise stated. Tibialis anterior protein extracts from a mouse that did not receive puromycin at sacrifice was included on all puromycin gels as a negative control. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences) was used to visualize the antibody-antigen interactions. Immunoblot images were collected using a digital imager (SynGene GBox) and quantified by densitometry using imaging software (Image J; NIH). Each gel contained samples from all groups and data was normalized to the respective control group (e.g. B6 control).

**Plasma Interleukin-6 Concentration**

Plasma IL-6 concentrations were determined as previously described (Hetzler et al., 2015). A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences and the manufacturer’s protocol was followed. Briefly, clear 96-well plates were coated and incubated overnight with an IL-6 capture antibody. The next morning the plate was blocked with assay diluent buffer, washed, and equal volumes of standards and plasma samples were added in duplicate. After a 2h incubation the plate was washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read at 450 nm using an iMark microplate absorbance reader (Bio-Rad Laboratories).
Statistical Analysis

Results are reported as the means ± standard error. A repeated-measures two-way ANOVA was performed to determine differences between cancer cachexia and ECC in B6 and MIN mice. Post-hoc analyses were performed with Student-Newman-Keuls methods when appropriate. Students t-test was used to determined differences between two groups when appropriate. The accepted level of significance was set at p<0.05 for all analysis. Statistical analysis and figure generation were performed using Prism 5 for Mac OS X (GraphPad Software Inc).

3.4 RESULTS

Systemic and muscle cachectic phenotype

In order to determine if wasting skeletal muscle could respond to a novel ECC bout we first established the cachectic phenotype in two separate cohorts of male MIN mice. MIN mice displayed several key features of severe cachexia which included body weight loss, muscle atrophy, adipose tissue depletion, high tumor burden, elevated plasma IL-6 levels, and hypogonadal features (levator ani-bulbocavernosus [LABC] and seminal vesicle atrophy) (Table 3.1). Body weights at sacrifice were lower when compared to B6 mice, and significant body weight loss from peak measurement was observed within MIN mice. Body weight loss was accompanied by reduced TA muscle mass and epididymal fat loss in MIN mice. Cachexia increased spleen weight and plasma IL-6 compared to B6 mice (Table 3.1). There were no differences in tibia length between B6 and MIN mice.

To further characterize the cachectic phenotype protein expression related to protein turnover, inflammation and metabolism was examined in the non-stimulated TA
muscle (Figure 3.1). Cachectic muscle demonstrated disrupted protein turnover regulation. Akt/mTORC1 signaling was disrupted in cachectic muscle. While Akt was activated, downstream mTORC1 signaling was suppressed (Figure 3.1A). Furthermore, the E3 ligases Atrogin-1 and MuRF1 were induced in cachectic muscle, which coincided with increased expression of total ubiquitinated proteins (Figure 3.1A). We have extended our previous observations that basal Akt activation is disconnected from mTORC1 and FOXO3 signaling, which may be related to decreased mTORC1 formation and shift towards mTORC2 formation. However, further investigation is needed to determine the precise mechanisms for perturbed signaling in cachectic skeletal muscle. Cachexia activated inflammatory / mechanical related proteins (STAT3, NFκB, ERK1/2, and P38) (Figure 3.1B). Lastly, cachexia altered the expression of proteins related to mitochondrial content (PGC-1α, COXIV) and metabolic regulation (AMPK, ACC), respectively (Figure 3.1C). Overall, these findings demonstrate that severe cachexia in this cohort of mice was associated with high circulating IL-6, enhanced muscle inflammatory signaling, and disrupted anabolic and metabolic regulation.

Mechanical signaling response to ECC

Having established the severe cachectic phenotype in this cohort of male MIN mice, we then examined mechano-sensitive pathways immediately following a single ECC bout (Figure 3.2A). First, we examined two mitogen-activated protein kinases (MAPKs) which have been shown to be activated immediately post-ECC (Nader & Esser, 2001; O'Neil et al., 2009). While cachexia increased basal P38 and ERK1/2 phosphorylation in the non-stimulated TA muscle, ECC-induced their activation irrespective of cachexia (Figure 3.2B). While absolute P38 and ERK1/2 phosphorylation were greater following ECC
during cachexia, there were no differences in the degree of activation from the non-contracted TA muscle. Given that ECC are a potent stimulator of Akt/mTORC1 signaling (Nader & Esser, 2001; O'Neil et al., 2009; Thomson, Fick, & Gordon, 2008), we next determined Akt/mTORC1 activation through phosphorylation of Akt and the downstream mTORC1 target P70S6K. While cachexia increased basal Akt phosphorylation in the non-stimulated TA muscle, ECC-induced its activation irrespective of cachexia (Figure 3.2C). Interestingly, while cachexia decreased basal P70S6K phosphorylation in the non-stimulated TA muscle, ECC-induced its activation irrespective of cachexia (Figure 3.2C). While absolute P70S6K phosphorylation was decreased following ECC during cachexia, there were no differences in the degree of activation from the non-stimulated TA muscle. We further validated P70S6K activation through the phosphorylation of the direct P70S6K target RPS6. Similarly, cachexia decreased basal RPS6 phosphorylation in the non-stimulated TA muscle, while ECC-induced its activation irrespective of cachexia (Figure 3.2C). Collectively, these findings demonstrate that mechano-sensitive pathways were induced immediately post-ECC despite disrupted basal Akt/mTORC1 signaling in cachectic MIN mice.

Proteolytic, metabolic, and inflammatory signaling response to ECC

Given that the ubiquitin-proteasome pathway has been implicated in the proteolytic response to acute resistance exercise (Fry et al., 2013), we examined the expression of two E3 ligases immediately following a single ECC bout. While ECC-induced Atrogin-1 and MuRF1 protein expression in B6 mice, this was not observed in MIN mice (Figure 3.2D). Given the potential role of metabolic stress to inhibit anabolic processes, we next examined AMPK and its direct downstream target Acetyl-CoA carboxylase (ACC) in response to
ECC. Interestingly, while the absolute AMPK and ACC phosphorylation were greater immediately post-ECC during cachexia, there were no differences in the degree of activation from the non-contracted TA muscle (Figure 3.2E). Lastly, we examined muscle inflammatory signaling pathways which have been implicated in cachexia and muscle contraction. Interestingly, STAT3 and NFκB were activated immediately post-ECC in B6 (Figure 3.2F). While basal STAT3 and NFκB were induced by cachexia, only NFκB was further induced immediately post-ECC in MIN mice (Figure 3.2F). Collectively, these findings demonstrate that mechano-sensitive pathways remained intact, and was not associated with an exacerbated metabolic and proteolytic response immediately post-ECC in cachectic MIN mice.

*Cachectic muscle anabolic signaling response to ECC*

Having established the cachectic muscle’s mechano-sensitive response to a single ECC bout, we then determined if cachexia disrupted ECC-induced protein synthesis and mTORC1 signaling. Therefore, anabolic signaling was examined 3h after a single ECC bout (Figure 3.3A). While cachexia suppressed protein synthesis, ECC activated protein synthesis irrespective of cachexia. Although the relative induction by ECC was not altered by cachexia, the absolute protein synthesis rate remained suppressed relative to B6 mice (Figure 3.3B). We then examined several upstream regulators and downstream targets implicated in the mechanical activation of mTORC1 signaling. Interestingly, while cachexia increased basal P38 and ERK1/2 phosphorylation, these mechano-sensitive signaling molecules were not altered 3h post-ECC irrespective of cachexia (Figure 3.3C). We then examined Akt/mTORC1 activation in response to ECC. While Akt was not altered by ECC regardless of cachexia, there was a strong trend (P=0.07) for ECC to
decrease Akt phosphorylation in B6 mice (Figure 3.3D). While cachexia decreased the phosphorylation of P70S6K, ECC-induced its activation irrespective of cachexia (Figure 3D). ECC also increased RPS6 phosphorylation irrespective of cachexia (Figure 3.3D). Collectively, these findings demonstrate that protein synthesis and mTORC1 signaling was induced 3h post-ECC in severely cachectic MIN mice.

*Cachectic muscle proteolytic, metabolic, and inflammatory response to ECC*

Muscle protein breakdown, metabolic dysfunction, and enhanced inflammatory signaling have established roles during cancer-induced muscle wasting, and these same signaling pathways are perturbed in response to muscle contraction (Begue et al., 2013; Serrano et al., 2008; Washington et al., 2011; White et al., 2009). ECC did not alter the expression of Atrogin-1 and MuRF1 irrespective of cachexia (Figure 3.4A). We have previously shown that the sustained activation of AMPK coincided with suppressed mTORC1 signaling 3h after a single bout of concentric muscle contractions (Puppa, Murphy, et al., 2014). Interestingly, while AMPK and ACC were activated by cachexia, ECC decreased their activation irrespective of cachexia (Figure 3.4B). Lastly, we found that both STAT3 and NFκB phosphorylation were induced by cachexia, and were further increased by ECC irrespective of cachexia (Figure 3.4C). Collectively, these data demonstrate that muscle metabolic and inflammatory signaling molecules were sensitive to both cachexia and contraction.

*Muscle inflammatory signaling regulation of basal and ECC-induced protein synthesis.*

Given that muscle STAT3 and NFκB signaling was induced by cachexia and ECC, we next examined its involvement in the regulation of basal and ECC-induced protein
synthesis in cachectic MIN mice. To accomplish this, we used an established pharmacological approach to lower basal muscle inflammatory signaling prior to ECC (Narsale et al., 2016; Puppa, Murphy, et al., 2014). This experimental paradigm has previously been used by our laboratory to lower muscle STAT3/NFκB signaling prior to a single bout of low-frequency electrical stimulation (Puppa, Murphy, et al., 2014). Therefore, we administered PDTC 24h prior to a single ECC bout in cachectic MIN mice (Figure 3.5A). As expected, PDTC decreased basal STAT3 and NFκB phosphorylation in the non-stimulated TA muscle (Figure 3.5B). There was no effect of PDTC on basal P38, ERK1/2, or Akt in cachectic skeletal muscle (data not shown). Interestingly, PDTC induced basal protein synthesis in cachectic muscle, and was further increased 3h post-ECC (Figure 3.5C). While there was a trend (P=0.08) for PDTC to increase basal P70S6K phosphorylation, there was a robust induction 3h post-ECC. Similarly, PDTC increased basal RPS6 phosphorylation, and was further increased 3h post-ECC in cachectic skeletal muscle (Figure 3.5D). As previously observed, the activation of mechano-sensitive pathways (P38, ERK1/2, Akt) were not altered 3h post-ECC (data not shown). Collectively, these data demonstrate that acute muscle STAT3 and NFκB inhibition improved basal and ECC-induced protein synthesis in cachectic MIN mice.

_Proteolytic, metabolic, and inflammatory signaling response to PDTC and ECC._

Lastly, we examined the proteolytic, metabolic, and inflammatory signaling response to PDTC and ECC. Neither PDTC nor ECC altered the expression of Atrogin-1 and MuRF1 in MIN mice (Figure 3.5E). While PDTC did not alter basal AMPK and ACC activation in cachectic skeletal muscle, these molecules were further suppressed 3h post-ECC (Figure 3.5F). Interestingly, while PDTC suppressed basal muscle STAT3 and NFκB
signaling, it did not block the induction 3h post-ECC (Figure 3.5G). Altogether, these data demonstrate that improved ECC-induced protein synthesis by PDTC corresponded to suppressed metabolic signaling, but was independent to altered proteolytic E3 ligase expression in cachetic MIN mice.

3.5 DISCUSSION

Healthy skeletal muscle stimulates protein synthesis in response to anabolic stimuli associated with daily living, which can include physical activity and feeding. While basal muscle protein synthesis and mTORC1 signaling is suppressed in tumor-bearing mice and some human cancer patients (Emery et al., 1984; White, Baynes, et al., 2011), the capacity for cachetic muscle to respond to an anabolic stimulus is not well understood. This knowledge could have significant ramifications for the treatment of the cachectic cancer patient. Resistance exercise consisting of ECC is a potent stimulator of protein synthesis and muscle growth (Chesley et al., 1992; Eliasson et al., 2006; Fry et al., 2011). We have previously found that repeated ECC bouts after the initiation of cachexia attenuated myofiber atrophy and was accompanied by suppressed muscle inflammatory signaling. However, the capacity to activate mTORC1 signaling and protein synthesis by ECC has not been investigated. Therefore, we examined if cachetic muscle maintained the ability to induce mechano-sensitive pathways and protein synthesis in response to a single ECC bout. We report that ECC-induced mechanical signaling was maintained in cachetic muscle, but the capacity for increased protein synthesis was attenuated. This finding demonstrates an unexpected uncoupling between the activation of known mechano-sensitive regulators of anabolic signaling and the absolute protein synthesis induction. Therefore, we also examined if the cachectic environment involving muscle inflammatory
signaling could regulate ECC-induced protein synthesis. Interestingly, both cachexia and ECC-induced muscle STAT3 and NFκB signaling. However, muscle STAT3/NFκB inhibition by PDTC increased basal and ECC-induced protein synthesis in cachectic MIN mice. These findings demonstrate that mechano-sensitive signaling is responsive to ECC, and highlight muscle inflammatory signaling’s role in the altered regulation of both basal and ECC-induced protein synthesis during cancer cachexia.

While exercise training has been discussed as a potential therapy to mitigate muscle atrophy during cancer cachexia, there is currently a limited understanding of the acute response and training adaptation to exercise. Whole-body treadmill exercise prevented muscle mass loss in tumor-bearing mice (Deuster, Morrison, & Ahrens, 1985; Penna et al., 2011; Salomao, Toneto, Silva, & Gomes-Marcondes, 2010), and blocked the disruption of muscle oxidative metabolism regulation at the initiation of cachexia (Puppa et al., 2012; White et al., 2012). However, the inability of severely cachectic mice to perform voluntary exercise remains a consistent barrier and has limited our understanding of the muscle response to exercise during refractory cachexia. To address this, we have reported that cachexia disrupted the metabolic and anabolic signaling response to a single bout of stimulated low-frequency concentric contractions (Puppa, Murphy, et al., 2014), which mimics low intensity, endurance type exercise. Given that exercise involves muscle contractions that can vary in overall intensity and metabolic demand, the molecular responses related to growth and metabolism are distinct between contraction types. Furthermore, the response of cachectic muscle to high force contractions is not well established. Therefore, we first examined the mechanical and metabolic response to a single ECC bout. We found that several contraction and mechano-sensitive kinases
(MAPKs, Akt, P70S6K) were induced by ECC in cachectic muscle. The activation of P38, ERK1/2, and Akt was transient, which extends previous observations in mouse skeletal muscle (Nader & Esser, 2001). In addition, P70S6K activation by ECC remained elevated irrespective of cachexia, which is in contrast to our previous observations using concentric muscle contractions (Puppa, Murphy, et al., 2014). Interestingly, we also observed a transient AMPK induction by ECC, which was not associated with mTORC1 signaling inhibition. Collectively, we provide initial evidence that the mechanical and metabolic plasticity of muscle to ECC is maintained despite the presence of a systemic cachectic environment.

There is considerable interest in understanding the mechanisms that serve to repress cachectic muscle anabolic signaling. We previously reported that repeated ECC bouts performed after the initiation of cancer cachexia could attenuate myofiber atrophy (Hardee et al., 2016), however this study did not determine whether these improvements were related to the induction of muscle growth or the attenuation of muscle breakdown. While our current study has extended these findings to demonstrate that mechano-signaling in cachectic muscle is maintained, the ability to synthesize protein remained dramatically suppressed. These findings further demonstrate that the capacity for either basal or contraction-induced muscle protein synthesis is suppressed by cachexia. The chronic activation of AMPK in cachectic skeletal muscle from tumor-bearing mice has been implicated as a potential mechanism for mTORC1 and protein synthesis suppression (White, Baynes, et al., 2011; White, Puppa, Gao, et al., 2013). Interestingly, our current study demonstrates the induction of protein synthesis after ECC coincides with reduced AMPK activation in tumor-bearing mice. Furthermore, reduced AMPK activation after a
single ECC bout has also recently been observed in castrated mice (Steiner et al., 2017). These findings are in contrast to our previous observations using low-frequency electrical stimulation, and point to the differential regulation of muscle metabolic signaling by different types of contraction. We previously found a sustained AMPK activation following a single bout of low-frequency, concentric contractions in cachectic skeletal muscle (Puppa, Murphy, et al., 2014). The specificity of the responses induced by different types of contractions may be related to metabolic stress as cachectic muscle develops mitochondrial dysfunction (Antunes et al., 2014; Tzika et al., 2013; White, Baltgalvis, et al., 2011). The cachectic muscle’s anabolic and metabolic response to different contraction types will require further investigation. Nonetheless, our findings suggest ECC may be a potential therapeutic treatment to promote muscle anabolism during cancer cachexia progression.

Muscle signaling related to inflammation, energy status, and proteostasis are disrupted during cachexia progression, and have been implicated in the regulation of muscle wasting (Carson, Hardee, & VanderVeen, 2016; K. C. Fearon, Glass, & Guttridge, 2012). Interestingly, many of these same pathways are induced by muscle contraction and exercise (B. Egan & Zierath, 2013). IL-6 and muscle STAT3 signaling through the gp130 receptor are activated during the progression of cachexia (Bonetto et al., 2012; Bonetto et al., 2011; White, Baltgalvis, et al., 2011), and are associated with mTORC1 signaling and protein suppression in preclinical cachexia models (White, Baynes, et al., 2011; White, Puppa, Gao, et al., 2013). Inhibition of muscle IL-6 signaling through either direct STAT3 inhibition or gp130 loss can attenuate wasting in mouse models of cachexia (Bonetto et al., 2012; Puppa, Gao, et al., 2014). We have previously found that short-term PDTC treatment
attenuated the suppression of mTORC1 signaling and protein synthesis while concomitantly reducing muscle STAT3 and NFκB activation in MIN mice (Narsale et al., 2016; Puppa, Murphy, et al., 2014). We extend these findings by demonstrating a single PDTC dose improved basal protein synthesis. It has recently been suggested that intermittent cycles of pathway inhibition / activation may be required to combat muscle wasting during cancer cachexia (Au, Desai, Koniaris, & Zimmers, 2016). Indeed, many cytokine-related signaling pathways have established roles in myogenesis (Costamagna, Costelli, Sampaolesi, & Penna, 2015) and load-induced muscle growth and remodeling (Serrano et al., 2008; Spangenburg & Booth, 2006; White et al., 2009). Therefore, we utilized an experimental paradigm that lowered chronic muscle inflammatory signaling, but did not block contraction-induced signaling (Puppa, Murphy, et al., 2014). Importantly, we found that PDTC treatment increased both basal and ECC-induced protein synthesis in cachectic muscle. These findings demonstrate that cachectic muscle retains the anabolic capacity to increase protein synthesis, and inflammatory signaling contributes to the suppression of these processes. Additional research is warranted to determine the specific mechanisms related to STAT3 and NFκB that serve to diminish the capacity for protein synthesis in cachectic muscle. Moreover, further research is also required to determine the effect of muscle inflammatory signaling on metabolic remodeling in response to repeated contraction bouts, which could dramatically impact health outcomes related to exercise.

Conclusion

In summary, we examined cachectic muscle’s ability to induce mTORC1 signaling and protein synthesis in response to ECC, and determined the role of muscle inflammatory
signaling involving STAT3 and NFκB on ECC-induced anabolic signaling. We found that mechano-sensitive signaling pathways related to P38, ERK1/2 and Akt were not altered by the cachectic environment in wasting muscle. While cachexia did not attenuate the ECC induction of mTORC1 signaling, the capacity for protein synthesis remained suppressed compared to healthy controls. Interestingly, we found that reducing muscle STAT3/NFκB signaling improved basal and ECC-induced protein synthesis during severe cachexia. These studies demonstrate that mechano-sensitive signaling pathways are maintained in skeletal muscle, but STAT3/NFκB signaling serves to attenuate basal and ECC-induced protein synthesis. Further work is necessary to determine whether intermittent anti-inflammatory therapies combined with exercise training may be useful to alleviate suppressed muscle protein synthesis during cancer cachexia.

ACKNOWLEDGEMENTS

The authors thank Gaye Christmus and Dr. Bradley Gordon for editorial review of the manuscript. This work was supported by National Institutes of Health Grants R01 CA-121249 (National Cancer Institute) and P20 RR-017698 (National Institute of General Medical Science) to J.A.C, SPARC Graduate Research Grant from the Office of the Vice President for Research at the University of South Carolina to J.P.H, and an ACSM Foundation Research Grant from the American College of Sports Medicine Foundation to J.P.H. The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia and Muscle (von Haehling, Morley, Coats, & Anker, 2015).
Table 3.1. B6 and MIN mice that performed a single ECC bout.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>MIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>26.7 ± 0.7</td>
<td>24.7 ± 0.4</td>
</tr>
<tr>
<td>ECC</td>
<td>26.7 ± 0.7</td>
<td>20.7 ± 0.4*†</td>
</tr>
<tr>
<td>% Change</td>
<td>0 ± 0</td>
<td>-16 ± 0.6*</td>
</tr>
<tr>
<td>Tibialis anterior, mg</td>
<td>49 ± 0.7</td>
<td>28 ± 0.9*</td>
</tr>
<tr>
<td>Epididymal fat, mg</td>
<td>361 ± 44</td>
<td>3 ± 2*</td>
</tr>
<tr>
<td>Spleen, mg</td>
<td>71 ± 4</td>
<td>506 ± 21*</td>
</tr>
<tr>
<td>Testes, mg</td>
<td>201 ± 7</td>
<td>103 ± 8*</td>
</tr>
<tr>
<td>LABC, mg</td>
<td>88 ± 2</td>
<td>31 ± 2*</td>
</tr>
<tr>
<td>Seminal vesicle, mg</td>
<td>261 ± 8</td>
<td>32 ± 3*</td>
</tr>
<tr>
<td>Plasma IL-6, pg/ml</td>
<td>0 ± 0</td>
<td>43 ± 3*</td>
</tr>
<tr>
<td>Tumor number</td>
<td>0 ± 0</td>
<td>87 ± 4*</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>16.9 ± 0.1</td>
<td>16.8 ± 0.1</td>
</tr>
</tbody>
</table>

Data are means ± standard error. There were no differences between control and stimulated TA muscles, therefore the average is presented. A two-way repeated measures ANOVA was used to determine differences between body weight at peak and immediately prior to ECC. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Students t-test was used to determine differences in all other variables. Statistical significance was set at p<0.05. Abbreviations: ECC, Eccentric contractions. No., number. g, grams. mg, milligrams. pg, picogram. ml, milliliter. mm, millimeter. LABC, levator ani / bulbocavernosus muscle. * = significantly different from B6. † = significantly different from difference from peak body weight.
Figure 3.1. Cachectic muscle phenotype. A) Protein turnover regulation in B6 and MIN mice. B) Inflammatory and mechano-sensitive pathways in B6 and MIN mice. C) Metabolic signaling regulation in B6 and MIN mice. Tibialis anterior (TA) protein
expression was examined in the non-stimulated muscle. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to B6 Control values. Dotted lines indicate images were cropped for representative purposes. Data are means ± standard error. N = 15 B6; N=17 MIN. Students t-test was used to determine differences between B6 and MIN mice. Statistical significance was set at p<0.05. * = significantly different from B6.
A) ~20 wk of age
Fasted
TA
ECC
Sacrifice
(immed post)
-120 ~22 (min)
-60 0 ~22 (min)

B) C57BL/6 Control  ApcMin/+ Control
C57BL/6 ECC  ApcMin/+ ECC

Protein expression
(normalized to C57BL/6 Control)
pERK1/2
pP38

C) C57BL/6 Control  ApcMin/+ Control
C57BL/6 ECC  ApcMin/+ ECC

Protein expression
(normalized to C57BL/6 Control)
pAKT
pP70S6K
pRPS6

C57BL/6
MIN
ECC
- + + - + +
- + + - + +
pERK1/2
ERK1/2
pP38
p38

C57BL/6
MIN
ECC
- + + - + +
- + + - + +
pAKT
AKT
pP70S6K
P70S6K
pRPS6
RPS6
Figure 3.2. Muscle mechanical signaling immediately post-ECC. A) Experimental Design. B6 and MIN mice were sacrificed immediately post-ECC. Mice were fasted 2h prior to contraction. B) Muscle mitogen activated protein kinase (MAPK) signaling regulation by ECC in B6 and MIN mice. C) Muscle Akt/mTORC1 signaling regulation by ECC in B6 and MIN mice. D) Muscle proteolytic regulation by ECC in B6 and MIN mice. E) Muscle metabolic signaling regulation in B6 and MIN mice. F) Muscle
inflammatory signaling regulation by ECC in B6 and MIN mice. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to B6 Control values. Dotted lines indicate images were cropped for representative purposes. Data are means ± standard error. N = 6 B6; N=7 MIN. A two-way repeated measures ANOVA was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. & = main effect of ECC. # = main effect of MIN.
Figure 3.3. Muscle protein synthesis and mTORC1 signaling 3h post-ECC. A) Experimental Design. B6 and MIN mice were sacrificed 3h post a single bout of ECC. Mice were fasted 2h prior to contraction and remained fasted during the 3h recovery until sacrifice. Mice were injected with puromycin 30min prior to sacrifice. B) Muscle protein
synthesis regulation by ECC in B6 and MIN mice. C) Muscle mitogen activated protein kinase (MAPK) signaling regulation by ECC in B6 and MIN mice. D) Muscle Akt/mTORC1 signaling regulation by ECC in B6 and MIN mice. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to B6 Control values. Dotted lines indicate images were cropped for representative purposes. Data are means ± standard error. N = 9 B6; N=10 MIN. A two-way repeated measures ANOVA was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Kneumnan-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. & = main effect of ECC. # = main effect of MIN.
Figure 3.4. Muscle proteolytic, metabolic, and inflammatory signaling 3h post-ECC.  
A) Muscle proteolytic regulation by ECC in B6 and MIN mice.  B) Muscle metabolic signaling regulation by ECC in B6 and MIN mice.  C) Muscle inflammatory signaling regulation by ECC in B6 and MIN mice.  The activation of signaling molecules was
determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to B6 Control values. Dotted lines indicate images were cropped for representative purposes. Data are means ± standard error. N = 9 B6; N=10 MIN. A two-way repeated measures ANOVA was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. & = main effect of ECC. # = main effect of MIN.
A) ~20 wk of age

PDTC Fasted TA ECC Sacrifice

-24 0 1 2 3 4 5 (hour)

B) ApcMin/+ ApcMin/+ PDTC

Protein expression (normalized to ApcMin/+ Control)

pSTAT3 pNFkB

MIN MIN PDTC

pSTAT3 STAT3 pNFkB NFkB

C) ApcMin/+ Control ApcMin/+ PDTC Control ApcMin/+ ECC ApcMin/+ PDTC ECC

Puromycin Incorporation (normalized to ApcMin/+ Control)

ApMin/+ ApMin/+ PDTC

MIN MIN PDTC

ECC - + + - - + NC

Puromycin Incorporation

D) ApcMin/+ Control ApcMin/+ PDTC Control ApcMin/+ ECC ApcMin/+ PDTC ECC

Protein expression (normalized to ApcMin/+ Control)

p70S6K pP70S6K P70S6K pRPS6 RPS6

MIN MIN PDTC

ECC - + + - - + NC

p70S6K P70S6K pRPS6 RPS6
Figure 3.5. Muscle inflammatory signaling regulation of ECC-induced protein synthesis in MIN mice. A) Experimental Design. MIN mice were sacrificed 3h post-ECC. A cohort of MIN mice were given a single PDTC treatment (10 mg/kg body weight)
24h prior to ECC. Mice were fasted 2h prior to ECC and remained fasted during the 3h recovery until sacrifice. Mice were injected with puromycin 30min prior to sacrifice. B) Muscle STAT3 and NFκB signaling in the non-stimulated muscle following a single PDTC treatment. C) Muscle protein synthesis regulation by PDTC and ECC in MIN mice. D) Muscle mTORC1 signaling regulation by PDTC and ECC in MIN mice. E) Muscle proteolytic regulation by PDTC and ECC in MIN mice. F) Muscle metabolic signaling regulation by PDTC and ECC in MIN mice. G) Muscle inflammatory signaling regulation by PDTC and ECC in MIN mice. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to MIN Control values. Dotted lines indicate images were cropped for representative purposes. Data are means ± standard error. N = 10 MIN; N=10 MIN PDTC. A two-way repeated measures ANOVA was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. & = main effect of ECC. # = main effect of PDTC.
CHAPTER 4

SYSTEMIC IL-6 AND MUSCLE GP130 REGULATION OF BASAL AND ECCENTRIC CONTRACTION-INDUCED PROTEIN SYNTHESIS

Hardee JP, Fix DK, Wang X, Goldsmith EC, Koh HJ, and JA Carson. Systemic IL-6 and Muscle GP130 Regulation of Basal and Eccentric Contraction-Induced Protein Synthesis. To be submitted to *Front Physiol.*
4.1 ABSTRACT

Mechanical loading is a potent stimulator of mechanistic target of rapamycin (mTORC1) signaling and muscle growth. While repeated ECC bouts during cachexia progression attenuated muscle mass loss in tumor-bearing mice, we have found that tumor-derived cachectic factors disrupted stretch-induced protein synthesis in cultured myotubes. The ability of tumor-derived cachectic factors and cytokines to disrupt mechanical signaling during muscle wasting has not been well described. Paradoxically, IL-6 signaling through the muscle gp130 receptor has been implicated in muscle wasting with cancer cachexia and muscle mass regulation with contraction and loading. Therefore, we examined long-term IL-6 regulation of mechanically induced protein synthesis in atrophic muscle. First, we determined if long-term IL-6 (24h, 100ng/mL) treatment could disrupt stretch-induced protein synthesis in differentiated C2C12 myotubes. Next, we determined if 2-wks systemic IL-6 overexpression could disrupt ECC-induced protein synthesis in tumor bearing ApcMin/+ (MIN) mice. Lastly, we examined basal and ECC-induced protein synthesis regulation by systemic IL-6 overexpression in mice lacking muscle gp130 receptor. The stimulated tibialis anterior (TA) muscle performed ECC while the non-stimulated TA served as intra-animal control. Long-term IL-6 exposure induced inflammatory signaling pathways related to GP130, STAT3, and NFkB, and suppressed myotube stretch-induction of protein synthesis and mTORC1 signaling. Muscle inflammatory signaling pathways related to GP130, STAT3, and NFkB were also induced by systemic IL-6 overexpression in both healthy C57BL/6 (B6) and MIN mice. Systemic IL-6 overexpression suppressed basal muscle protein synthesis in both B6 and MIN mice. While systemic IL-6 overexpression disrupted ECC-induced protein synthesis in MIN
mice, this response was maintained in B6 mice. Interestingly, ECC-induced mTORC1 signaling was not altered by systemic IL-6 overexpression regardless of cachexia mice. Lastly, muscle gp130 receptor blocked systemic IL-6 suppression of basal protein synthesis. However, mice lacking muscle gp130 receptor demonstrated an attenuate protein synthesis response to ECC. These studies demonstrate that IL-6 signaling through gp130 can regulate basal and mechanical activation of protein synthesis in skeletal muscle.

**Keywords:** Mechanical Signaling, Eccentric contractions, Muscle Protein Synthesis, Cancer Cachexia

### 4.2 INTRODUCTION

Cancer cachexia, a wasting syndrome characterized by skeletal muscle depletion, contributes to increased patient morbidity and mortality (K. C. Fearon, 1992; Tisdale, 2009). Additional consequences of skeletal muscle mass depletion in cancer patients can include reduced anticancer therapy tolerance, increased susceptibility to treatment toxicity, and decreased patient quality of life (Barret et al., 2014; Evans et al., 2008; H. W. Jung et al., 2015). Therefore, it is recognized that the maintenance of skeletal muscle mass and metabolic function are critically important during cancer patient treatment and survival. Skeletal muscle mass is regulated through the balance between protein synthesis and breakdown processes. The protein synthetic rate is controlled by translational efficiency and capacity, which is regulated by the mechanistic target of rapamycin complex 1 (mTORC1). mTORC1 has been implicated in the regulation of cap-dependent initiation of translation through the phosphorylation of substrates such as eukaryotic initiation factor (eIF) 4E binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (P70S6K).
While cachexia-induced wasting is linked to disrupted mTORC1 signaling and suppressed protein synthesis (Eley & Tisdale, 2007; Lima, Sato, Enos, Baynes, & Carson, 2013; Lopes, Black, Ashford, & Pain, 1989; White, Baynes, et al., 2011), the systemic drivers regulating cancer-induced wasting have not been fully elucidated.

Several members of the interleukin-6 (IL-6) family of cytokines (e.g., IL-6, IL-11, leukemia inhibitory factor [LIF]) have been implicated in muscle metabolism and growth regulation (Pal, Febbraio, & Whitham, 2014; Serrano et al., 2008; White et al., 2009). In general, IL-6 family of cytokines signal through gp130 by forming either heterodimer or homodimer complexes consisting of the cytokine, ligand specific transmembrane receptor α-subunit, and the transmembrane type I cytokine receptor β-subunit gp130 (Ernst & Jenkins, 2004). Signal transduction through gp130 dimerization leads to the activation of several intracellular pathways, which include JAK/STAT, Ras/ERK, and PI3K/Akt (Ernst & Jenkins, 2004; Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). Circulating levels of these cytokines are elevated during wasting in patients and preclinical models (Bonetto et al., 2011; Tazaki et al., 2011), and have been implicated in muscle wasting through the activation of gp130/STAT3 signaling during cancer cachexia (Bonetto et al., 2012; Bonetto et al., 2011; S. J. Lee & Kim, 2017; Puppa, Gao, et al., 2014; White, Baynes, et al., 2011). While systemic IL-6 overexpression can disrupt mTORC1 signaling and protein synthesis in tumor-bearing mice (White, Puppa, Gao, et al., 2013), blocking muscle IL-6 signaling through direct STAT3 inhibition or gp130 receptor loss attenuated wasting independent to protein synthesis activation (Bonetto et al., 2012; Puppa, Gao, et al., 2014). Whether cachectic muscle retains the ability to induce protein synthesis in the presence of
elevated systemic IL-6 and muscle gp130/STAT3 signaling has not been fully explored, which could have therapeutic implications for the cachectic cancer patient.

Exercise is a non-pharmacological treatment approach that improves indices of health related to muscle and systemic function in healthy individuals and also those with chronic disease (Hurley, Hanson, & Sheaff, 2011; Zinna & Yarasheski, 2003). Skeletal muscle mass is highly influenced by mechanically induced changes in protein synthesis, and the activation of mTORC1 is required for increased protein synthesis in response to mechanical loading (Bodine, Stitt, et al., 2001; Drummond, Fry, et al., 2009; Goodman, Frey, et al., 2011). ECC-induced by high-frequency electrical stimulation have been used to examine signaling associated with muscle hypertrophy in rodents (Baar & Esser, 1999; Nader & Esser, 2001; Witkowski et al., 2010), and have demonstrated great utility for improving our mechanistic understanding of contraction-induced protein synthesis and mTORC1 signaling (Jacobs et al., 2013; O'Neil et al., 2009; West et al., 2016). Related to cancer cachexia, there is evidence that repeated ECC bouts can attenuate muscle and myofiber atrophy in tumor-bearing mice (al-Majid & McCarthy, 2001; Hardee et al., 2016). While we have found that repeated ECC bouts could attenuate myofiber atrophy independent to changes in plasma IL-6 levels (Hardee et al., 2016), significant gaps remain in our understanding of protein synthesis activation by ECC during cancer cachexia. Moreover, we have found that tumor-derived cachectic factors blocked the mechanical activation of protein synthesis in vitro (Gao & Carson, 2016), which could have significant ramifications on protein synthesis activation by ECC. Thus, the interaction between mechanical signaling and systemic inflammatory cytokines on muscle protein synthesis regulation requires further investigation.
While the suppression of basal protein synthesis may not fully account for the severe muscle wasting that accompanies cancer (Horstman et al., 2016), it has been recognized that the inability to stimulate protein synthesis in response to anabolic stimuli (e.g., anabolic resistance) can have significant long-term ramifications on skeletal muscle homeostasis (Fry & Rasmussen, 2011; Walker et al., 2011). However, our understanding of muscles ability to induce protein synthesis following mechanical stimuli in the presence of a systemic cachectic environment has not been fully elucidated. Therefore, the purpose of this study was to determine IL-6 regulation of basal and mechanically induced protein synthesis and mTORC1 signaling during cancer cachexia. The interaction between IL-6 and mechanical stimulation was examined in C2C12 myotubes and skeletal muscle from MIN and skeletal-muscle specific gp130 KO mice. We hypothesized that IL-6 would block the mechanical activation of protein synthesis in atrophic muscle. To test this hypothesis, we first examined long-term IL-6 regulation of myotube stretch-induced protein synthesis. We then examined systemic IL-6 overexpression regulation of ECC-induced protein synthesis in B6 and MIN mice. Lastly, we examined IL-6 overexpression regulation of ECC-induced protein synthesis in gp130 KO mice. We provide evidence that elevated STAT3 and AMPK activation by IL-6 disrupts mechanically induced protein synthesis in skeletal muscle.

4.3 METHODS

Animals

Male MIN mice were originally purchased from Jackson Laboratories (Bar Harbor, ME), bred at the University of South Carolina’s Center for Colon Cancer Research Mouse Core, and obtained from the investigators breeding colony for the current experiments.
Littermates lacking the Apc mutation (B6) were used as controls. In addition, male LoxP-flanked (floxed) mice for IL-6ST gene (provided by Dr. Colin Stewart in collaboration with Dr. Lothar Hennighausen) were crossed with heterozygous myosin light chain If (Mlc)-Cre mice (provided by Dr. Steven Burden; ref (Bothe, Haspel, Smith, Wiener, & Burden, 2000)). The offspring positive for Cre were crossed with IL-6STlox/lox to obtain breeding pairs for generation of the mice used in the current study: IL-6STlox/lox Mlc-Cre+/−, which lack gp130 expression in muscle cells (herein referred to as gp130 KO), and IL-6STlox/lox Mlc-Cre−/−, which express gp130 in muscle cells (herein referred to as gp130 WT). All mice were on a B6 background and genotyped by PCR analyses using tail genomic DNA as previously described (Mehl, Davis, Berger, & Carson, 2005; Puppa, Gao, et al., 2014). Mice were individually housed, kept on a 12:12-h light-dark cycle, and had access to standard rodent chow (cat#8604 Rodent Diet; Harlan Teklad) and water ad libitum. Body weight and food measurements were taken weekly, and the percentage body weight loss from peak body weight was calculated (B6 and MIN experiments). The University of South Carolina’s Institutional Animal Care and Use Committee approved all animal experimentation in this study.

Systemic IL-6 overexpression

In vivo intramuscular electroporation of an IL-6 overexpression plasmid was used to increase circulating IL-6 levels in mice as previously described (White, Puppa, Gao, et al., 2013). The quadriceps muscle was used as a vessel to produce IL-6 and secrete it into circulation and was not used for any analyses in the study. The TA muscle used in the study was not subjected to electroporation. Briefly, mice were injected with 50 ug of the IL-6 plasmid driven by the CMV promoter, or empty control vector, into the quadriceps
muscle. Mice were anesthetized with a 2% mixture of isoflurane and oxygen (1 L/min). The leg was shaved and a small incision was made over the quadriceps muscle. Fat was dissected away from the muscle, and the plasmids were injected in a 50-μl volume of phosphate-buffered saline (PBS). A series of eight 50-ms, 100-V pulses was used to promote uptake of the plasmid into myofibers, and then the incision was closed with a wound clip. Both vector control (N=10/genotype) and IL-6 (N=10/genotype) groups received the appropriate plasmid starting at 12 wks. of age, and a second electroporation was performed at 13 wks. of age to maintain elevated plasma IL-6 levels. At 14 wks. of age mice performed a single ECC bout and were sacrificed 3h post-ECC.

Eccentric contractions (ECC)

ECC of the TA muscle was induced by high-frequency electrical stimulation of the sciatic nerve as previously described with slight modifications (Baar & Esser, 1999; Hardee et al., 2016). Mice were anesthetized via isoflurane (2% in O₂ with 1.5% maintenance), the stimulated leg was shaved at the hip region, and two needle electrodes were placed subcutaneously to stimulate the sciatic nerve. Tetanic muscle contractions of one hind limb were generated using a Grass Stimulator (Grass Instruments) for 10 sets of 6 repetitions (100 Hz, 6-12V, 1ms duration). Ten seconds of rest was given between stimulations and 50 seconds of rest was given between sets. The stimulation protocol recruits all motor units and results in net plantar flexion of the ankle (Wong & Booth, 1988, 1990). The dorsiflexors (TA and EDL) undergo ECC while the plantar flexors (gastrocnemius, soleus, and plantaris) perform concentric muscle contractions. In all experiments, the stimulated TA performed ECC while the non-stimulated TA served as intra-animal control. Our laboratory and others have demonstrated repeated ECC bouts, but not concentric
contractions, can induce muscle and myofiber growth in rodents (al-Majid & McCarthy, 2001; Baar & Esser, 1999; Hardee et al., 2016; Wong & Booth, 1988). Therefore, the TA was examined in all experiments. Mice were given an intraperitoneal injection of warm saline following the stimulation procedure and returned to cages upon complete recovery.

*Tissue Collection*

Mice received an intraperitoneal injection of puromycin (0.04 umol/kg body weight) 30 minutes prior to sacrifice (Goodman, Mabrey, et al., 2011; Narsale et al., 2016). Mice were anesthetized with a subcutaneous injection of ketamine / xylazine / acepromazine cocktail (1.4 ml/kg body weight) at the time of sacrifice. Muscles and organs were rapidly excised, cleared of excess connective tissue, rinsed in phosphate-buffered saline (PBS), dried on blotting paper, weighed, and snap frozen in liquid nitrogen. Immediately prior to dissection blood was collected via retro-orbital sinus with heparinized capillary tubes, placed on ice, and centrifuged (10,000 x g for 10 min at 4°C). The supernatant was removed and stored for plasma IL-6 analysis. Plasma and tissue samples were stored at -80°C until analysis.

*C2C12 cell culture*

C2C12 myoblasts purchased from the American Type Culture Collection (Manassas, VA) were cultured in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 ug/ml streptomycin. Myoblasts were then suspended (1x10^6 cells/mL) and plated onto Silastic membrane mounted in a stretching device (~1.5–2x10^5 cells/stretching device). The cells were grown to ~95% confluence and differentiated into myotubes. To induce differentiation, C2C12 myoblasts were incubated in DMEM supplemented with 2% heat-
inactivated horse serum, 50 U/ml penicillin, and 50 ug/ml streptomycin. After 96 h
differentiation myotubes were treated with control (DMSO) or recombinant IL-6 (100
ng/mL, Sigma) for 24 h. To induce 5% stretch, screw nuts on both axles of the stretching
device were rotated by 1.5 cycles using sterile forceps after 20h of IL-6 exposure.
Myotubes were constantly stretched during the last 4h of IL-6 exposure. Protein was
collected as previously described (Gao & Carson, 2016; White, Puppa, Gao, et al., 2013).

**Western Blotting**

Western blot analysis was performed as previously described [36]. Briefly, frozen
TA muscle was homogenized in Mueller buffer and protein concentration was determined
by the Bradford method. Crude TA muscle homogenates and myotube extracts were
fractionated on 7-15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred
to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau
red to verify equal loading and transfer. Membranes were then blocked at room
temperature (RT) for 1-2h in 5% non-fat milk Tris-buffered saline with 0.1% Tween-20
(TBST). Primary antibodies for puromycin (Millipore, cat#MABE343, 1:2000), phospho
P70S6K (T389) (cat#9205, 1:1000), total P70S6K (cat#2708, 1:1000), RPS6 (S240/244)
(cat#2215, 1:500), total RPS6 (cat#2708, 1:1000), phospho Akt (S473) (cat#4060, 1:1000),
total Akt (cat#9272, 1:2000), phospho NFκB (S563) (cat#3033, 1:500), total NFκB
(cat#4764, 1:2000), phospho STAT3 (Y750) (cat# 9145, 1:1000), total STAT3 (cat#4904,
1:2000), phospho AMPK (T172) (cat#, 1:2000), total AMPK (cat#2603, 1:1000), phospho
ACC (S59) (cat#3661, 1:1000), total ACC (cat#3662), and GAPDH (cat#2118, 1:10000)
were incubated overnight in 5% TBST milk. Membranes were then incubated in 5% milk-
TBST containing anti-rabbit (cat#7074, 1:5000) or anti-mouse (cat#7076, 1:5000) IgG
horseradish-peroxidase conjugated secondary antibodies for 1h at RT. Exceptions to the aforementioned procedures were that for puromycin incorporation 1% BSA-TBST was used for primary antibody and horseradish-peroxidase conjugated rabbit anti-mouse IgG2a antibody (LifeTechnologies, cat#610220, 1:5000) in 5% milk-TBST was used for secondary antibody. All antibodies were from Cell Signaling Technology unless otherwise stated. Tibialis anterior protein extracts from a mouse that did not receive puromycin at sacrifice was included on all puromycin gels as a negative control. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences) was used to visualize the antibody-antigen interactions. Immunoblot images were collected using a digital imager (SynGene GBox) and quantified by densitometry using imaging software (Image J; NIH). Each gel contained samples from all groups and data was normalized to the respective control group (e.g. B6 control).

Plasma Interleukin-6 Concentration

Plasma IL-6 concentrations were determined as previously described [37]. A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences and the manufacturer’s protocol was followed. Briefly, clear 96-well plates were coated and incubated overnight with an IL-6 capture antibody. The next morning the plate was blocked with assay diluent buffer, washed, and equal volumes of standards and plasma samples were added in duplicate. After a 2h incubation the plate was washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read at 450 nm using an iMark microplate absorbance reader (Bio-Rad Laboratories).
**Statistical Analysis**

Results are reported as the means ± standard error. Data were analyzed by two-way ANOVA with Student-Newman-Keuls methods post hoc when appropriate. The accepted level of significance was set at p<0.05 for all analysis. Statistical analysis was performed using GraphPad (Prism 5 for Mac OS X, La Jolla, CA).

**4.4 RESULTS**

*IL-6 regulation of C\textsubscript{2}C\textsubscript{12} myotube stretch-induced muscle protein synthesis*

We have previously found that long-term IL-6 treatment suppressed mTORC1 signaling and recapitulated wasting mechanisms observed during cancer cachexia progression in MIN mice (White, Puppa, Gao, et al., 2013; White et al., 2012). Moreover, tumor-derived factors could disrupt the mechanical activation of protein synthesis in stretched myotubes (Gao & Carson, 2016). Therefore, we sought to determine the potential interaction between long-term IL-6 exposure and mechanical signaling in C\textsubscript{2}C\textsubscript{12} myotubes (Fig. 4.1A). Activation of IL-6 signaling was evident by increased total gp130 expression and the phosphorylation of STAT3, NFkB, and AMPK (Figure 4.1B). There was no effect of stretch alone or when combined IL-6 on total gp130 expression or the phosphorylation of STAT3, NFkB, and AMPK (Figure 4.1B). Activation of mechanical signaling was evident by increased Akt/mTORC1 signaling, as stretch induced the phosphorylation of Akt, P70S6K, and RPS6 (Figure 4.1C). In contrast, IL-6 treatment suppressed basal and blocked the stretch induction of Akt/mTORC1 signaling (Figure 4.1C). Similarly, while stretch activated protein synthesis, IL-6 suppressed basal and blocked the stretch induction
These data demonstrate that long-term IL-6 exposure was sufficient to disrupt the mechanical activation of mTORC1 signaling and protein synthesis, and was associated with the sustained activation of STAT3 and AMPK in myotubes.

**IL-6 regulation of ECC-induced protein synthesis in tumor bearing mice**

We have previously found that systemic IL-6 overexpression can induce cachexia and suppress mTORC1 signaling in male tumor bearing MIN mice (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Puppa et al., 2012). Therefore, we then examined if systemic IL-6 was sufficient to disrupt the mechanical activation of protein synthesis. To accomplish this, mice performed a single ECC bout following 2 wks. systemic IL-6 overexpression (Figure 4.2A). Systemic IL-6 overexpression increased plasma IL-6 levels in both B6 and MIN mice (Figure 4.2B; Table 4.1), however IL-6-induced body weight loss in MIN mice (Figure 4.2C; Table 4.1). Systemic IL-6 did not affect body weight, muscle mass, or epididymal fat in WT mice (Table 4.1). In contrast, systemic IL-6 overexpression decreased tibialis anterior and epididymal fat mass in MIN mice (Table 4.1), and plasma IL-6 levels were negatively correlated with tibialis anterior muscle mass in MIN mice (Figure 4.2D). Interestingly, both systemic IL-6 and the presence of tumors increased spleen weight (Table 4.1). Tibia length, an index of body size, was lower in MIN mice (Table 4.1). Collectively, these results establish systemic IL-6 overexpression was sufficient to induce cachexia in tumor bearing MIN mice.

We then examined muscle inflammatory signaling activation by IL-6 and ECC in B6 and MIN mice. The activation of muscle IL-6 signaling was confirmed by increased phosphorylation of STAT3, NFkB, AMPK, and ACC in B6 and MIN mice (Figure 4.1C).
Muscle STAT3 and NFκB activation by IL-6 was further induced by ECC in B6 mice (Figure 4.3B) induced STAT3 and NFκB signaling while decreases AMPK and ACC signaling. Muscle AMPK and ACC activation was decreased by ECC regardless of IL-6 activation in B6 mice (Figure 4.3B). IL-6 activation of muscle STAT3 was not affected by ECC in MIN mice (Figure 4.3D), while NFκB activation by IL-6 was further induced by ECC (Figure 4.3D). IL-6 activation of muscle AMPK and ACC activation was not altered by ECC in MIN mice (Figure 4.3D).

We then examined if IL-6 was sufficient to block the mechanical activation of protein synthesis and mTORC1 signaling in B6 and MIN mice. Mechanical activation of protein synthesis was evident in both B6 and MIN mice (Figure 4.4A,C). While systemic IL-6 overexpression suppressed protein synthesis regardless of tumor presence (Figure 4.4A,C), ECC-induced protein synthesis in B6 mice (Figure 4.4A). Interestingly, IL-6 overexpression blocked ECC-induced protein synthesis in MIN mice (Figure 4.4C). The activation of mechanical signaling was evident by increased P70S6K and RPS6 signaling in both B6 and MIN mice (Figure 4.4B,D). While systemic IL-6 overexpression reduced P70S6K and RPS6 signaling, ECC-induced P70S6K and RPS6 signaling in both B6 and MIN mice (Figure 4.4B,D). These findings demonstrate that IL-6 can regulate basal protein synthesis regardless of cancer, and disrupted the mechanical activation of protein synthesis in MIN mice.

IL-6 regulation of ECC-induced protein synthesis in gp130 KO mice.

To elucidate possible mechanisms involved in IL-6 suppression of basal and ECC-induced mTORC1 signaling and protein synthesis, we generated mice lacking the gp130
receptor specifically in skeletal muscle. Mice lacking gp130 (gp130 KO) and their littermates (gp130 WT) were subjected to identical experimental procedures as previously described, and were sacrificed 3h post a single ECC bout following 2-wks. systemic IL-6 overexpression. There was no effect of gp130 loss on several animal characteristics such as body weight or muscle mass (Table 4.2), as we have previously observed (Puppa, Gao, et al., 2014). Furthermore, while systemic IL-6 overexpression increased plasma IL-6 levels compared to vector controls, it did not induce body weight loss or muscle atrophy in WT or KO mice (Table 4.2). To verify muscle gp130 loss in these mice, we examined gp130 protein expression in the non-stimulated TA muscle. As expected, gp130 protein was reduced in KO mice (Figure 4.5A). Interestingly, there was a trend to increase gp130 protein expression with systemic IL-6 overexpression in WT mice (Figure 4.5A). In contrast to our previous observation, systemic IL-6 overexpression suppressed basal protein synthesis, and blocked its induction by ECC in WT mice (Figure 4.5B). However, muscle gp130 loss blocked basal protein synthesis suppression by IL-6, and attenuated the ECC induction (Figure 4.5C). Collectively, these findings demonstrate that gp130 can regulate IL-6 suppression of basal protein synthesis, and alter the protein synthesis induction by ECC.

4.5 DISCUSSION

Tumor-derived factors and cytokines have been implicated in cancer-induced skeletal muscle wasting through disrupted protein turnover regulation (Aversa et al., 2016; Pettersen et al., 2017; White, Baynes, et al., 2011). While we have previously found that tumor-derived cachectic factors blocked the mechanical activation of protein synthesis in vitro (Gao & Carson, 2016), cachectic muscle’s ability to stimulate protein synthesis in the
presence of systemically elevated IL-6 has not been widely examined. Therefore, the purpose of this study was to determine IL-6 regulation of basal and mechanically induced protein synthesis and mTORC1 signaling during cancer cachexia. The interaction between IL-6 and mechanical activation of protein synthesis and mTORC1 signaling was examined in C2C12 myotubes and in skeletal muscle from MIN mice. We found that the sustained activation of inflammatory / metabolic signaling pathways (GP130, STAT3, NFkB, AMPK) coincided with suppressed myotube stretch-induction of protein synthesis and mTORC1 signaling. Second, we examined systemic IL-6 regulation of ECC-induced protein synthesis in B6 and MIN mice. As observed in culture, muscle inflammatory / metabolic signaling pathways related to STAT3, NFkB, and AMPK were activated following systemic IL-6 overexpression, which coincided with suppressed basal protein synthesis regardless of cancer. However, the induction of skeletal muscle wasting was only observed in tumor bearing MIN mice, as we have previously observed. While ECC-induced protein synthesis following systemic IL-6 overexpression in B6 mice, this response was blocked in MIN mice. Interestingly, mTORC1 signaling induction by ECC was not altered by systemic IL-6 overexpression regardless of cachexia, which demonstrates that the mechanical activation of mTORC1 signaling remained intact. Lastly, muscle gp130 receptor blocked systemic IL-6 suppression of basal protein synthesis. However, mice lacking muscle gp130 receptor demonstrated an attenuate protein synthesis response to ECC. Collectively, these results demonstrate that systemic IL-6 can regulate basal and mechanical activation of protein synthesis in skeletal muscle.

Disrupted muscle protein synthesis and mTORC1 signaling coincides with increasing levels of circulating plasma IL-6 during cancer cachexia progression (White,
Moreover, systemic IL-6 overexpression in weight stable tumor bearing mice can induce cachexia and suppress protein synthesis, and long-term IL-6 exposure can stimulate myotube atrophy associated with disrupted mTORC1 signaling (White, Puppa, Gao, et al., 2013). We extend these findings by demonstrating that long-term IL-6 suppression of protein synthesis and mTORC1 signaling coincides with the activation of inflammatory / metabolic signaling pathways (STAT3, NFkB, AMPK) in both skeletal muscle and cultured myotubes. Interestingly, muscle atrophy following systemic IL-6 overexpression was only observed in tumor-bearing mice despite similar plasma IL-6 levels and muscle inflammatory / metabolic signaling activation. These findings are in agreement with previous research demonstrating preserved muscle mass and myofiber area despite elevated IL-6 levels in healthy mice (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; J. L. Chen et al., 2016). While speculative, it is feasible that other cytokines such as LIF or TNFα could work in concert with IL-6 to disrupt similar or distinct pathways that synergize the wasting process. Furthermore, cancer can promote a cachectic environment that may sensitize muscle to systemic inflammatory insult, however the mechanisms involved in this cellular response have not been determined. We have recently found that while female MIN mice are protected against IL-6-induced wasting during the initiation of cachexia, however the loss of ovarian function was associated with severe cachexia and increased sensitivity to muscle inflammatory signaling (Hetzler, Hardee, LaVoie, Murphy, & Carson, 2017). While we have found that male MIN mice are susceptible to IL-6-induced cachexia across all stages of tumor burden, there is a strong relationship between cachexia severity and muscle STAT3/GP130 expression. Further
research is required to determine if the link between systemic factors and intrinsic muscle processes requires muscle GP130 signaling.

Nonetheless, we have found strong relationship between systemic IL-6 and muscle AMPK in skeletal muscle (White, Baynes, et al., 2011). AMPK activation has been implicated in skeletal muscle metabolism and in the suppression of mTORC1 signaling (Bolster et al., 2002; Jorgensen, Richter, & Wojtaszewski, 2006). Short-term activation of AMPK is associated with glucose homeostasis and the metabolic benefits of exercise, whereas the long-term activation of AMPK can suppress mTORC1 activity (Mounier, Theret, Lantier, Foretz, & Viollet, 2015). Indeed, AMPK inhibits mTORC1 through the phosphorylation of TSC2 and Raptor (Gwinn et al., 2008; Inoki et al., 2003; Shaw, 2009). The phosphorylation of TSC2 by AMPK inhibits mTORC1 and S6K1 activity (Inoki et al., 2002; Inoki et al., 2003). Moreover, phosphorylation of the mTORC1 binding partner Raptor by AMPK at two highly conserved serine residues (S722 and S792) leads to the suppression of mTORC1 kinase activity (Gwinn et al., 2008). Interestingly, while systemic IL-6 overexpression induced AMPK activation and suppressed protein synthesis, this was not associated with muscle wasting in healthy mice. Whether the suppression of mTORC1 signaling was associated with the induction of autophagy or ubiquitin proteasome activation should be investigated in future studies. A negative protein balance would need to be achieved to induce wasting, however indices of these processes were not examined. Related to cachexia, we extend previous findings and demonstrate that systemic IL-6 overexpression is associated with STAT3/AMPK activation and muscle atrophy in tumor-bearing MIN mice. We then examined if cachectic muscle could respond to mechanical stimuli after long-term IL-6 exposure in vitro and in vivo. We found that long-term IL-6
blocked the mechanical activation of protein synthesis in atrophic myotubes and mouse skeletal muscle. Interestingly, while myotubes demonstrated a blunted mechanical response, mouse skeletal muscle retained the ability to activate mTORC1 signaling. We have also observed similar responses using LLC conditioned media in culture and in LLC tumor bearing mice. While protein synthesis activation was blocked, we have found that the mTORC1 signaling activation by ECC is maintained in cachectic muscle from LLC tumor-bearing mice (unpublished observations). Further, LLC tumor derived cachectic factors disrupted myotube stretch induced protein synthesis was blocked in culture (Gao & Carson, 2016). Interestingly, the current study found a similar cachectic muscle response to ECC as we have observed in LLC mice, which is in contrast to previous observations during the natural progression of cachexia in MIN mice. This is of interest, as while the cancer environments may be different, the rate of wasting may also be a predictive measure of the ability to respond to mechanical stimuli. We have found that cachectic muscle during the natural progression (e.g., 6-8 wks. of wasting) remained plastic to protein synthesis induction by ECC, whereas the rapid induction by IL-6 overexpression blocked this activation. While we have not mechanistically determined the differences why this occurs, future investigations should determine if the rate of wasting determines a muscles ability to induce anabolic processes. Given that overall mass is tightly regulated by fluctuations between anabolic and catabolic processes, it is imperative that we define how protein synthesis can be modulated in response to various cachectic stimuli.

**Conclusion**

In summary, we examined IL-6 regulation of basal and ECC-induced protein synthesis and mTORC1 signaling during cancer cachexia. We found that IL-6 suppressed
basal protein synthesis and mTORC1 signaling in skeletal muscle and cultured myotubes. Furthermore, IL-6 blocked the mechanical activation of protein synthesis in atrophic skeletal muscle and myotubes. While long-term IL-6 blocked myotube stretch-induced mTORC1 signaling, cachectic muscle remained responsive to ECC in tumor bearing mice. Interestingly, muscle gp130 loss blocked IL-6 suppression of basal protein synthesis, but also attenuated the protein synthesis response to ECC. Collectively, these studies demonstrate that systemic IL-6 and muscle GP130 disrupt basal and ECC-induced protein synthesis. Further work is necessary to determine if AMPK activation by IL-6/GP130 signaling is required for protein synthesis suppression.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grants R01 CA-121249 (National Cancer Institute) and P20 RR-017698 (National Institute of General Medical Science) to J.A.C.
Table 4.1. B6 and MIN mice that performed a single ECC bout following 2 wk systemic IL-6 overexpression.

<table>
<thead>
<tr>
<th></th>
<th>B6 Vector</th>
<th>B6 IL-6</th>
<th>MIN Vector</th>
<th>MIN IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>10</td>
<td>12</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>24.7 ± 0.3</td>
<td>24.9 ± 0.5</td>
<td>23.1 ± 0.7</td>
<td>22.0 ± 0.6</td>
</tr>
<tr>
<td>Post</td>
<td>26.2 ± 0.3†</td>
<td>25.8 ± 0.5†</td>
<td>22.9 ± 0.8</td>
<td>20.2 ± 0.6†</td>
</tr>
<tr>
<td>% Change from Pre</td>
<td>5.9 ± 1.0</td>
<td>3.7 ± 0.9</td>
<td>-0.7 ± 1.7</td>
<td>-8.2 ± 1.0</td>
</tr>
<tr>
<td>Tibialis anterior, mg</td>
<td>47 ± 1.0</td>
<td>46 ± 0.8</td>
<td>39 ± 1.9†</td>
<td>32 ± 2.0†</td>
</tr>
<tr>
<td>Epididymal fat, mg</td>
<td>298 ± 15</td>
<td>292 ± 16</td>
<td>207 ± 29†</td>
<td>100 ± 37†</td>
</tr>
<tr>
<td>Spleen, mg</td>
<td>77 ± 8</td>
<td>129 ± 6</td>
<td>299 ± 45†</td>
<td>428 ± 34†</td>
</tr>
<tr>
<td>Testes, mg</td>
<td>197 ± 5</td>
<td>212 ± 4</td>
<td>192 ± 7†</td>
<td>154 ± 13†</td>
</tr>
<tr>
<td>LABC, mg</td>
<td>82 ± 3</td>
<td>81 ± 2</td>
<td>67 ± 5†</td>
<td>48 ± 5†</td>
</tr>
<tr>
<td>Seminal vesicle, mg</td>
<td>239 ± 12</td>
<td>229 ± 9</td>
<td>154 ± 23</td>
<td>86 ± 20</td>
</tr>
<tr>
<td>Plasma IL-6, pg/ml</td>
<td>0 ± 0</td>
<td>112 ± 17 &amp;</td>
<td>31 ± 5*</td>
<td>130 ± 26 &amp;</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>16.9 ± 0.1</td>
<td>16.9 ± 0.1</td>
<td>16.6 ± 0.1#</td>
<td>16.6 ± 0.1#</td>
</tr>
</tbody>
</table>

Data are means ± standard error. There were no differences in the non-stimulated and ECC muscles, therefore the average tibialis anterior muscle is shown. A two-way ANOVA with Student-Knewman-Keuls post hoc methods were performed when appropriate. Statistical significance was set at p<0.05. Abbreviations: ECC, Eccentric contractions. No., number. g, grams. mg, milligrams. pg, picogram. ml, milliliter. mm, millimeter. LABC, levator ani / bulbocavernosus muscle. * = significantly different from B6. † = significantly different to all groups. # = main effect of MIN. & = main effect of IL-6.
Table 4.2. Muscle GP130 WT and KO mice that performed a single ECC bout following 2 wk systemic IL-6 overexpression.

<table>
<thead>
<tr>
<th></th>
<th>WT Vector</th>
<th>WT IL-6</th>
<th>KO Vector</th>
<th>KO IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>24.8 ± 0.5</td>
<td>24.9 ± 0.4</td>
<td>26.3 ± 0.8</td>
<td>25.6 ± 0.6</td>
</tr>
<tr>
<td>Post</td>
<td>25.7 ± 0.4†</td>
<td>25.5 ± 0.2†</td>
<td>27.0 ± 0.8†</td>
<td>26.4 ± 0.5†</td>
</tr>
<tr>
<td>% Change from Pre</td>
<td>3.9 ± 1.9</td>
<td>2.8 ± 0.8</td>
<td>2.8 ± 0.6</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>Tibialis anterior, mg</td>
<td>47 ± 0.6</td>
<td>47 ± 1.6</td>
<td>49 ± 1.4</td>
<td>48 ± 1.1</td>
</tr>
<tr>
<td>Epididymal fat, mg</td>
<td>274 ± 59</td>
<td>270 ± 23</td>
<td>295 ± 26</td>
<td>292 ± 26</td>
</tr>
<tr>
<td>Spleen, mg</td>
<td>87 ± 5</td>
<td>110 ± 11&amp;</td>
<td>76 ± 4</td>
<td>129 ± 23&amp;</td>
</tr>
<tr>
<td>Testes, mg</td>
<td>185 ± 9</td>
<td>182 ± 5</td>
<td>211 ± 4#</td>
<td>224 ± 7#</td>
</tr>
<tr>
<td>LABC, mg</td>
<td>91 ± 2</td>
<td>84 ± 4</td>
<td>83 ± 2</td>
<td>84 ± 0.9</td>
</tr>
<tr>
<td>Seminal vesicle, mg</td>
<td>259 ± 12</td>
<td>256 ± 14</td>
<td>257 ± 15</td>
<td>273 ± 16</td>
</tr>
<tr>
<td>Plasma IL-6, pg/ml</td>
<td>0 ± 0</td>
<td>210 ± 43&amp;</td>
<td>0 ± 0</td>
<td>203 ± 42&amp;</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>16.8 ± 0.1</td>
<td>16.9 ± 0.1</td>
<td>17.0 ± 0.1</td>
<td>16.9 ± 0.1</td>
</tr>
</tbody>
</table>

Data are means ± standard error. There were no differences in the non-stimulated and ECC muscles, therefore the average tibialis anterior muscle is shown. A two-way ANOVA with Student-Knewman-Keuls post hoc methods were performed when appropriate. Statistical significance was set at p<0.05. Abbreviations: ECC, Eccentric contractions. No., number. g, grams. mg, milligrams. pg, picogram. ml, milliliter. mm, millimeter. LABC, levator ani / bulbocavernosus muscle. † = significantly different from pre body weight. # = main effect of KO. & = main effect of IL-6.
Figure 4.1. Long-term IL-6 regulation of myotube stretch-induced protein synthesis. A) Experimental Design. B) Myotube inflammatory signaling regulation by IL-6 and stretch. C) Myotube mTORC1 signaling and protein synthesis regulation by IL-6 and stretch. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to Control values. Dotted lines indicate images were cropped for representative purposes. Data are means ± standard error. A two-way ANOVA was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knnewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. # = main effect of IL-6.
Figure 4.2. Systemic IL-6 regulation of cachexia initiation in tumor-bearing MIN mice. A) Experimental Design. B) Plasma IL-6 levels following 2 wks systemic IL-6 overexpression. C) Body weight loss following 2 wks systemic IL-6 overexpression. D) Correlation between tibialis anterior muscle mass and plasma IL-6 levels following 2 wks systemic IL-6 overexpression. Data are means ± standard error. A two-way ANOVA was
used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Linear regression was performed to determine the relationship between tibialis anterior muscle mass and plasma IL-6 levels. Statistical significance was set at p<0.05. * = significantly different to C57BL/6 Vector. † = significantly different to all groups. ‡ = significantly different to all groups.
Figure 4.3. Systemic IL-6 regulation of muscle inflammatory signaling in tumor-bearing MIN mice. A) Muscle inflammatory signaling regulation by IL-6 in B6 mice. B) Muscle inflammatory signaling regulation by IL-6 and ECC in B6 mice. C) Muscle
inflammatory signaling regulation by IL-6 in MIN mice. D) Muscle inflammatory signaling regulation by IL-6 and ECC in MIN mice. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to Control values. Data are means ± standard error. Student’s t-test or two-way ANOVA was used to determine differences between treatment groups when appropriate. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. * = significantly different to Vector within genotype. Different letters are statistically different.
Figure 4.4. Systemic IL-6 regulation of muscle mTORC1 signaling and protein synthesis in tumor-bearing MIN mice. A) Muscle protein synthesis regulation by IL-6 and ECC in B6 mice. B) Muscle mTORC1 signaling regulation by IL-6 and ECC in B6 mice.
mice. C) Muscle protein synthesis regulation by IL-6 and ECC in MIN mice. D) Muscle mTORC1 signaling regulation by IL-6 and ECC in MIN mice. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to Control values. Data are means ± standard error. A repeated measures two-way ANOVA was used to determine differences between treatment groups when appropriate. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. # = main effect of pIL-6. $ = main effect of ECC.
Figure 4.5. Systemic IL-6 and muscle gp130 regulation of muscle mTORC1 signaling and protein synthesis in tumor free mice. A) Muscle gp130 protein synthesis in WT and KO mice. B) Muscle mTORC1 signaling regulation by IL-6 and ECC in WT mice. C) Muscle protein synthesis regulation by IL-6 and ECC in KO mice. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to Control values. Data are means ± standard error. A repeated measures two-way ANOVA was used to determine differences between treatment groups when appropriate. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical
significance was set at p<0.05. Different letters are statistically different. # = main effect of pIL-6. $ = main effect of ECC.
CHAPTER 5

TRAINING REGULATION OF ECCENTRIC CONTRACTION-INDUCED PROTEIN SYNTHESIS IN CACHECTIC SKELETAL MUSCLE

5.1 ABSTRACT

Exercise training has been postulated to attenuate cancer-induced skeletal muscle wasting, the acute responses and training adaptations to ECC in cachectic muscle have not been fully elucidated. We have found that while a single ECC bout could stimulate mTORC1 signaling in cachectic muscle, the protein synthesis induction remained suppressed compared to healthy controls. Interestingly, muscle STAT3/NFκB inhibition increased basal and ECC-induced protein synthesis in cachectic MIN mice. We have also found that ECC training could attenuate myofiber atrophy which coincided with suppressed muscle inflammatory / metabolic signaling and improved oxidative capacity. However, whether training improves the regulation of basal and ECC-induced protein synthesis during cancer cachexia has not been investigated. Therefore, the purpose of this study was to determine if training could improve basal and ECC-induced protein synthesis and mTORC1 signaling during cancer cachexia. Male MIN mice initiating cachexia performed repeated ECC bouts over 2 wks. and were sacrificed either 3h (acute response) or 48 (training response) after the last bout. The stimulated tibialis anterior (TA) muscle performed contractions while the non-stimulated TA served as the intra-animal control. Age-matched B6 mice served as healthy controls. ECC training improved basal protein synthesis and mTORC1 regulation in MIN mice, which coincided with improved oxidative capacity, altered autophagy flux, and suppressed STAT3/AMPK activation. Further, the activation of protein synthesis and mTORC1 signaling by a single ECC bout was maintained following training in B6 and MIN mice. Collectively, these results demonstrate that training can improve basal and ECC-induced protein synthesis and mTORC1
Keywords: ApcMin/++; Cancer Cachexia; Eccentric contractions; Glycoprotein 130; Interleukin-6; Muscle Protein Synthesis

5.2 INTRODUCTION

Cancer cachexia is a complex, metabolic wasting syndrome characterized by the loss of skeletal muscle, and accounts for 40% of all cancer-related deaths (Bruera, 1997; Tisdale, 2009). Adverse consequences of skeletal muscle depletion include reduced anticancer therapy tolerance, increased susceptibility to treatment toxicity, and decreased patient quality of life (Barret et al., 2014; Evans et al., 2008; H. W. Jung et al., 2015). While many pharmaceutics have been proposed to treat this wasting condition, many have failed to improve mass and metabolic quality due to the singular specificity and adverse side effects (S. Cohen et al., 2015). Exercise is a non-pharmacological treatment approach that improves indices of health related to muscle function and metabolism in healthy individuals (Hurley et al., 2011; Zinna & Yarasheski, 2003), and has demonstrated benefits on treatment and survival outcomes in cancer patients (Brown, Winters-Stone, Lee, & Schmitz, 2012; Hardee, Porter, Sui, et al., 2014; Hojman, Gehl, Christensen, & Pedersen, 2017). However, whether these outcomes can be achieved in the cachectic cancer patient has not yet been investigated (Grande, Silva, & Maddocks, 2015; Grande et al., 2014a). Thus, there are significant gaps that remain in our understanding of the interactions between exercise and the cachectic cancer environment, which hinders the use of exercise as an effect treatment strategy.
Despite the limitations in our mechanistic understanding of physical activity based therapies in cachectic cancer patients, the acute responses and training adaptations to exercise are actively being examined in preclinical cancer cachexia models. Exercise feasibility becomes a significant barrier when studying cachectic tumor-bearing mice as they are unable to perform vigorous exercise (e.g., treadmill exercise, ladder climbing). Therefore, our laboratory has utilized electrical stimulation of the sciatic nerve to examine the molecular and cellular responses to muscle contraction during severe cachexia. We have found that cancer cachexia disrupted the metabolic and anabolic signaling response to a single concentric muscle contraction bout induced by low-frequency electrical stimulation (Puppa, Murphy, et al., 2014). In contrast, ECC-induced by high-frequency electrical muscle stimulation stimulated protein synthesis in cachectic muscle, however the absolute response remained suppressed compared to healthy skeletal muscle (Hardee et al., 2017). Moreover, repeated ECC bouts started at the time of tumor implantation or after cachexia development attenuated muscle and myofiber atrophy, respectively (al-Majid & McCarthy, 2001; Hardee et al., 2016). However, neither of these ECC training studies examined the cellular processes related to growth, nor significant gaps remain in our understanding of the regulation of protein synthesis by ECC during cancer cachexia.

Skeletal muscle mass is maintained through daily fluctuations in protein synthesis and protein breakdown (Schiaffino & Mammucari, 2011), often referred to as protein turnover or proteostasis. While protein breakdown has an established role in muscle wasting, less is known related to the regulation of protein synthesis during cancer cachexia. In healthy skeletal muscle, protein synthesis is highly responsive to nutrients, physical activity level, and growth factors/hormones (Kimball et al., 2002; Schiaffino &
Mammucari, 2011). Protein kinase B (PKB, also known as Akt) and the mechanistic target of rapamycin complex 1 (mTORC1) have established roles for the integration of upstream signaling pathways to regulate protein synthesis (Kimball et al., 2002; Laplante & Sabatini, 2009; Schiaffino & Mammucari, 2011). While growth factors such as insulin and insulin-like growth factor-1 (IGF-1) stimulate mTORC1 signaling through the activation of Akt (Kimball et al., 2002; Schiaffino & Mammucari, 2011), muscle contraction and mechanical loading can induce Akt/mTORC1 signaling independent of classical IGF-1 receptor and phosphatidylinositol 3 kinase (PI3K) activation (Witkowski et al., 2010). mTORC1 activation promotes protein synthesis by phosphorylating the eukaryotic initiation factor 4E (4E-BP1) and the p70 ribosomal S6 kinase (S6K1). The hyperphosphorylation of 4E-BP1 prevents binding to eukaryotic initiation factor 4E (eIF4E) and the formation of 4E-BP1-eIF4E complex, resulting in the assembly of the eIF4F complex and translation initiation (Kimball et al., 2002). In addition, S6K1 activation by mTORC1 has been implicated in cap-dependent translation, translation elongation, and ribosomal biogenesis (Bentzinger et al., 2013; Bentzinger et al., 2008; Laplante & Sabatini, 2009). Cancer cachexia disrupts Akt/mTORC1 signaling leading to the suppression of basal protein synthesis (Samuels et al., 2001; Smith & Tisdale, 1993; White, Baynes, et al., 2011). Moreover, cachectic mice demonstrate an impaired response to nutrients and muscle contraction (Puppa, Murphy, et al., 2014; White, Puppa, Gao, et al., 2013). Given that ECC can induce protein synthesis and mTORC1 signaling in healthy skeletal muscle (Baar & Esser, 1999), this may be a viable treatment approach to improve basal protein synthesis regulation and improve the acute response to anabolic stimuli.
While evidence suggest a single ECC bout can stimulate protein synthesis (Hardee et al., 2017) and repeated ECC can prevent muscle and myofiber atrophy in tumor-bearing mice (al-Majid & McCarthy, 2001; Hardee et al., 2016), it is currently unknown if ECC training improves basal and ECC-induced protein synthesis and mTORC1 signaling during cancer cachexia. Therefore, the purpose of this study was to determine if ECC training could improve basal protein synthesis and mTORC1 regulation, and improve the anabolic response to a single ECC bout. We hypothesized that ECC training would improve suppressed basal ECC-induced protein synthesis and mTORC1 signaling during severe cancer cachexia. To test this hypothesis, MIN mice that had initiated cachexia performed repeated ECC bouts (8 total sessions) and were sacrificed either 48h (basal training effect) or 3h (acute ECC effect) after the last ECC bout. Our findings demonstrate that ECC training improved basal and ECC-induced protein synthesis and mTORC1 regulation in MIN mice.

5.3 METHODS

Animals

Male MIN mice on a B6 (B6) background were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at the University of South Carolina’s Animal Resource Facility. All mice used in the current study were obtained from the investigators breeding colony within the Center for Colon Cancer Research Mouse Core. Mice were individually housed, kept on a 12:12-h light-dark cycle, and had access to standard rodent chow (#8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. Body weight measurements were taken weekly, and the percentage body weight
loss from peak body weight (~10-14 weeks of age) was calculated. Mice lacking the Apc allele mutation served as controls for all experiments. Body weight measurements were taken weekly, and the percentage body weight loss from peak body weight was calculated. The University of South Carolina’s Institutional Animal Care and Use Committee approved all animal experimentation in this study.

**Experimental Designs**

Experiment 1:  B6 (N=8) and MIN (N=8) mice were subjected to repeated ECC bouts (8 total sessions) after the initiation of cachexia. Each ECC bout was separated by at least 48h (e.g., Monday, Wednesday, Friday) and each stimulation session was performed as previously described (Hardee et al., 2016). Mice were sacrificed 48h after the last ECC bout. We have found no effect of a single ECC bout on protein synthesis at this time point (data not shown), and thus any alteration would represent the muscles adaptation to training.

Experiment 2:  B6 (N=8) and MIN (N=9) mice were subjected to repeated ECC bouts (8 total sessions) after the initiation of cachexia. Each ECC bout was separated by at least 48h (e.g., Monday, Wednesday, Friday) and each stimulation session was performed as previously described (Hardee et al., 2016). Mice were sacrificed 3h after the last ECC bout. We have previously found that a novel single ECC bout induced protein synthesis in both B6 and MIN mice at this time point (Hardee et al., 2017).

**Eccentric contractions (ECC)**

ECC of the tibialis anterior (TA) muscle was induced by high-frequency electrical stimulation of the sciatic nerve as previously described with slight modifications (Baar &
Esser, 1999; Hardee et al., 2016). Mice were anesthetized via isoflurane (2% in O\textsubscript{2} with 1.5% maintenance), the stimulated leg was shaved at the hip region, and two needle electrodes were placed subcutaneously to stimulate the sciatic nerve. Tetanic muscle contractions of one hind limb were generated using a Grass Stimulator (Grass Instruments) for 10 sets of 6 repetitions (100 Hz, 6-12V, 1ms duration). Ten seconds of rest was given between stimulations and 50 seconds of rest was given between sets. The stimulation protocol recruits all motor units and results in net plantar flexion of the ankle (Wong & Booth, 1988, 1990). The dorsiflexors (TA and EDL) undergo ECC while the plantar flexors (gastrocnemius, soleus, and plantaris) perform concentric muscle contractions. In all experiments, the stimulated TA performed ECC while the non-stimulated TA served as intra-animal control. Our laboratory and others have demonstrated repeated ECC bouts, but not concentric contractions, can induce muscle and myofiber growth in rodents (al-Majid & McCarthy, 2001; Baar & Esser, 1999; Hardee et al., 2016; Wong & Booth, 1988). Therefore, the TA was examined in all experiments. Mice were given an intraperitoneal injection of warm saline following the stimulation procedure and returned to cages upon complete recovery.

Tissue Collection

Mice were anesthetized by a subcutaneous injection of ketamine / xylazine / acepromazine cocktail (1.4 ml/kg body weight) at the time of sacrifice. The TA muscles were rapidly excised, cleared of excess connective tissue, rinsed in PBS, weighed, and snap frozen in liquid nitrogen. The TA muscle was cut at the mid-belly, and frozen in liquid nitrogen. Blood was collected prior to muscle collection via retro-orbital eye bleed with heparinized capillary tubes, placed on ice, and centrifuged (10,000 x g for 10 min at 4°C).
The supernatant was removed and stored for plasma IL-6 analysis. Plasma and tissue samples were stored at -80°C until further analysis.

**Western Blotting**

Western blot analysis was performed as previously described (Hetzler et al., 2014). Frozen TA muscle was homogenized in ice-cold Mueller buffer and protein concentration was determined by the Bradford method. Non-stimulated and stimulated TA muscles from B6 and MIN mice were run on the same gels. Crude muscle homogenates were fractionated on 6-5% SDS-polyacrylamide gels and transferred to PVDF membranes overnight. Membranes were stained with Ponceau red to verify equal loading and transfer for each gel. Membranes were blocked at room temperature for 1-2 h in 5% Tris-buffered saline with 0.1% Tween-20 (TBST) milk. Primary antibodies for puromycin (Millipore, cat#MABE343, 1:2000), phospho P70S6K (T389) (cat#9205, 1:1000), total P70S6K (cat#2708, 1:1000), RPS6 (S240/244) (cat#2215, 1:500), total RPS6 (cat#2708, 1:1000), phospho Akt (S473) (cat#4060, 1:1000), total Akt (cat#9272, 1:2000), phospho NFκB (S563) (cat#3033, 1:500), total NFκB (cat#4764, 1:2000), phospho STAT3 (Y750) (cat#9145, 1:1000), total STAT3 (cat#4904, 1:2000), phospho AMPK (T172) (cat#, 1:2000), total AMPK (cat#2603, 1:1000), PGC-1α (Abcam, cat#ab54481, 1:1000), COXIV (cat#4844, 1:1000), and GAPDH (cat#2118, 1:10000) were incubated overnight in 5% TBST milk. Membranes were then incubated in 5% milk-TBST containing anti-rabbit (cat#7074, 1:5000) or anti-mouse (cat#7076, 1:5000) IgG horseradish-peroxidase conjugated secondary antibodies for 1h at RT. Exceptions to the aforementioned procedures were that for puromycin incorporation 1% BSA-TBST was used for primary antibody and horseradish-peroxidase conjugated rabbit anti-mouse IgG2a antibody
(LifeTechnologies, cat#610220, 1:5000) in 5% milk-TBST was used for secondary antibody. All antibodies were from Cell Signaling Technology unless otherwise stated. Tibialis anterior protein extracts from a mouse that did not receive puromycin at sacrifice was included on all puromycin gels as a negative control. Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences) was used to visualize the antibody-antigen interactions. Immunoblot images were collected using a digital imager (SynGene GBox) and quantified by densitometry using imaging software (Image J; NIH). Each gel contained samples from all groups and data was normalized to the respective control group (e.g. B6 control).

**Succinate dehydrogenase activity**

Succinate dehydrogenase (SDH) enzyme activity was performed as previously described to determine muscle oxidative capacity (Hardee, Puppa, et al., 2014). Briefly, frozen cross-sections were air-dried for 10 minutes, followed by incubation in a solution containing 0.2M phosphate buffer (pH 7.4), 0.1M MgCl₂, 2.4 mM nitroblue tetrazolium (NBT), and 0.2M succinic acid for 45 minutes at 37°C. Sections were then washed in dH₂O for 3 minutes, dehydrated in 50% ethanol for 2 minutes, and mounted for viewing with mounting media. Digital photographs were taken from each section at X25 magnification, and fibers were manually traced with imaging software (Image J; NIH). Whole TA muscle cross-sections were examined since fibers with high oxidative capacity are more abundant in the deep region of the muscle compared to the superficial region (Pullen, 1977). The images were converted to 8-bit gray scale (range of gray levels 0-255) images, and an integrated optical density was created by subtracting the background intensity from each myofiber. Thresholds corresponding to high SDH enzyme activity
were set manually and uniformly across all images, and myofibers were classified as having high or low SDH enzyme activities. The whole muscle containing glycolytic and oxidative regions were included in analysis. The cross-sectional area of high and low SDH enzyme activity myofibers were quantified. The analyses were performed by an investigator blinded to the treatment groups.

*Plasma Interleukin-6 Concentration*

Plasma IL-6 concentrations were determined as previously described (Hetzler et al., 2015). A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences and the manufacturer’s protocol was followed. Briefly, clear 96-well plates were coated and incubated overnight with an IL-6 capture antibody. The next morning the plate was blocked with assay diluent buffer, washed, and equal volumes of standards and plasma samples were added in duplicate. After a 2h incubation the plate was washed and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added and the reaction was developed for 20 minutes. The reaction was stopped with sulfuric acid and absorbance was read at 450 nm using an iMark microplate absorbance reader (Bio-Rad Laboratories).

*Statistical Analysis*

Results are reported as the means ± standard error. Either a Students t-test or two-way repeated measures ANOVA with Student-Knewman-Keuls post hoc methods were performed when appropriate. The accepted level of significance was set at p<0.05 for all analysis. Statistical analysis and figure generation were performed using Prism 5 for Mac OS X (GraphPad Software Inc).
5.4 RESULTS

Animal characteristics (Experiment 1)

Male B6 and MIN mice performed repeated ECC bouts over ~2 wks. and were sacrificed 48 h the last bout. MIN mice had initiated cachexia prior to the first ECC bout and continued to lose body weight during the training period (Table 5.1). MIN mice displayed several key features of severe cachexia which included body weight loss, adipose tissue depletion, muscle atrophy, elevated plasma IL-6 levels, and hypogonadal features (levator ani-bulbocavernosus [LABC] and seminal vesicle atrophy) (Table 5.1). MIN mice had smaller TA and gastrocnemius muscle mass (-33% and -37%, respectively) at sacrifice compared to B6 mice. ECC increased TA muscle mass regardless of cachexia, however there was no effect of concentric contractions on gastrocnemius muscle mass (Table 5.1). Plasma IL-6 and tumor number was elevated in MIN mice at sacrificed (Table 5.1). There were no differences in tibia length, a measure of body size, between B6 and MIN mice.

Cachexia and ECC on TA muscle cachectic signature in MIN mice (Experiment 1)

Cachectic signaling related to muscle wasting was examined in the non-stimulated TA muscle of B6 and MIN mice. Cachectic MIN muscle demonstrated enhanced inflammatory / metabolic (STAT3, AMPK) signaling; however, ECC training reduced STAT3 and AMPK activation regardless of cancer (Figure 5.1). Indices of mitochondrial content and autophagy / mitophagy were disrupted in cachectic MIN muscle (Figure 5.1B). PGC-1α and COXIV protein were reduced in MIN mice, however ECC training increased COXIV protein expression in MIN mice (Figure 5.1B). Interestingly, cachectic MIN muscle demonstrated enhanced autophagy / mitophagy (LC3-II, P62) regulation, however
ECC training reduced LC3-II and P62 protein expression regardless of cancer (Figure 5.1B). While cytochrome c oxidase (COX) enzyme activity was reduced in cachectic MIN muscle, ECC training increased COX enzyme activity regardless of cancer (Figure 5.1C). These findings demonstrate that ECC training improved muscle inflammatory / metabolic signaling regulation, which coincided with improved muscle oxidative capacity and autophagy / mitophagy regulation.

In a separate cohort of cachectic MIN mice, we have previously demonstrated that repeated ECC attenuated myofiber atrophy that coincided with improved succinate dehydrogenase enzyme activity (Hardee et al., 2016). Therefore, we examined post hoc if these alterations were related to improved cross-sectional area of myofibers containing high and low succinate dehydrogenase enzyme activity. We found that while cachexia decreased myofiber area of both high and low succinate dehydrogenase enzyme activity myofibers, ECC increased both high and low succinate dehydrogenase enzyme activity myofiber area regardless of cancer (Figure 5.2A). While cachexia resulted in a leftward shift in the disruption of high and low succinate dehydrogenase enzyme activity myofiber area, ECC-induced a rightward shift in the disruption of high and low succinate dehydrogenase enzyme activity myofiber area (Figure 5.2B,C). These findings demonstrate that ECC training attenuated atrophy of both myofibers containing high and low succinate dehydrogenase enzyme activity.

Given that a single ECC bout is a potent stimulator of protein synthesis and Akt/mTORC1 signaling (Nader & Esser, 2001; O'Neil et al., 2009; Thomson et al., 2008), we next determined if ECC training could improve basal protein synthesis and Akt/mTORC1 signaling in cachectic muscle. There was no effect of ECC training on Akt
regardless of cancer (Figure 5.3A). Interestingly, while cachexia decreased basal P70S6K phosphorylation, ECC training improved P70S6K activation in cachectic MIN muscle (Figure 5.3A). We further validated P70S6K activation through the phosphorylation of the direct P70S6K target RPS6. Similarly, cachexia decreased basal RPS6 phosphorylation, whereas ECC training increased RPS6 activation in cachectic MIN mice (Figure 5.3A). Interestingly, ECC training increased basal protein synthesis regardless of cancer (Figure 5.3B). Collectively, these findings demonstrate ECC training improved basal mTORC1 signaling and protein synthesis in cachectic MIN muscle.

*Animal characteristics (Experiment 2)*

Similar to the previous experiment, male B6 and MIN mice performed repeated ECC bouts over 2 wks. and were sacrificed 3h after the last bout. MIN mice had initiated cachexia prior to the first ECC bout and continued to lose body weight during the training period (Table 5.2). MIN mice displayed several key features of severe cachexia which included body weight loss, adipose tissue depletion, muscle atrophy, elevated plasma IL-6 levels, and hypogonadal features (levator ani-bulbocavernosus [LABC] and seminal vesicle atrophy) (Table 5.2). MIN mice had smaller TA and gastrocnemius muscle mass (-38% and -41%, respectively) at sacrifice compared to B6 mice. ECC increased TA muscle mass regardless of cachexia, however there was no effect of concentric contractions on gastrocnemius muscle mass (Table 5.2). Plasma IL-6 and tumor number was elevated in MIN mice at sacrificed (Table 5.2). There were no differences in tibia length, a measure of body size, between B6 and MIN mice.
Training regulation of ECC-induced mTORC1 signaling and protein synthesis (Experiment 2)

Having established the cachectic muscle’s response to repeated ECC bouts, we then determined if training improved ECC-induced protein synthesis and mTORC1 signaling. Therefore, anabolic signaling was examined 3h after a single ECC bout following training. ECC activated protein synthesis after training regardless of cancer. Although the relative induction by ECC was not altered by cachexia, the absolute protein synthesis rate remained suppressed relative to healthy B6 mice (Figure 5.4A). While Akt was increased by cachexia in MIN muscle, there was no effect of ECC regardless of cancer (Figure 5.4B). While cachexia decreased the phosphorylation of P70S6K, ECC-induced its activation regardless of cancer cachexia (Figure 5.4B). ECC also increased RPS6 phosphorylation regardless of cancer cachexia (Figure 5.4B). Collectively, these findings demonstrate that protein synthesis and mTORC1 signaling was induced 3h after a single ECC bout following training in cachectic MIN mice.

5.5 DISCUSSION

Physical activity and exercise interventions have clear therapeutic implications for treating and preventing muscle wasting associated with cancer cachexia. Whole-body treadmill exercise can prevent muscle mass loss in tumor bearing mice (Deuster et al., 1985; Penna et al., 2011; Salomao et al., 2010), and is sufficient to rescue suppressed muscle oxidative metabolism at the initiation of cachexia (Puppa et al., 2012; White et al., 2012). While evidence suggest a single ECC bout can stimulate protein synthesis (Hardee et al., 2017) and repeated ECC can prevent muscle and myofiber atrophy in tumor-bearing
mice (al-Majid & McCarthy, 2001; Hardee et al., 2016), it is currently unknown if ECC training improves basal and ECC-induced protein synthesis and mTORC1 signaling during cancer cachexia. Therefore, the purpose of this study was to determine if ECC training improved basal protein synthesis and mTORC1 regulation, and improved the anabolic response to a single ECC bout. We hypothesized that ECC training would improve suppressed basal ECC-induced protein synthesis and mTORC1 signaling during severe cancer cachexia. To test this hypothesis, MIN mice that had initiated cachexia performed repeated ECC bouts (8 total sessions) and were sacrificed either 48h (basal training effect) or 3h (acute ECC effect) after the last ECC bout. We found that ECC training improved protein synthesis which coincided with the enhanced oxidative metabolism and reduced inflammatory / metabolic activation. In addition, the acute ECC induction of protein synthesis and mTORC1 signaling was maintained after training in cachectic muscle. Our findings demonstrate that ECC training improved basal and ECC-induced protein synthesis and mTORC1 regulation in cachectic MIN muscle.

While the effects of exercise in the cachectic cancer patient have not been investigated, preclinical models have examined the interaction between muscle contraction and the cachectic environment. We have previously demonstrated that the cachectic environment disrupted concentric contraction induced anabolic and metabolic signaling in MIN mice (Puppa, Murphy, et al., 2014). In contrast, we found that a single ECC bout could induce protein synthesis, however the absolute induction remained suppressed compared to healthy skeletal muscle (Hardee et al., 2017). Interestingly, repeated ECC (e.g., training) attenuated myofiber atrophy that coincided with reduced inflammatory signaling and improved succinate dehydrogenase enzyme activity (Hardee et al., 2016).
We extend these findings demonstrating that ECC training improved basal protein synthesis regulation, which was linked to improved oxidative capacity and indices of autophagy/mitophagy control. Interestingly, hypertrophic growth induced by resistance exercise was accompanied by improved mitochondrial function and content (Porter et al., 2015), which is in contrast to commonly discussed training adaptations (e.g., hypertrophy, strength) associated with growth (B. Egan & Zierath, 2013). However, emerging evidence suggests that oxidative metabolism is linked to muscle protein synthesis via mTORC1 signaling. Indeed, a reciprocal relationship between mTORC1 and oxidative metabolism regulation has been observed across multiple cell types (Cunningham et al., 2007; Polak et al., 2008; Schieke et al., 2006). Inhibition of mTORC1 signaling reduces mitochondrial respiration (coupled and uncoupled), impairs TCA cycle activity, and lowers ATP production capacity (Morita et al., 2013). Related to skeletal muscle, reduced mitochondrial content and function has been observed in muscle-specific mTOR or Raptor knockout mice (Bentzinger et al., 2008; Risson et al., 2009). Interestingly, mTORC1 inactivation has also been associated with altered systemic metabolic homeostasis and a muscle atrophy/dystrophic phenotype (Bentzinger et al., 2008; Risson et al., 2009). While protein synthesis may be suppressed prior to disrupted mitochondrial function during cancer cachexia (White, Baynes, et al., 2011; White et al., 2012), future research is needed to determine the precise interactions between these cellular processes during cachexia progression. Nonetheless, the current findings demonstrate that ECC improved basal protein synthesis regulation that coincided with enhanced oxidative capacity.

Anabolic resistance, which is defined as an attenuated response to anabolic stimuli such as nutrition and exercise (Burd, Gorissen, & van Loon, 2013; Fry & Rasmussen,
has been implicated in muscle wasting associated with cancer (Horstman et al., 2016). We have found an attenuated response to glucose injection and contraction during severe cachexia (Puppa, Murphy, et al., 2014; White, Puppa, Gao, et al., 2013). While evidence suggest exercise training may be beneficial for improvements in basal protein synthesis regulation, the response to anabolic stimuli after training has not been well described. We are the first to demonstrate that the acute anabolic response to ECC was maintained after training in cachectic muscle. Interestingly, it has been postulated that the protein synthesis induction following a novel exercise bout is related to skeletal muscle remodeling rather than hypertrophic growth, whereas the response favors growth in the trained state (Damas et al., 2016). This would suggest that the protein synthesis induction by ECC following training would be partitioned towards skeletal muscle growth in cachectic muscle. While the acute anabolic response to resistance exercise is diminished in the trained state (Damas, Phillips, Vechin, & Ugrinowitsch, 2015), we did not observe this in cachectic muscle. Moreover, we have not observed indices of muscle damage following an acute bout (SS and JAC unpublished) or training in cachectic muscle (Hardee et al., 2016). Therefore, the protein synthesis and mTORC1 signaling response to ECC appears to be related to growth, however future investigations are needed to determine the precise mechanisms associated with this growth response. It is also possible that the protein synthesis induction following exercise could be replacing damaged or dysfunctional proteins, which is a positive exercise adaptive response. Nonetheless, our results collectively demonstrate that the activation of protein synthesis following training was associated with improved muscle oxidative metabolism and reduced inflammatory signaling in cachectic muscle.
In summary, we demonstrate that cachectic skeletal muscle retains the anabolic and metabolic plasticity to improve basal and ECC-induced protein synthesis in response to training. We report that ECC training improved protein synthesis which coincided with enhanced oxidative metabolism and reduced STAT3/AMPK activation. In addition, we also found that the acute ECC induction of protein synthesis and mTORC1 signaling was maintained after training in cachectic muscle. Interestingly, these data suggest that cachectic skeletal muscle remains plastic to exercise despite the presence of a systemic cachectic environment. While further research is needed to determine whether these initial improvements can be sustained over time, it appears cachectic skeletal muscle retains the capacity to adapt to ECC throughout the progression of cancer cachexia. Whether exercise training is also associated with an enhanced anabolic response to feeding or anabolic stimuli will improve our efforts for treating the cachectic cancer patient.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grants R01 CA-121249 (National Cancer Institute) and P20 RR-017698 (National Institute of General Medical Science) to J.A.C.
Table 5.1. B6 and MIN mice that performed ECC training.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>MIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>27.5 ± 0.7</td>
<td>25.5 ± 0.5</td>
</tr>
<tr>
<td>Pre</td>
<td>27.6 ± 0.3</td>
<td>23.5 ± 0.5*†</td>
</tr>
<tr>
<td>Post</td>
<td>27.7 ± 0.3</td>
<td>22.6 ± 0.6*†‡</td>
</tr>
<tr>
<td>% change from peak</td>
<td>1.1 ± 0.5</td>
<td>-11 ± 1.0*</td>
</tr>
<tr>
<td>Tibialis anterior, mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>47.7 ± 1.0</td>
<td>32.6 ± 1.3#</td>
</tr>
<tr>
<td>ECC</td>
<td>49.8 ± 1.0&amp;</td>
<td>36.4 ± 1.0#&amp;</td>
</tr>
<tr>
<td>Epididymal fat, mg</td>
<td>309 ± 27</td>
<td>68 ± 17*</td>
</tr>
<tr>
<td>Spleen, mg</td>
<td>72 ± 2</td>
<td>593 ± 30*</td>
</tr>
<tr>
<td>Testes, mg</td>
<td>198 ± 3</td>
<td>136 ± 14*</td>
</tr>
<tr>
<td>LABC, mg</td>
<td>90 ± 2</td>
<td>46 ± 4*</td>
</tr>
<tr>
<td>Seminal vesicle, mg</td>
<td>234 ± 18</td>
<td>53 ± 7*</td>
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<tr>
<td>Plasma IL-6, pg/ml</td>
<td>0 ± 0</td>
<td>35 ± 5*</td>
</tr>
<tr>
<td>Tumor number</td>
<td>0 ± 0</td>
<td>68 ± 8*</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>16.9 ± 0.1</td>
<td>16.8 ± 0.1</td>
</tr>
</tbody>
</table>

Data are means ± standard error. Either a Students t-test or two-way repeated measures ANOVA with Student-Knewman-Keuls post hoc methods were performed when appropriate. Statistical significance was set at p<0.05. Abbreviations: ECC, Eccentric contractions. No., number. g, grams. mg, milligrams. pg, picogram. ml, milliliter. mm, millimeter. LABC, levator ani / bulbocavernosus muscle. * = significantly different from B6. † = significantly different from peak body weight. ‡ = significantly different from pre body weight. # = main effect of MIN. & = main effect of ECC.
Table 5.2. B6 and MIN mice that performed a single ECC bout after training.

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>MIN</th>
</tr>
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<tbody>
<tr>
<td>No. of mice</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>26.5 ± 0.9</td>
<td>25.3 ± 0.4</td>
</tr>
<tr>
<td>Pre</td>
<td>26.5 ± 0.9</td>
<td>22.4 ± 0.6*†</td>
</tr>
<tr>
<td>Post</td>
<td>27.7 ± 0.3</td>
<td>21.3 ± 0.5*†‡</td>
</tr>
<tr>
<td>% change from peak</td>
<td>0.8 ± 0.9</td>
<td>-16 ± 1.4*</td>
</tr>
<tr>
<td>Tibialis anterior, mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45.4 ± 1.5</td>
<td>28.0 ± 1.1#</td>
</tr>
<tr>
<td>ECC</td>
<td>49.4 ± 1.0&amp;</td>
<td>30.6 ± 1.2#&amp;</td>
</tr>
<tr>
<td>Epididymal fat, mg</td>
<td>293 ± 39</td>
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<tr>
<td>Spleen, mg</td>
<td>66 ± 2</td>
<td>598 ± 42*</td>
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<tr>
<td>Testes, mg</td>
<td>187 ± 4</td>
<td>87 ± 10*</td>
</tr>
<tr>
<td>LABC, mg</td>
<td>90 ± 3</td>
<td>30 ± 1*</td>
</tr>
<tr>
<td>Seminal vesicle, mg</td>
<td>261 ± 19</td>
<td>28 ± 2*</td>
</tr>
<tr>
<td>Plasma IL-6, pg/ml</td>
<td>0 ± 0</td>
<td>51 ± 6*</td>
</tr>
<tr>
<td>Tumor number</td>
<td>0 ± 0</td>
<td>80 ± 9*</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>16.9 ± 0.1</td>
<td>16.8 ± 0.1</td>
</tr>
</tbody>
</table>

Data are means ± standard error. Either a Students t-test or two-way repeated measures ANOVA with Student-Knewman-Keuls post hoc methods were performed when appropriate. Statistical significance was set at p<0.05. Abbreviations: ECC, Eccentric contractions. No., number. g, grams. mg, milligrams. pg, picogram. ml, milliliter. mm, millimeter. LABC, levator ani / bulbocavernosus muscle. * = significantly different from B6. † = significantly different from peak body weight. ‡ = significantly different from pre body weight. # = main effect of MIN. & = main effect of ECC.
Figure 5.1. ECC training regulation of muscle STAT3 and AMPK signaling. A) Muscle STAT3 and AMPK regulation by ECC training and cancer cachexia. B) Muscle oxidative capacity regulation by ECC training and cancer cachexia. C) Muscle cytochrome c oxidase (COX) enzyme activity regulation by ECC training and cancer cachexia. The activation of signaling molecules was determined by the phosphorylated...
and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to Control values. Data are means ± standard error. A two-way ANOVA was used to determine differences between treatment groups when appropriate. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. & = main effect of ECC training. # = main effect of MIN.
Figure 5.2. ECC training regulation of succinate dehydrogenase enzyme activity myofiber area. A) High and low succinate dehydrogenase enzyme activity myofiber area. B) High succinate dehydrogenase enzyme activity myofiber area distribution. C) Low succinate dehydrogenase enzyme activity myofiber area distribution. High and low
succinate dehydrogenase enzyme activity myofiber area was examined post hoc on a separate cohort of B6 and MIN previously described (Hardee et al., 2016). Data are means ± standard error. A two-way ANOVA was used to determine differences between treatment groups when appropriate. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. & = main effect of ECC training. # = main effect of MIN.
Figure 5.3.  ECC training regulation of muscle mTORC1 signaling and protein synthesis.  A)  Muscle Akt/mTORC1 signaling regulation by ECC training and cancer cachexia.  B)  Muscle protein synthesis regulation by ECC training and cancer cachexia.  The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate.  For protein expression, values were corrected for equal protein loading using GAPDH.  All samples were run on the same gel and normalized to Control values.  Data are means ± standard error.  A two-way ANOVA was used to determine differences between treatment groups when appropriate.  Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate.  Statistical significance was set at p<0.05.  Different letters are statistically different.  & = main effect of ECC training.  # = main effect of MIN.
Figure 5.4. ECC-induced protein synthesis and mTORC1 signaling following training. A) Muscle protein synthesis activation by ECC following training. B) Muscle Akt/mTORC1 signaling activation by ECC following training. The activation of signaling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to Control values. Data are means ± standard error. A two-way ANOVA was used to determine differences between treatment groups when appropriate. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at p<0.05. Different letters are statistically different. & = main effect of ECC training. # = main effect of MIN.
CHAPTER 6

DISCUSSION
Cachexia, a complex metabolic syndrome that involves the unintentional loss of bodyweight which cannot be reversed by nutritional intervention, directly impacts patient survival and life quality (K. Fearon et al., 2011). Cancer cachexia accounts for approximately 20% of all cancer related deaths, and approximately 40% of deaths related to colon cancer (Bruera, 1997; Tisdale, 2002). Human cancer patients and preclinical models share several key features of cachexia which include body weight loss, muscle atrophy, adipose tissue depletion, elevated plasma IL-6 levels, and hypogonadism (Evans et al., 2008; K. Fearon et al., 2011; White, Puppa, Narsale, et al., 2013). Skeletal muscle mass depletion associated with cancer cachexia directly contributes to increased patient morbidity and mortality (K. C. Fearon, 1992; Tisdale, 2009). Tumor-derived factors and cytokines have been implicated in muscle wasting associated with cancer, and high levels of circulating IL-6 have been reported in both cachectic cancer patients (Argiles, Busquets, & Lopez-Soriano, 2003; Burney et al., 2012; Kuroda et al., 2005; Kuroda et al., 2007; Scott, McMillan, Crilly, McArdle, & Milroy, 1996) and preclinical models (Bonetto et al., 2011; Strassmann et al., 1993; Strassmann, Fong, Kenney, & Jacob, 1992). While significant progress has been made in our understanding of disrupted protein regulation by circulating factors such as IL-6 during cancer, it is currently unknown if suppressed muscle protein synthesis and mTORC1 signaling can be activated in the presence of the cachectic environment. The overall purpose of this dissertation was to determine how cancer-induced inflammation affects wasting muscles ability to respond to ECC. We hypothesized that chronic muscle inflammatory signaling would attenuate the anabolic response to ECC. Key findings from the experiments accompanying this dissertation demonstrate: (1) cachexia did not disrupt the ECC induction of mechano-sensitive pathways and mTORC1
signaling, (2) cachexia did not disrupt ECC-induced protein synthesis, but the capacity for protein synthesis remained suppressed; (3) short-term muscle STAT3/NFκB inhibition prior to contraction improved basal and ECC-induced protein synthesis; (4) elevated IL-6 levels could disrupt mechanical activation of protein synthesis in atrophic myotubes and MIN skeletal muscle; (5) muscle gp130 regulated IL-6 suppression of basal protein synthesis and ECC-induced protein synthesis, (6) ECC training improved basal mTORC1 regulation which was related to improved oxidative capacity and suppressed AMPK activity; and (7) exercise training maintained ECC-induced protein synthesis and mTORC1 signaling in cachectic MIN muscle. These findings provide preliminary mechanistic insight to the use of ECC as a potential therapeutic to combat skeletal muscle wasting during cancer cachexia.

**Cachectic muscle response to a single ECC bout in male MIN mice**

Disrupted muscle protein synthesis and mTORC1 signaling parallel increased plasma IL-6 levels during cancer cachexia progression (White, Baynes, et al., 2011). Moreover, we have previously found that systemic IL-6 overexpression can suppress protein synthesis in tumor-bearing mice, and long-term IL-6 exposure can stimulate myotube atrophy associated with disrupted mTORC1 signaling (White, Puppa, Gao, et al., 2013). The present study extends these findings establishing severe wasting coincided with a cachectic muscle signature exhibiting enhanced inflammatory signaling, disrupted proteostasis regulation, and perturbed metabolic signaling regulation. Indeed, we found that basal Akt/mTORC1 signaling was disrupted in MIN mice. In healthy skeletal muscle, binding of insulin/IGF1 to its respective cell surface receptor initiates tyrosine kinase activity and PI3K-dependent activation of Akt Thr308 through PDK1. The rapamycin-
insensitive mTORC2 has also been implicated in growth factor signaling through the phosphorylation of Akt Ser473 (Fayard et al., 2005), and this site is required for full Akt activation. Subsequent downstream targets of Akt which can control muscle protein synthesis include TSC2, GSK3B, and PRAS40 (Laplante & Sabatini, 2009; Schiaffino & Mammucari, 2011). While Akt stimulates protein synthesis by activating mTORC1 in healthy skeletal muscle, male MIN mice demonstrated elevated Akt Ser473 phosphorylation despite reduced mTORC1 signaling and protein synthesis. It has been suggested that this robust Akt activation in cachectic muscle may be related to the redistribution of mTOR to mTORC2 formation when mTORC1 signaling is suppressed. Indeed, our data demonstrate Akt activation coincided with reduced phosphorylation of downstream mTORC1 targets P70S6K and RPS6, which has previously been observed by our laboratory (White, Puppa, Gao, et al., 2013). Interestingly, the activation of mTORC1 signaling by ECC was not sufficient to relieve the robust activation of Akt in cachectic skeletal muscle. Future research is warranted to determine the precise role of mTOR complex formation in cancer-induced skeletal muscle wasting.

While our understanding of disrupted proteostasis during cachexia has increased dramatically (Bonaldo & Sandri, 2013; Sandri, 2013; White, Baynes, et al., 2011), we have a more limited understanding of how the cachectic environment affects muscle’s ability to stimulate protein synthesis in response to an anabolic stimulus. Resistance exercise is a potent stimulator of mechano-sensitive pathways that interact with mTORC1 signaling to induce protein synthesis in healthy adults (Fry et al., 2011; Walker et al., 2011). However, no studies to date have examined the cachectic muscle response to exercise in cancer patients (Grande et al., 2014b; Granger, McDonald, Parry, Oliveira, & Denehy, 2013), and
the cachectic muscle’s acute response and training adaptations to exercise has only recently been examined in preclinical models. While there is evidence that repeated ECC bouts after the initiation of cachexia attenuated muscle and myofiber atrophy in tumor-bearing mice (al-Majid & McCarthy, 2001; Hardee et al., 2016), the capacity to activate mechano-sensitive pathways, mTORC1 signaling, and protein synthesis by ECC has not been investigated. The present study found that cachexia did not disrupt ECC-induced mechano-sensitive pathways and mTORC1 signaling when examined immediately or 3h post-ECC. In fact, the induction of mechano-sensitive and mTORC1 signaling pathways were either induced to a similar or greater extent than healthy skeletal muscle. While the stimulation model used in the current studies provides maximal activation of motor units and subsequent muscle contraction, these data would suggest increased sensitivity to contraction in cachectic MIN mice. Given that severe cachexia is associated with profound reductions in voluntary physical activity (Narsale et al., 2016; Puppa, Murphy, et al., 2014), this may serve to increase cachectic muscle’s sensitivity to contraction. Therefore, the induction of mechano-sensitive pathways in cachectic muscle to this stimulation paradigm may be more related to reduced physical activity levels prior to contraction, rather than an enhanced activation of mechano-sensitive pathways and mTORC1 signaling. However, force production during each stimulation session was not examined, and would provide clearer insight to the degree of activation, total work produced, and fatigue accumulation during each contraction bout. There is also the potential that cachexia would increase the susceptibility to muscle damage during high-force contractions. We and others have not observed evidence of muscle damage at the acute time points utilized in the current study or following repeated ECC bouts (Hardee et al., 2016; McLoughlin, Mylona, Hornberger,
Esser, & Pizza, 2003). Nonetheless, it appears that cachectic muscle retains the capacity to induce mechano-sensitive and mTORC1 signaling pathways in response to a single ECC bout.

We found that cachectic muscle could induce protein synthesis, however the overall capacity for this induction remained suppressed to healthy skeletal muscle. While cachectic muscle can initiate an anabolic response to a single ECC bout, whether these repeated bouts are sufficient to account for the improved myofiber area following ECC training will require future investigation. While a similar protein synthesis response was observed 3h post-ECC, we have not determined the time course for the magnitude and duration of this protein synthesis induction by ECC. This stimulation paradigm has been shown to induce protein synthesis for up to 18-24h in healthy, rodent skeletal muscle (West et al., 2016). However, it is plausible that either the magnitude or duration of protein synthesis induction by ECC may be altered by the cachectic environment during cancer cachexia. Indeed, an attenuated mTORC1 signaling response has been observed 6h post-ECC in aged skeletal muscle (Funai, Parkington, Carambula, & Fielding, 2006). Furthermore, both the magnitude and duration of mTORC1 activation by ECC was disrupted in obese Zucker rats compared to lean counterparts (Katta et al., 2009). While these studies did not directly measure protein synthesis rates per se, they demonstrate conditions associated with muscle metabolic dysfunction can alter the response to a single ECC bout. Future studies are warranted to determine if cachexia disrupts either the magnitude or duration of the protein synthesis induction by ECC. Similarly, nutrient availability can interact with muscle contraction to augment the duration and absolute protein synthesis induction in human and rodent skeletal muscle (Drummond, Dreyer, Fry,
Glynn, & Rasmussen, 2009), the fed response should be examined in future studies. Recently, resistance exercise has been shown to improve the anabolic response to feeding in cancer patients received androgen deprivation therapy (Hanson et al., 2017). It has been suggested that resistance exercise may also enhance the nutrient sensitivity of muscle for up to 24h post-contraction (Burd, Tang, Moore, & Phillips, 2009). Whether single and repeated exercise bouts improve muscle’s sensitivity to nutrients has yet to be examined with cancer cachexia, which could have significant ramifications on muscle mass regulation during cancer. Collectively, future studies are warranted to determine the extent and duration of the anabolic response to ECC in both the fasted and fed state.

We have previously found that systemic IL-6 overexpression can suppress protein synthesis in tumor-bearing mice, and long-term IL-6 exposure can stimulate myotube atrophy associated with disrupted mTORC1 signaling (White, Puppa, Gao, et al., 2013). Interestingly, muscle STAT3/NFkB inhibition increased basal and ECC-induced protein synthesis in cachectic MIN muscle. Interestingly, while these findings are consistent with the anabolic properties of PDTC treatment in male MIN mice (Narsale et al., 2016; Puppa, Murphy, et al., 2014), they are in contrast to previous studies which have not observed improved protein synthesis regulation by inflammatory signaling inhibition (Puppa, Gao, et al., 2014; White, Baynes, et al., 2011). Given that PDTC has both anti-inflammatory and anti-oxidant properties (Shi, Leonard, Wang, & Ding, 2000), additional studies are needed to determine the precise molecular mechanisms associated with improve anabolism by PDTC treatment. Nonetheless, the current findings highlight a role for muscle inflammatory signaling in the regulation of basal and ECC-induced protein synthesis during cancer cachexia.
Systemic IL-6 regulation of ECC-induced protein synthesis in MIN mice

The male MIN mouse is an IL-6-dependent model of cancer cachexia that develops a slow onset of cachexia (Baltgalvis, Berger, Pena, Davis, & Carson, 2008; Carson & Baltgalvis, 2010). During the natural progression of cachexia plasma IL-6 is associated with tumor burden (polyp number and polyp size), body weight and muscle loss, and suppressed protein synthesis and mTORC1 signaling (Baltgalvis, Berger, Pena, Davis, & Carson, 2008; McClellan et al., 2012; White, Baynes, et al., 2011). Furthermore, systemic IL-6 overexpression can induce cachexia (e.g., body weight and muscle mass loss, disrupted protein turnover) in weight stable MIN mice (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Baltgalvis et al., 2009; Puppa et al., 2012; White, Puppa, Gao, et al., 2013; White et al., 2012). Interestingly, the rapid induction of cachexia by IL-6 in weight stable MIN mice recapitulates the cachectic phenotype observed during the natural progression of cancer cachexia. Whole-body treadmill exercise prevented systemic IL-6-induced muscle wasting and disrupted mTORC1 signaling in MIN mice (Puppa et al., 2012; White, Puppa, Gao, et al., 2013), which occurred independent to alterations in inflammatory signaling. Therefore, we examined the protein synthesis and mTORC1 signaling response to a single ECC bout after 2 wks. systemic IL-6 overexpression. In contrast to our previous observations, systemic IL-6 overexpression blocked the mechanical activation of protein synthesis in MIN mice. We also found that long-term IL-6 disrupted myotube stretch-induced protein synthesis and mTORC1 signaling. These findings extend our previous observations in stretched myotubes exposed to LLC conditioned media (Gao & Carson, 2016). Interestingly, the discrepancy between the activation of protein synthesis responses by ECC between studies may be related to the rate
and duration of cachexia development (2 wks. vs 6 wks.). We have previously observed the cachectic environment blocked ECC-induced protein synthesis in LLC tumor-bearing mice, despite a robust activation of mTORC1 signaling (unpublished observations). Thus, the ability to induce protein synthesis in response to anabolic stimuli may be related to rate of wasting induced by the cachectic environment. We also observed systemic IL-6 overexpression suppressed basal protein synthesis in healthy B6 mice independent to alterations in muscle mass, which provides evidence of a role for systemic IL-6 in protein synthesis suppression. Collectively, these studies demonstrate that circulating cachectic factors such as IL-6 can regulate basal and mechanical activation of protein synthesis in atrophic myotubes and muscle.

**Exercise training regulation of protein synthesis and mTORC1 regulation in MIN mice**

While preliminary evidence suggest that cachectic muscle retains the plasticity to adapt to mechanical loading and muscle contraction (al-Majid & McCarthy, 2001; Hardee et al., 2016; Norton et al., 1979; Otis et al., 2007), it is unclear whether these training adaptations improve metabolic regulation and function. Related to the cachectic muscle phenotype, muscle STAT3 and AMPK activation coincides with suppressed protein synthesis and disrupted mitochondrial quality control in preclinical models (White, Baltgalvis, et al., 2011; White, Baynes, et al., 2011; White, Puppa, Gao, et al., 2013; White et al., 2012). Moreover, severe cachexia is associated disrupted metabolic homeostasis and functional deficits (Baltgalvis, Berger, Pena, Davis, Muga, et al., 2008; Puppa et al., 2011). Several lines of evidence suggest an intricate relationship between protein synthesis, mitochondrial function, and metabolic plasticity in skeletal muscle (Bentzinger et al., 2008;
Cunningham et al., 2007; Risson et al., 2009). While not commonly appreciated as an adaptation to high-force muscle contractions, recent evidence suggest that resistance exercise can improve mitochondrial function in healthy skeletal muscle (Porter et al., 2015). Related to cancer cachexia, we have previously found that repeated ECC bouts after the initiation of cachexia attenuated myofiber atrophy that coincided with improved oxidative capacity (e.g., percent fibers with high succinate dehydrogenase enzyme activity) and suppressed chronic AMPK activation in MIN mice (Hardee et al., 2016). In the current study, we found the ECC training improved whole muscle cytochrome c oxidative (COX) enzyme activity, suppressed chronic AMPK activation, and improved basal protein synthesis. Furthermore, we demonstrate that improved oxidative capacity coincided with the induction of autophagy / mitophagy processes. Unfortunately, it is currently unknown if these changes were associated with improved muscle function. Critical to the treatment of the cachectic cancer patient is improvements in muscle function. Indeed, several clinical trials have been terminated due to lack of functional improvements (S. Cohen et al., 2015). We have recently found that several functional deficits that occur with cancer cachexia are associated with increased muscle inflammatory signaling, and muscle fatigability is elevated prior to cachexia development in male MIN mice (VanderVeen, Hardee, Fix, & Carson, 2017). Future studies are warranted to determine if the observed changes related to muscle inflammatory signaling, oxidative capacity, and mitochondrial quality control in the current study are associated with improved muscle function.

While the current studies were isolated to a single eccentrically contracted muscle, we provide initial mechanistic insight into the potential role of muscle contraction during cancer. Interestingly, we found that ECC training improved basal protein synthesis and
mTORC1 regulation, and found that the protein synthesis induction by ECC was maintained in trained skeletal muscle. Experimental evidence in humans suggests that the protein synthesis induction following a novel resistance exercise bout may be highly influenced by the resolution of acute muscle damage, whereas the acute protein synthesis response in the trained state may be more related to growth (Damas et al., 2016). Given that we have not previously observed indices of muscle damage following the completion of ECC training (Hardee et al., 2016), these data collectively suggest that the acute protein synthesis induction by ECC following training is partitioned towards growth. However, a significant barrier to our fundamental understanding of muscle proteostasis in both basal and stimulated conditions is a precise measurement of protein breakdown in rodent skeletal muscle. While accelerated protein breakdown has established roles in skeletal muscle atrophy, this process is also stimulated by exercise and muscle contraction. It has been suggested that targeting aberrant protein degradation processes may be a potential treatment for cachexia (S. Cohen et al., 2015), however it is conceivable that inhibiting protein degradation processes may be detrimental to systemic metabolism and muscle function. Indeed, inhibition of ER stress and unfolding protein response pathways has been shown to exacerbate skeletal muscle wasting during cancer cachexia (Bohnert et al., 2016). Muscle protein breakdown processes (e.g., autophagy, mitophagy, proteasome activity) are also stimulated by both acute endurance and resistance exercise (C. He et al., 2012; Vainshtein, Tryon, Pauly, & Hood, 2015; Wolfe, 2006a), and it has been suggested that the positive benefits of exercise may be related to the clearance of damaged or dysfunctional proteins and organelles. Mostly driven by methodology issues, whether acute exercise can favorably accelerate protein turnover in cachectic skeletal muscle has not been
investigated. Furthermore, additional studies are required to determine if the anabolic responses observed in the current study promote a positive net protein balance in cachectic muscle.

Summary

In summary, cachectic muscle maintained the ability to induce mechano-sensitive pathways and mTORC1 signaling in response to a single ECC bout, however the capacity to induce protein synthesis remained suppressed. Interestingly, short-term muscle STAT3/NFkB inhibition prior to contraction improved basal and ECC-induced protein synthesis. Elevated IL-6 levels could disrupt mechanical activation of protein synthesis in cultured myotubes and MIN skeletal muscle. IL-6 suppression of basal protein synthesis required muscle gp130 signaling. ECC training improved basal mTORC1 regulation which coincided with improved oxidative capacity and suppressed AMPK activity. Lastly, ECC training maintained ECC-induced protein synthesis and mTORC1 signaling in cachectic muscle.

Table 6.1. Summary of findings.

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<th>Outcome</th>
<th>Cancer cachexia</th>
<th>Acute ECC</th>
<th>ECC training</th>
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<tbody>
<tr>
<td>Muscle mass</td>
<td>Decreased</td>
<td>No change</td>
<td>Increased</td>
</tr>
<tr>
<td>Protein synthesis</td>
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<td>Increased</td>
<td>Increased</td>
</tr>
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<td>mTORC1 signaling</td>
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<td>Increased</td>
<td>Increased</td>
</tr>
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<td>Decreased</td>
</tr>
<tr>
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<td>Decreased</td>
</tr>
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<td>Not measured</td>
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