**Effects of low dose leucine supplementation on gastrocnemius muscle mitochondrial content and protein turnover in tumor bearing mice**

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Title: Effects of low dose leucine supplementation on gastrocnemius muscle mitochondrial content and protein turnover in tumor bearing mice

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Running Title: Leucine supplementation alters muscle signaling in tumor bearing mice
Abstract

Many forms of cancer are associated with loss of lean body mass, commonly attributed to decreased protein synthesis and stimulation of proteolytic pathways within the skeletal muscle. Leucine has been shown to improve protein synthesis, insulin signaling, and mitochondrial biogenesis, key signaling pathways influenced by tumor signaling. The purpose of this study was to examine the effects of leucine supplementation on mitochondrial biogenesis and protein turnover in tumor bearing mice. Twenty male C57BL/6 mice were divided into four groups (n=5): Chow, leucine (Leu), Lewis lung carcinoma (LLC) implant, LLC+Leu. At 9-10 weeks of age, mice were inoculated and supplemented with 5% leucine (w/w) in the diet. C2C12 myotubes were treated with 2.5mM leucine and 25% LLC conditioned media to further elucidate the direct influence of the tumor and leucine on the muscle. Measures of protein synthesis, mitochondrial biogenesis, and inflammation in the gastrocnemius were assessed via western blot analysis. Gastrocnemius mass was decreased in LLC+Leu relative to LLC (p=0.040). Relative protein synthesis rate was decreased in LLC mice (p=0.001). No change in protein synthesis was observed in myotubes. Phosphorylation of STAT3 was decreased in the Leu group relative to the control in both mice (p=0.019) and myotubes (p=0.02), but did not significantly attenuate the inflammatory effect of LLC implantation (p=0.619). LLC decreased markers of mitochondrial content; however, PGC-1α was increased in LLC+Leu relative to LLC (p=0.001). While leucine supplementation was unable to preserve protein synthesis or mitochondrial content associated with LLC implantation, it was able to increase mitochondrial biogenesis signaling.

Keywords: leucine, skeletal muscle, protein turnover, mitochondrial content, mitochondrial biogenesis, cancer
**New and Noteworthy:** This study provides novel insights on the effect of leucine supplementation on mitochondrial biogenesis and protein turnover in Lewis Lung Carcinoma bearing mice. While no beneficial effects of leucine on muscle mass maintenance were identified in this population, increased signaling for mitochondrial biogenesis was seen in the skeletal muscle. Additionally we noted that leucine supplementation decreased inflammatory signaling in skeletal muscle.
Introduction

Cancer is one of the leading causes of death. Loss of lean mass is an indicator of increased mortality in various pathological states and is commonly associated with cancer (Nanri et al. 2010; Pocock et al. 2008). The loss of lean mass induced by cancer is commonly attributed to decreased skeletal muscle protein synthesis and stimulation of proteolytic pathways, potentially modulated by a state of chronic inflammation (Fearon et al. 2006). Impaired mitochondrial function in the skeletal muscle is often associated with the loss of muscle mass and function (Julienne et al. 2012; White et al. 2011; White et al. 2012). Mitochondrial dysfunction in the skeletal muscle can lead to decreases in muscle mass, maintenance, and function (Argilés et al. 2015; Romanello and Sandri 2015). Due to the relationship between mitochondrial disruption and muscle loss, preservation of mitochondrial function may be a vital mechanism to prevent further disruption of metabolic pathways already affected by cancer and mitigate muscle wasting.

Clinical strategies to address cancer associated weight loss including nutritional interventions, have been utilized to increase food intake, preserve body mass, and improve quality of life, with varying degrees of success (Baldwin et al. 2012; Balstad et al. 2014). However, due to differences in methodology, results from these studies do not demonstrate consistent preservation of body mass or lowered mortality (Baldwin et al. 2012; Balstad et al. 2014). These findings suggest the need for additional strategies for managing weight loss induced by cancer cachexia. One potential strategy may be the supplementation of the branched chain amino acid (BCAA) leucine, which has been established as a stimulator of protein synthesis through increased activity of the mammalian target of rapamycin (mTOR) pathway (Suryawan et al. 2012). In tumor bearing mice, leucine supplementation mitigated the loss of
lean mass and increased protein synthesis, without increasing tumor mass (Eley et al. 2007; Gomes-Marcondes et al. 2003). Thus, the therapeutic signaling produced from leucine supplementation may counter balance cachectic signaling induced by the development of cancer. However, Peters et al. demonstrated that leucine supplementation was unable to preserve body weight (Peters et al. 2011). Leucine supplementation has been shown to attenuate protein degradation, increase mitochondrial function, and modulate insulin signaling (Di Camillo et al. 2014; Liang et al. 2014; Sugawara et al. 2009). However, whether leucine preserves or modulates mitochondrial function in a tumor bearing model has yet to be elucidated. Therefore, the purpose of this paper is to determine if low dose leucine supplementation can preserve mitochondrial content, increase protein synthesis, and attenuate markers of protein degradation in the skeletal muscle of tumor bearing mice.

**Materials and Methods**

**Animals**

All animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of Memphis.

Twenty C57BL/6 male mice, aged 7-8 weeks, were purchased from ENVIGO (Indianapolis, IN) and individually housed in a climate controlled room on a 12:12-h light-dark cycle. Upon arrival, all mice were allowed two weeks to acclimate to the new housing facilities before being divided into 4 groups: Chow n=5, Leucine enriched diet (Leu) n=5, Lewis Lung Carcinoma implanted (LLC) n=5, and LLC+Leu n=5. The mice were allocated such that each group had an equivalent average weight. At 9-10 weeks of age, LLC groups received the
subcutaneous injection of 1x10^6 LLC cells suspended in phosphate buffered saline (PBS) while control groups received an equivalent volume of PBS. Prior to tumor implantation, LLC cells were rinsed with PBS. Each mouse received a 100 µl, subcutaneous injection of 1 x10^6 cells in their right flank or 100ul of PBS control injection. Food was switched from standard chow to their respective experimental diet with Leu group diets being supplemented with Leucine 5% w/w. Diets were purchased from Research Diets (New Brunswick, NJ) control: D10001 (1.6% leucine), Leucine: D16121405 (6.6% leucine); macronutrient composition of the diets can be found in table 1. Mice were monitored over the course of 28 days. Food intake and body weight were measured every 48h and mice were checked daily for overall health. As has been previously done, mice were fasted 5h prior to euthanasia and injected with puromycin (0.04 µM/g BW sigma, St. Louis, MO) 30 minutes prior to tissue collection for assays measuring protein synthesis (Puppa et al. 2014). Immediately before euthanasia, mice were anesthetized with isoflurane and tissues were harvested. The tumor, organs, and muscles of the hind limb were removed, weighed, snap frozen in liquid nitrogen, and stored at -80 °C for further analysis.

Cell Culture

The Lewis Lung Carcinoma (LLC) cell line utilized for this experiment was provided as a gift from James Carson (University of South Carolina). The LLC cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin, refreshing media every 48h.

To further understand if the effects on muscle are from a tumor secreted factor or systemic changes in the mouse, C2C12 myoblasts (ATCC, Manassas, VA) were plated and differentiated in 2% Horse serum for 72h. Upon differentiation cells were treated with differentiation media supplemented with either 25% C2C12 conditioned media or 25% LLC
conditioned media as previously done (Puppa et al. 2014). Based on data from Eley et al. and Sun et al, 2.5mM leucine was administered in media for 2h prior to harvest {Eley, 2007 #11; Sun, 2007 #39}. Cells were rinsed in sterile PBS 3 times before being harvested in radioimmunoprecipitation assay (RIPA) buffer and stored for protein analysis.

Western Blot

A portion of the excised gastrocnemius muscle was homogenized using a Kontes glass homogenizer in a 10:1 v/w ratio in ice cold Muller buffer containing protease and phosphatase inhibitors. The total protein concentration of this lysate was quantified using the Bradford protein assay. Samples were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 4-15% gradient gel (Bio-Rad, Hercules, California), and subsequently transferred to a PVDF membrane. The blot was then blocked with 5% BSA for 1 h before being incubated in primary antibody (OxPhos cocktail and PGC-1α 1:2000, Abcam, Cambridge, MA ; puromycin 1:5000, Millipore, St. Louis Missouri; P-STAT3, STAT3, P-mTOR, mTOR, Ubiquitin, P-FOXO, FOXO 1:2000 cell signaling, Beverly, MA) and HRP-conjugated secondary antibodies (1:5000, cell signaling, Beverly, MA in 5% BSA for 2 h). Blots were washed in TBST before being visualized with a chemiluminescent agent and imaged using a Fotodyne® 60-7020 bench top imager. All bands were quantified via densitometry via ImageJ.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 7® software. All data are presented as means ± standard error of mean (SEM). Statistical significance was set at α = 0.05. Western blot data were analyzed via two-way analysis of variance (ANOVA). Pre-planned t-tests
were used to examine the effect of LLC, the effect of leucine in the control condition, and the effect of leucine in the LLC condition.

Results

Body composition and food intake

All mice continued to grow throughout the duration of the study. Leucine supplementation had no effect on tumor mass after 4 weeks (p=0.82, Table 2). There was no significant difference in tumor free body weight at the time of euthanasia (p=0.14, Table 2). There was no effect of leucine or tumor on food intake throughout the duration of the study (Chow: 3.04±0.09, LLC: 3.07±0.09, Leu: 3.23±0.17, LLC+Leu: 3.07±0.15). While there was no direct effect of cancer or leucine supplementation alone on gastrocnemius mass, a pre-planned t-test shows leucine decreased muscle mass in tumor bearing mice by 12% compared to tumor bearing controls (p=0.03, Fig 1). Although cachexia is typically associated with both loss of lean and fat mass, we detected no change in epidydimal fat mass across treatment groups (p=0.17, Table 2) suggesting that the mice were in a state of pre-cachexia.

Inflammation

Cancer is associated with an increased inflammatory state. To examine the effects of cancer and leucine supplementation on inflammation we looked at spleen mass and muscle signal transducer and activator of transcription 3 (STAT3) phosphorylation, a marker of tissue level inflammation. There was a main effect of LLC implantation to cause splenomegaly (p=0.002, Table 2). At the level of the muscle, phosphorylation of STAT3 was increased in tumor bearing mice (p=0.03). A pre-planned t-test showed a 75% decrease with leucine
supplementation in tumor free mice, while there was no effect of leucine on STAT3 phosphorylation in tumor bearing mice (Figure 2).

To further examine the influence of a tumor environment and leucine supplementation on muscle inflammatory markers we supplemented C2C12 myotubes with 25% LLC conditioned media to mimic the tumor environment and supplemented cells with 2.5mM leucine. Similar to \textit{in vivo} results, leucine decreased p-STAT3 in the control cells and there was a main effect of leucine to decrease STAT3 phosphorylation (p=0.01, Fig 2B). Unlike the \textit{in vivo} results, there was no effect of LLC conditioned media to increase p-STAT3 (p=0.36, Fig 2B). These data suggest that leucine can decrease basal muscle STAT3 phosphorylation, while the presence of a tumor environment may attenuate the leucine suppression of p-STAT3.

Muscle Protein Synthesis

Leucine is a known stimulator of protein synthesis; therefore, we examined the effects of leucine supplementation on markers of skeletal muscle protein synthesis in tumor bearing mice. There was no effect of leucine or tumor on mTOR phosphorylation in tumor bearing mice (Fig 3A). Interestingly, there was a main effect of the tumor to decrease protein synthesis measured by 30 minutes of puromycin incorporation p=0.001; however, there was no effect of leucine, similar to the mTOR findings (Fig 3B).

To further examine these effect of the tumor environment and leucine on skeletal muscle directly, we examined phosphorylation of mTOR in C2C12 cells treated with LLC conditioned media and leucine. \textit{In vitro} there was a trend for leucine to increase mTOR phosphorylation in the absence of LLC conditioned media, p=0.09 (Fig 3C). However, there was no effect of LLC conditioned media on mTOR activation. These data suggest that prior to muscle mass loss in...
LLC tumor bearing mice, protein synthesis was not affected by the tumor or leucine supplementation; however, protein synthesis may be suppressed through an mTOR independent mechanism.

Muscle Protein Degradation

Muscle protein degradation can occur through numerous pathways including the ubiquitin proteasomal pathway as well as through autophagy. We examined the effects of leucine supplementation on ubiquitination of proteins, a precursor step to proteasomal degradation, and the activation of FOXO3a, a regulator of skeletal muscle degradation and autophagy. There was a trend for increased ubiquitination of proteins after 4 weeks with LLC tumor implantation, p=0.09 (Fig 4A). There was no effect of four weeks of leucine supplementation on ubiquitination of proteins in mice (Fig 4A). Interestingly, phosphorylation of FOXO3a was decreased in tumor bearing mice, regardless of leucine supplementation. Additionally, leucine decreased FOXO3a phosphorylation independently of tumor (Fig 4B).

To examine the effects of the tumor secreted environment on markers of degradation we used C2C12 myotubes treated with LLC conditioned media and leucine. In C2C12 myotubes there was a main effect of leucine to decrease ubiquitination of proteins; however, there was no effect of tumor conditioned media on ubiquitination of muscle proteins (Fig 4C). LLC conditioned media and leucine supplementation had no effect on FOXO3a phosphorylation in C2C12 myoblasts (Fig 4D).

Mitochondrial Biogenesis and Content

We next examined markers of mitochondrial content and biogenesis as disruptions in mitochondria are closely linked to alterations in protein synthesis and degradation and systemic
health. There was a main effect for tumor bearing mice to have decreased markers of mitochondrial content including cytochrome c, $p=0.01$, while there was no effect of leucine (Fig 5A). Additionally, tumor bearing mice had reductions in ATP5a, $p=0.04$, as well as UQ CRC2, $p=0.006$ (Fig 5B-C), components of electron transport chain complexes five and three, respectively, independently of leucine. To examine alterations in mitochondrial biogenesis signaling we looked at protein expression of PGC1α. In tumor bearing mice, there was an effect of leucine to increase PGC1α protein expression, $p=0.02$. This effect was not seen in the control mice (Fig 5D). To examine the effects of the tumor microenvironment on muscle mitochondrial content and biogenesis we examined C2C12 myotubes treated with LLC conditioned media and leucine. In C2C12 myotubes, cytochrome C expression was decreased with LLC media ($p=0.04$) (Fig 5E). There was no change in PGC-1α protein expression in C2C12 myotubes with LLC conditioned media or leucine supplementation (Fig 5F).

**Discussion**

Cancer is commonly associated with muscle loss that contributes to increased morbidity and mortality. There are currently no treatments for this muscle atrophy and therapeutic approaches are needed to attenuate or prevent this process. We used leucine supplementation as a preventative therapeutic measure in an effort to preserve muscle mass in tumor bearing mice. Leucine in combination with LLC tumor implantation resulted in decreased muscle mass, which was independent of alterations in mTOR phosphorylation. Leucine decreased phosphorylation of STAT3 independently of LLC implantation, while both LLC tumor implantation and leucine resulted in significant decreases in pFOXO3a. LLC implantation decreased markers of mitochondrial content which were unaffected by leucine.
In our experiments LLC implantation did not independently affect tumor free body weight, fat mass, or muscle mass which is in contrast to previous studies that have shown LLC-induced decreases in tumor free body mass and gastrocnemius weight when compared to controls (Puppa et al. 2014). Interestingly, leucine supplementation in the LLC condition resulted in a decrease of muscle mass contrasting other reports showing preserved muscle mass in different models of cancer cachexia (Eley et al. 2007; Peters et al. 2011). However, our results were similar with Peters et al where a low dose leucine supplementation did not result in preservation of body or muscle mass. These data suggest that a higher leucine dosage may be needed to prevent cancer induced weight loss.

At physiological levels leucine works with insulin to activate anabolic pathways when both amino acids and food are available (Garlick 2005). Additionally, leucine can increase energy expenditure in mice (Argiles et al. 1996; Freudenberg et al. 2012; She et al. 2007; Zhang et al. 2007). Cancer is also known to increase the metabolic rate and energy expenditure (Falconer et al. 1994; Porporato 2016). The combination of leucine and cancer may increase metabolic rate significantly and without increased food intake may result in excessive atrophy. Cancer is associated with both a reduction in amino acids as well as insulin resistance, which may contribute to the atrophy. Further work needs to be done to better understand alterations in energy expenditure with LLC induced cachexia and leucine supplementation.

Tumor implantation impaired skeletal muscle protein synthesis, measured by puromycin incorporation, coinciding with previous research (Puppa et al. 2014; Smith and Tisdale 1993). Leucine supplementation not only failed to attenuate this loss but, the LLC+Leu groups experienced a significant decrease relative to the control, suggesting interference in leucine’s normal anabolic stimulation in vivo. Interestingly, mTOR phosphorylation was unaltered with
LLC tumor implantation in vivo or LLC conditioned media in vitro despite lower relative protein synthesis rates in vivo, suggesting inhibition downstream of mTOR or suppression of alternative pathways such as MAPK/eIF4E (Engelke et al. 2016). In vitro, Lang et al. demonstrated that in a state of induced sepsis, which is seen in many models of cancer, stimulation of mTOR by leucine supplementation was blocked (Lang and Frost 2004). Additionally, excess glucocorticoids, which are known to be elevated in cancer models (Barber et al. 2004), can also induce a state of leucine resistance (Rieu et al. 2004). These findings suggest that under inflammatory conditions, skeletal muscle may not respond to anabolic signaling by leucine supplementation.

Protein degradation is controlled by two main pathways, the ubiquitin-proteosomal and autophagy-lysosomal pathways. We saw no effects of LLC tumors to alter muscle protein ubiquitination, suggesting a limited role for the ubiquitin proteosomal pathway. Leucine can decrease muscle proteolysis (Mitch and Clark 1984; Nagasawa et al. 2002). In mice fed a protein free diet, leucine supplementation increased atrogin-1 expression, but decreased markers of autophagy (Nagasawa et al. 2002). Similar to Peters et al., we show that leucine did not affect markers of the ubiquitin proteasome pathway in tumor bearing mice (Peters et al. 2011). Both leucine and tumor decreased FOXO3a activation, a key regulator of autophagy. While we did not measure autophagy in these tissues, inflammation can regulate FOXO3a. Inhibition of STAT3 signaling through muscle gp130 deletion decreases FOXO3a phosphorylation (Puppa et al. 2014). Because leucine decreased STAT3 phosphorylation in control muscle this may explain the decrease in FOXO phosphorylation.

Despite leucine having no effect on protein synthesis, leucine did improve mitochondrial biogenesis signaling as PGC-1α was increased in the LLC+Leu group relative to the LLC group in vivo; however, this did not result in increases in mitochondrial content. There was no effect of
LLC or leucine in vitro. Liang et al demonstrated that leucine supplementation in the media significantly increased PGC-1α expression in C2C12 myotubes after 48h, suggesting that a longer time period may be needed to see the effects of leucine on C2C12 myotubes (Liang et al. 2014). Wang et al. utilized a muscle specific overexpression of PGC-1α to explore influences of mitochondrial content in muscle preservation under cachexia. The MCK-PGC-1α strain maintained upregulation of PGC-1α under tumor burden, resulting in increased mitochondrial biogenesis and content within the skeletal muscle. Despite increased mitochondrial function, the MCK-PGC-1α groups experienced no preservation of skeletal muscle (Wang et al. 2012). These results resonate with ours, as leucine increased PGC-1α without preserving muscle mass or increasing protein synthesis under tumor burden.

While the current study is limited by the sample size, we were able to demonstrate that leucine supplementation can decrease inflammatory signaling in the skeletal muscle as well as increase markers of mitochondrial biogenesis signaling; however, this was insufficient to preserve muscle mass in tumor bearing mice. Future studies are needed to better understand the mechanisms through with leucine is promoting this signaling in the skeletal muscle. Studies should also be implemented looking at leucine in the context of a treatment and not just a prevention.

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**Conflict of interest:** The authors have no conflicts of interest to report.
References:


Table 1: Macronutrient composition of experimental diets.

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<tr>
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<td>g (%) kcal (%)</td>
</tr>
<tr>
<td>Protein</td>
<td>20 21</td>
<td>24 25</td>
</tr>
<tr>
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<td>66 68</td>
<td>63 64</td>
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<tr>
<td>Fat</td>
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<td>5 11</td>
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<tr>
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Table 2: Characteristic data of control and tumor bearing mice with and without leucine supplementation.

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<th>LLC</th>
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<td></td>
<td>Chow (n=5)</td>
<td>Leucine (n=5)</td>
</tr>
<tr>
<td>BW @ Sac (g)</td>
<td>23.0 ± 0.6</td>
<td>23.9 ± 0.9</td>
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<tr>
<td>BW-tumor (g)</td>
<td>23.0 ± 0.7</td>
<td>23.9 ± 0.10</td>
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<tr>
<td>Gastrocnemius (mg)</td>
<td>119.3 ± 5.0</td>
<td>119.2 ± 5.0</td>
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<tr>
<td>Soleus (mg)</td>
<td>9.5 ± 0.3</td>
<td>8.8 ± 0.6</td>
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<tr>
<td>Tibialis Anterior (mg)</td>
<td>42.1 ± 1.8</td>
<td>42.8 ± 2.3</td>
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<tr>
<td>Rectus Femoris (mg)</td>
<td>87.9 ± 4.1</td>
<td>90.1 ± 4.0</td>
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<tr>
<td>Heart (mg)</td>
<td>151 ± 6</td>
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<tr>
<td>Epi Fat (mg)</td>
<td>363 ± 22</td>
<td>425 ± 22</td>
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<tr>
<td>Liver (g)</td>
<td>1.06 ± 0.04</td>
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<tr>
<td>Spleen (mg)</td>
<td>63.5 ± 1.8</td>
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<tr>
<td>Tibia Length (mm)</td>
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<td>17.2 ± 0.2</td>
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Values are expressed as mean ± SE. * Significant from Control Leucine, † Significant from Control Chow, ‡ Main effect of LLC. Significance was set at p<0.05.
Figure Legend:

**Figure 1. Gastrocnemius mass**

Average gastrocnemius weight normalized to tibia length were calculated. Data are represented as mean + SEM. Significance was set at p>0.05. τ significant base on t-test.

**Figure 2. Muscle inflammatory signaling**

Western blot analysis of A) STAT3 phosphorylation are presented as a ratio over its non-phosphorylated form in gastrocnemius of control and tumor bearing mice with and without leucine supplementation. Western blot analysis of B) STAT3 phosphorylation: total STAT3 in C2C12 myotubes treated with LLC conditioned media and leucine. Data are represented as mean ± SEM. Significance was set at p>0.05.* indicates significant difference calculated from a Two-Way ANOVA; τ indicates significant difference calculated by t-test. ME indicates main effect.

**Figure 3. Muscle protein synthesis signaling**

Western blot analysis of A) mTOR phosphorylation to total mTOR and B) puromycin incorporation in gastrocnemius of control and tumor bearing mice with and without leucine supplementation. Western blot analysis of C) mTOR phosphorylation: total mTOR in C2C12 myotubes treated with LLC conditioned media and leucine. Data are represented as mean ± SEM. Significance was set at p>0.05.* indicates significant difference calculated from a Two-Way ANOVA; ME indicates main effect.
Figure 4. Muscle protein degradation signaling

A) Western blot analysis of total ubiquitination and B) FOXO3a phosphorylation: total FOXO3a in gastrocnemius of control and tumor bearing mice with and without leucine supplementation. Western blot analysis of C) total ubiquitination and D) FOXO3a phosphorylation: total FOXO3a in C2C12 myotubes treated with LLC conditioned media and leucine. Data are represented as mean ± SEM. Significance was set at p>0.05.* indicates significant difference calculated from a Two-Way ANOVA; ME indicates main effect.

Figure 5. Muscle mitochondrial biogenesis signaling and content

Western blot analysis of A) Cytochrome C B) complex V ATP5a C) complex III UQCRC2 D) PGC-1α in gastrocnemius of control and tumor bearing mice with and without leucine supplementation. Western blot analysis of E) cytochrome c and F) PGC-1α in C2C12 myotubes treated with LLC conditioned media and leucine. Data are represented as mean ± SEM. Significance was set at p>0.05.* indicates significant difference calculated from a Two-Way ANOVA; τ indicates significant difference calculated by t-test. ME indicates main effect.
Figure 1
Figure 3

A.

![Graph showing P-mTOR/mTOR (Normalized IOD) for Chow and Leu in Control and LLC conditions.]

B.

![Graph showing Puromycin (Normalized IOD) for Chow and Leucine in Control and LLC conditions.]

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C.

![Graph showing PmTOR/mTOR normalized IOD for 25% C2C12 and 25% LLC cells with Control and Leucine conditions.](image)

![Western blots for P-mTOR and mTOR under different conditions: Control, Leu, Control+LLC, Leu+LLC.](image)
Figure 4

A. 

![Graph showing ubiquitin/GAPDH normalized IOD for different conditions: Control, Leu, Chow, and LLC.]

- **Chow**
- **Leu**

B. 

![Graph showing P-FOXO3a/FOXO3a normalized IOD for different conditions: Control, Leu, Chow+LLC, and Leu+LLC.]

- **Chow**
- **Leu**
- **Chow+LLC**
- **Leu+LLC**

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Figure 5.

A.  

Cytochrome C (Normalized IOD)

- **Control**
- **LLC**

<table>
<thead>
<tr>
<th>Chow</th>
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<th>ME: LLC</th>
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<tbody>
<tr>
<td>1.5</td>
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B.  

CV-ATP5a (Normalized IOD)

- **Control**
- **LLC**

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<thead>
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<td>1.5</td>
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C.

![Bar graph showing CIII-UQCR2 expression](image)

- **CIII-UQCR2** (Normalized IOD)
- **Chow** and **Leucine**
- ME: LLC

D.

![Bar graph showing PGC-1α expression](image)

- **PGC-1α** (Normalized IOD)
- **Chow** and **Leucine**

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E. Cytochrome C/tubulin

Control  Leucine  Control+LLC  Leu+LLC

Cytochrome C

Tubulin

F. PGC-1α/tubulin (Normalized IOD)

Control  Leucine  Control+LLC  Leu+LLC

PGC-1α

Tubulin