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Sex-Based Differences in ACVR2B and GRB10 Expression in Skeletal Muscle During Induced Cancer Cachexia and Disuse

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Sex-Based Differences in ACVR2B and GRB10 Expression in Skeletal Muscle During Induced Cancer Cachexia and Disuse

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This thesis is approved for recommendation to the Graduate Council.

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Abstract

Background: Muscle atrophy is the shrinkage of muscle fibers that are largely maintained by a balance of protein synthesis and degradation. Imbalances in protein synthesis and degradation are observed in different muscle atrophy disorders such as cancer cachexia and disuse. These forms of muscle atrophy produce distinct wasting pathologies that are influenced by biological sex. Activin receptor type-2B (ACVR2B) and Growth factor receptor-bound protein 10 (GRB10) have been identified as genes that potentially regulate muscle mass while also displaying sexually dimorphic differences. **Purpose:** To examine the influence of ACVR2B and GRB10 throughout the development of cancer cachexia and disuse-induced atrophy in male and female mice. **Methods:** Lewis Lung Carcinoma (LLC) was injected into the hind flank of male and female C57BL6/J mice at 8 weeks of age. Tumors were allowed to develop for 1, 2, 3, or 4 weeks and were compared to a PBS control. Disuse-induced male and female C57BL6/J mice were hindlimb unloaded for 24 hours, 48 hours, 72 hours, and 168 hours and were compared to cage control mice. RT-qPCR was used to measure mRNA content of ACVR2B and GRB10 of the gastrocnemius muscle. **Results:** LLC female mice had ~35% lower ACVR2B mRNA content compared to PBS control with a ~40% and ~30% lower content at LT and HT compared to 2-week LLC animals ($p<0.05$). LLC male ACVR2B mRNA contents were significantly lower by ~48%, ~56%, and ~36% at one, two, and four weeks, respectively, of tumor-bearing compared to PBS control mice ($p=0.0006$). No significant differences were observed in GRB10 mRNA content in LLC female mice across all cohorts. GRB10 mRNA content in LLC males was significantly lower by ~41% after one week of tumor implantation compared to LLC PBS control mice ($p=0.0094$). ACVR2B mRNA content in HU female mice was not significantly different across all cohorts ($p=0.192$). HU males displayed a ~two-fold lower ACVR2B mRNA

content after 3 days compared to cage control mice ($p=0.0233$) and 7 days of unloading ($p=0.015$). GRB10 mRNA content in HU males was significantly lower at 2 and 3 days with a ~51% lower content compared to control mice with also a ~two-fold decrease compared to 7 days of hindlimb unloading. **Conclusions:** ACVR2B and GRB10 content displayed differential responses during cancer cachexia and disuse-induced muscle atrophy that varied by sex. Data suggests that ACVR2B and GRB10 play a greater role in males during muscle atrophy than compared to females. Future experiments incorporating additional muscle groups are warranted to better understand the pathways of ACVR2B and GRB10 and their influence between sexes during cancer cachexia and disuse.

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I. Literature Review

Muscle Wasting Overview

Muscle atrophy is defined as the shrinkage of muscle fibers due to the loss of contractile proteins and organelles.[1] Skeletal muscle mass is largely maintained by a balance between protein synthesis and degradation contributing to overall muscle mass.[2] When an imbalance of protein synthesis and degradation occur, muscle atrophy can be observed as seen in disorders such as cancer and disuse.[3, 4] Cancer cachexia and muscle disuse produce distinct wasting pathologies and understanding the mechanisms and pathways associated with each can aid in the development of preventative and possibly, curative treatments.[2] Muscle atrophy is further associated with muscle weakness and fatigue contributing to a reduced quality of life.[5] Therefore, it is clinically important to further investigate the mechanisms in different pathologies that contribute to muscle atrophy and the preservation of muscle mass.

Looking specifically at cancer cachexia and disuse-induced muscle wasting, I will investigate the different patterns of wasting between male and female mice with a specific focus of the genes associated with muscle growth such as Activin receptor type-2B (ACVR2B) and Growth factor receptor-bound protein 10 (GRB10). Because recent evidence demonstrates sex-related differences in response to different types of muscle atrophies such as cancer cachexia and disuse-induced,[6] it is of great benefit to further investigate these differences as increased understanding can advance knowledge and therapeutic strategies in muscle atrophy. These genes have previously been shown to be significant regulators of muscle mass with prevalent dimorphic expression but have not been systematically investigated to determine their roles in muscle homeostasis during disuse and cancer cachexia-induced muscle atrophy.[7] This observed sex-related difference in expression will be further built upon by examining how cancer cachexia

and disuse-induced muscle atrophy influences the expression of ACVR2B and GRB10 in male and female mice.

Cancer Cachexia

Cancer cachexia is defined as the decrease of skeletal muscle and adipose tissue masses, with the development of cancer, that cannot be reversed by nutritional support alone.[8] Cancer cachexia contributes to the death of an estimated 2 million people worldwide each year.[5, 9] It is estimated that 1.8 million Americans will be diagnosed with cancer in the year 2020 while an estimate 606,520 will die from the disease.[10] Cachexia is estimated to be the primary cause of death for approximately 30% of cancer patients.[11] In addition, cancer cachexia is often associated with pancreatic, esophageal, lung, liver, stomach, and bowel cancer.[12]

There are multiple mechanisms involved in the development of cancer cachexia, many including physiologic responses driven by inflammation and pro-inflammatory cytokine activity which has been associated with muscle wasting.[3] A favored atrophy of Type II muscle fibers is observed with cancer cachexia displaying a fast-to-slow fiber type shift.[13] To date, there are no reversible treatments of cancer cachexia and therapy remains focused on supplying nutritional support while researchers investigate approaches that can target and reduce overactivation of cell injury and inflammation.[12]

Disuse-induced Atrophy

Prolonged muscle disuse is an inducible form of muscle wasting. This type of atrophy can be observed with prolonged bed rest, casting, immobilization, and limb unloading.[6, 14] Disuse can lead to a reduction in muscle mass, fiber size, strength, and function with the extent

varying on the length of disuse and the specific muscle.[14] Disuse atrophy is fiber type dependent, however, in contrast with cancer cachexia, disuse presents with a greater susceptibility of oxidative, Type I muscle fibers.[6] A greater percentage loss of fiber cross-sectional area is observed in Type I muscle fibers than compared to Type II muscle fibers affecting specific muscles differently based on fiber type composition.[4] Studies have shown that skeletal muscle mass and strength can decrease 6-24% following various types of disuse.[15] These values are dependent on the muscle type and extent of unloading.[4] Unlike cancer cachexia, disuse atrophy does not display an inflammatory action.[6]

There are many proposed pathways contributing to muscle atrophy observed with disuse. Molecular signal changes in pathways such as the Protein kinase B (also known as AKT)-Mechanistic target of rapamycin (mTOR) pathway and Myostatin pathway display skeletal muscle protein adaptations that occur through disuse. Activation of AKT via phosphorylation activates mTOR kinase leading to the activation of Ribosomal protein S6 kinase beta-1 (p70S6K), a translation initiation factor involved in regulating protein synthesis, remaining as an essential part of muscle protein synthesis.[15-17] AKT-mTOR pathway is not activated in disuse conditions leading to muscle atrophy.[15] While there is a decrease in basal protein synthesis, there is limited evidence of protein degradation observed in humans.[4] Treatments to combat disuse muscle atrophy remain minimal due to a lack of understanding of the molecular and cellular mechanisms responsible for atrophy. Therefore, further investigation on regulation of muscle protein turnover is needed to effectively prevent muscle wasting caused via disuse.

Muscle Sex Differences

Characteristics of muscle atrophy varies depending on the cause of wasting as well as biological sex. Sex hormones play a significant role in muscle homeostasis ranging from anabolism, regeneration, and inflammation, sometimes acting as protective factors.[18] Males largely express testosterone that actively promotes muscular regeneration and protein synthesis through androgen receptor signaling.[19] Females, having lower levels of testosterone, express estrogen which has a protective effect by mitigating inflammation in skeletal muscle.[18] Other studies have demonstrated a potential protective effect from female sex hormones in disuse atrophy.[20] Because of these sex differences and varying hormonal profiles, it is important to investigate the influence sex can have on different atrophy conditions. Cancer cachexia and disuse atrophy have markedly different characteristics associated with each which is influenced by biological sex.[21] While there are various sex-differences that influence muscle phenotype, function, and regulation,[6] little investigation has focused on the biological sex differences and mechanisms during wasting and warrants further investigation.[21]

Looking at the muscle composition between sexes, women have a larger relative composition of Type I muscle fibers and depend more on oxidative metabolism compared to males.[6] Conversely, males have a larger ratio of Type II glycolytic muscle fibers and a greater muscle mass.[6] This difference in muscle fiber type composition influences the ways males and females atrophy during disuse and cancer cachexia due to the two types of wasting differential responses.

Cancer cachexia favors the atrophy of Type II glycolytic muscle fibers and displays a fast-to-slow muscle fiber type switch.[13] Males experiencing cancer cachexia have been shown to lose a greater percent of body weight, rapidly losing muscle and fat, compared to females whose

fat mass declines initially but lose muscle more gradually.[18] Overall, females appear to be more protected from the inflammation induced muscle wasting which occurs during cancer cachexia when compared to males who have greater muscle losses.[6]

Disuse-induced favors atrophy of oxidative myofibers.[21] Muscles predominantly composed of slow twitch fibers, such as postural muscles, are highly susceptible to disuse and can display fiber shifts towards fast glycolytic phenotypes.[15] Because muscle composition varies between the sexes, it is important to consider sex when investigating disuse atrophy. As previously stated, females have a relatively larger composition of Type I oxidative muscle fibers.[6] Research has shown that atrophying skeletal muscle has an increase in oxidative stress and that estradiol can act as both a membrane stabilizer and antioxidant.[20] Few studies have specifically focused on the potential influence sex differences can have on disuse atrophy, but research has shown that women lose muscle faster compared to males.[6] Women have also been shown to display a greater loss of strength in isometric and concentric contractions compared to males after short-term immobilization.[20] Despite a previous study showing men and women having similar decreases in cross-sectional area after disuse-induced, men retained a greater degree of specific strength compared to females.[20]

While sex differences have been observed through disuse-induced atrophy, it is not clear whether these differences can be attributed to the favorable atrophy of Type I oxidative muscle fibers that are more abundant in females, or if there are other differences in muscle physiology between sexes.[6] Understanding the wasting characteristics of cancer cachexia and disuse-induced and the differences that are present between sexes create the foundation for understanding the potential dimorphic expression of ACVR2B and GRB10.

ACVR2B

ACVR2B is an activin and part of the TGF Beta family.[22] It is a protein coding gene and encodes a myostatin receptor involved in growth factor pathways known to regulate muscle mass.[7] Activin type II receptors are capable of binding myostatin, a negative regulator of muscle growth that blocks myogenic development, and other TGF Beta family members.[23] TGF Beta family receptors and ligands control progenitor activation and muscle growth formation.[24] Previous research has shown increased muscle mass and promoted skeletal muscle growth in mice following the administration of soluble ACVR2B.[25] The soluble form of ACVR2B leads to the suppression of myostatin.[24] Muscle mass increases of up to 60% have been observed in wild-type mice following soluble ACVR2B administration.[23] Further research and better understanding of ACVR2B may be clinically beneficial in the prevention of muscle atrophy and can aid in treatments of degenerative myopathies, like cancer cachexia. Pathways involving ACVR2B are yet to be fully understood whether they act primarily upon myofibers or target additional cell types.[24]

The binding of myostatin is proposed to signal through two activin receptors, ACVR2A and ACVR2B.[26] Activin type 1 receptors are activated and phosphorylated through the binding of myostatin to ACVR2B which leads to intracellular signaling of receptor-regulated proteins, Smad2 and Smad3.[25] Smad2, Smad3, and Smad4 are required for myostatin-induced transcription.[27] Administration of soluble ACVR2B has shown to inhibit muscle and adipose wasting in cachexia by targeting myostatin-family ligands.[28]

Inhibition of myostatin binding has previously been shown to be a therapeutic approach to preserving muscle mass in cancer cachexia.[28] A proposed mechanism of muscle wasting observed in cachexia is increased signaling of activin receptor ligands.[29] Blocking of these

ligands and their receptors can prevent muscle atrophy as well as increase of muscle mass. Soluble ACVR2B produced an increase of muscle mass by 23-40% in a study performed by Benny Klimek et al.[28] While this study effectively showed that ACVR2B promotes muscle preservation in cancer cachexia, the protective effect may be due to other myostatin targets and the underlying mechanisms are yet to be fully uncovered. Other studies have shown that blocking of activin receptor ligands can improve survival of cancer with improved limb and respiratory muscle mass.[29] In addition, Huot et al[30] demonstrated the complete preservation of cardiac and muscle function as well as preservation of adipose tissue, bone, and skeletal muscle in tumor hosts receiving ACVR2B/Fc. While there is previous research focused on ACVR2B's protective effect of muscle mass throughout cancer cachexia, research is not as prevalent on its impact in disuse-induced and potential differences in expression between sexes. Studies focused on cancer cachexia have briefly mentioned ACVR2B's protective effect on muscle mass in other myopathies often including disuse.

When examining the expression of ACVR2B between sexes, to my knowledge, one study has observed to be expressed at 1.6-fold greater in women compared to men using quantitative RT-PCR.[7] Difference in the expression of ACVR2B between sexes is scarce but few associations have been observed in regard to cancer treatments incorporating both sexes. A study on liver cancer investigating levels of ACVR2B-AS1 showing that liver cancer specimens contained significantly elevated levels of ACVR2B-AS1 and that women with higher levels had shorter relapse-free survival compared to those with lower ACVR2B-AS1 levels.[31] This study further showed that ACVR2B could be a beneficial therapeutic target in cancer treatment and prognosis. Further investigation is warranted to understand sexual dimorphic expression of

ACVR2B in cancer cachexia and disuse for the preservation of muscle mass and advanced treatment opportunities.

GRB10

Growth factor receptor-bound protein 10 is an adapter protein that interacts with the insulin receptor by binding to and suppressing signaling.[32] It is involved with many tyrosine-phosphorylated growth factor receptors and is highly expressed in muscle, pancreas, and adipose tissues that involve glucose metabolism and insulin action.[33] Variations in GRB10 expression alter insulin receptor protein levels.[32] GRB10 negatively regulates insulin signaling with the inhibition of insulin receptor kinase activity by facilitating degradation of the receptor. By controlling the receptor, activation of growth factors can be regulated.[34]

GRB10 has been shown to play an important role in the regulation of muscle size and muscle metabolism.[33] Studies involving mice with the loss of GRB10 resulted in increased signaling of the insulin receptor in muscle tissue and increased glucose uptake.[35] GRB10 knock out mice have also shown hyperplasia of muscle cells.[33] A study performed by Smith et al[35] compared muscle and adipose mass from birth to one-year of male and female wild-type mice and disrupted GRB10 mice. This study showed that male and female GRB10 mice were approximately 13% heavier with reduced adiposity at six months compared to wild-type littermates.[35]

In addition to insulin receptor signaling, GRB10 is also a substrate for mTOR complex 1.[36] Edick et al.[36] characterized the function of GRB10 and its regulation of mTORC1 in human muscle. This study proposed that mTORC1 controls phosphatidylinositide 3-kinase (PI3K) signaling by GRB10 through alteration of insulin receptor abundance. The PI3K/Akt pathway

plays great significance for metabolic actions of insulin including glucose uptake and glycogen synthesis in skeletal muscle. GRB10 silenced myotube showed increased insulin responsiveness with enhanced insulin-induced PI3K/Akt signaling with a higher abundance of the insulin receptor.[36] Emerging research into cancer cachexia has focused on the influence and contribution of mTOR and mTOR inhibitors.[37] GRB10 stabilizes mTORC1 which participates in cell proliferation and survival. With GRB10's role in regulating mTORC1 and its negative feedback on PI3K/Akt signaling not yet being fully established, research into the expression of GRB10 in cancer can contribute further insight.

Differences in insulin receptor gene expression has been observed to be higher in women by a 3-fold increase.[7] Because GRB10 expression can restrain IGF-1 signaling, Welle et al[7] proposes that a greater GRB10 expression may contribute to sex differences in muscularity. This hypothesis is supported from Smith et al[35] where GRB10 knockout mice exhibited increased muscularity. Similar to ACVR2B, literature of sexual dimorphic expression of GRB10 is limited and warrants further investigation. In addition, the influence GRB10 has on skeletal muscle makes it an important contender towards investigating changes in expression that may occur during muscle atrophy in cancer cachexia and disuse.

Purpose

Considering the potential interplay with muscle growth mechanism and the limited knowledge regarding the roles of ACVR2B and GRB10 during muscle atrophies between males and females, the purpose of this work is to better understand biological sex differences in ACVR2B and GRB10 expression during cancer cachexia and disuse-induced muscle atrophy.

This study will aid in improved treatment and preventative therapies to counteract the progression of muscle atrophy.

Objectives for Data Analysis

- Describe muscle atrophy characteristics observed between disuse-induced and cancer cachexia
- Describe difference of muscle wasting in males versus females during disuse-induced and cancer cachexia samples
- Assess differences in expression of ACVR2B and GRB10 during disuse-induced and cancer cachexia samples
- Determine differences in expression of ACVR2B and GRB10 in males and females
- Further experiments may be pursued as dictated by data outcomes

II. Methods

Animal experiments for this proposal were previously approved by the University of Arkansas Institutional Animal Care and Use Committee and performed by Dr. Greene's laboratory group. My work does not involve direct use of research animals but is limited to analysis of muscle tissue samples previously collected. This thesis involves analysis of collected tissue samples from two different models of muscle atrophy; hindlimb suspension for induced-disuse atrophy and Lewis Lung Carcinoma (LLC) implantation for induced cancer cachexia.

Animals and Intervention

The mice utilized for this study were a cohort of male and female C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animal experiments were performed at the University of Arkansas in Fayetteville, Arkansas and have been approved by the Institutional Animal Care and Use Committees of the University of Arkansas. These animals were housed to meet the requirements for hindlimb suspension as well as being placed in a temperature-controlled environment maintained on a 12:12 h light-dark cycle. The mice received *ad libitum* access to normal rodent chow and water. The mice utilized for induced cancer cachexia received LLC implantation at 8 weeks in the hind flank. I did not personal perform animal work in this thesis; all animal work was previously performed and I am solely working with tissue analyses.

Hindlimb Suspension

Induced muscle atrophy of the skeletal muscle was produced by hindlimb suspension as previously described.[38-40] Prior to hindlimb suspension, mice were acclimatized to handling by researchers for one week before being subjected to seven days of suspension. Animals' tails were first sterilized using ethanol wipes followed by iodine swabsticks. After which, tails were coated with benzoin to increase adhesiveness of the tail. Appropriate size of athletic tape was then wrapped around the base of the animals' tail and a small stainless-steel hook was then connected to the athletic tape and adjusted to allow normal blood circulation. Fishing string was then utilized to connect between the hook and a custom designed pulley system to allow for unloading of the hindlimb legs, allowing only forelimbs to have contact with the cage floor with a suspension angle of 30-45 degrees. The unloading system involved a single rod that reaches

across the cage allowing access to all areas but limiting contact with cage walls to prevent potential hindlimb loading. The cage floor was fitted with grid wire with only a small amount of bedding to prevent hindlimb loading from mounded up bedding. Animals were monitored daily to ensure unloading of hindlimbs as well as signs of tail necrosis or distress. With the mice being suspended for seven days to induce disuse atrophy, the bedding materials were not replaced through the course of the experiment. Disuse was allowed to develop over a time course to create the following experimental groups: cage control, 24 hours, 48 hours, 72 hours, and 168 hours of disuse. The length of disuse has been shown to induce disuse atrophy in both male and female animal models.[41-44]

Lewis Lung Carcinoma Cell Culture and Implantation

Lewis lung carcinoma cells (ATCC CRL-164) were used for this project. LLC cell culture and harvest were prepared as previously described by Brown et al.[45] Briefly, these cells were plated in 250 mL culture flasks in DMEM with 10% fetal bovine serum supplemented with 1% penicillin and streptomycin. Upon confluency, cells were trypsinized, suspended, counted, and diluted in PBS for implantation. At 8 weeks of age, mice were implanted with LLC (1×10^6 cells in 100 uL of PBS) subcutaneously in the hind flank where the tumor was allowed to develop for 1-4 weeks in separate cohorts of animal. A control group was given an equal volume of PBS at 8 weeks of age while also aged-matched with 4 weeks of tumor bearing mice. At the time of harvest, mice were 12 weeks in age. This method has been widely used to induce cancer cachexia and inducing cachectic phenotype.[45-47]

Tissue Collection and Sample Preparation

After the suspension and cancer-induced cachectic periods, animals were anesthetized under isoflurane. Tissue samples of the plantaris, gastrocnemius, soleus, extensor digitorum longus; EDL, tibialis anterior; TA, heart, tumor, lungs, spleen, epididymal fat, and plasma were collected, and mice were humanely euthanized while under anesthesia. Tissue sample weights were recorded and snap-frozen in liquid nitrogen. Tissues were stored at -80°C for further analyzation. For the purpose of this thesis, the gastrocnemius muscles are the only tissue sample being analyzed.

RNA isolation, cDNA synthesis, and qualitative real-time qPCR

Frozen gastrocnemius muscle samples were powdered, and 20 mg of samples were suspended in 1 mL of TRIzol reagent and then homogenized using a Polytron for ~5 seconds for five times. These samples were transferred into a 1.5 mL microtube on ice and 200 µL of chloroform was added. After 15 minutes, the samples were vortexed and centrifuged for 25 minutes. The supernatant liquid was removed and placed in a new tube where equal amounts of 70% of Diethyl Pyrocabonate (DEPC) treated ethanol was added as the sample was loaded into a RNeasy column. An RNA isolation kit (K145002; Invitrogen, Carlsbad, CA, USA), was used for RNA isolation. 30 uL of DNase treated RNA was collected and the purity of the RNA was verified by fluorometry using 260/280 nm ratios read on a Bio-Tek Power Wave XC microplate reader (BioTek Instruments Inc., Winooski, VT, USA) with Take3 microvolume plate and Gen5 software. Subsequently, using VILO Superscript reagent (11755050; Invitrogen, Carlsbad, CA, USA), 1 µg of RNA was reverse-transcribed into cDNA and diluted to 1:100 (10 ng/µL) through the process. Real-time polymerase chain reaction (PCR) was performed on the sample with the

use of TaqMan probe reagent, Step-One real-time RT-PCR instrumentation (Applied BioSystems, Foster City, CA, USA), and QuantStudio 3 Flex real-time RT-PCR instrumentation (Applied BioSystems, Foster City, CA). Ct values were analyzed following the manufacturer's protocols of 4 minutes of incubations, 45 cycles of denaturation, and annealing and extension at 95 °C, 60 °C, and 72°C. Target TaqMan probes for ACVR2B (Mm00431664) and GRB10 (Mm01180443) were purchased from Applied Biosystems (Life Technologies). The final gene expressions were calculated using the ΔΔCT method and the relative quantification was calculated as $2^{-\Delta\Delta CT}$ as described previously.[48] As a loading control, 18s Ct values did not differ between groups within each experiment.

Statistical Analysis

A One-way ANOVA will be used as the global analysis for each dependent variable. Where significant F-ratio is found, statistical differences among means were determined by Tukey's post hoc test. The comparison-wise error rate, α, is set at 0.05 for all statistical tests. All data will be analyzed using the Statistical Analysis System (SAS, version 9.3, Cary, NC, USA) and data expression as mean ± Standard error of the mean (SEM).

III. Results

Induced muscle atrophy by cancer cachexia and hindlimb unloading

Phenotypic characteristics of mice following induced cancer cachexia and hindlimb unloading have been previously reported.[45, 46, 49] Following tumor implantation, tissue wet weights of the gastrocnemius muscle were ~12% lower in 4-week male tumor-bearing mice compared to control mice ($p<0.05$)[45] while female gastrocnemius muscle were ~8% lower in 1-

week females compared to controls ($p<0.05$).[50] After hindlimb unloading, males and females both showed lower body and lower-limb muscle weights ($p<0.05$).[49] Lower overall muscle weights in females were significant after 24 hours of unloading while this effect was absent in males.[49]

ACVR2B and GRB10 mRNA Content in Lewis Lung carcinoma male and female mice

ACVR2B and GRB10 mRNA contents were not significantly different between biological sexes for PBS control LLC mice ($p>0.05$, Figure 1B & 1D). In LLC induced cachexia, female mice had a lower significant mRNA content of ~35% in ACVR2B in the low-tumor cohort compared to female PBS control mice. Females were also ~40% and ~30% lower at LT and HT compared to 2-week LLC animals, respectively ($p<0.05$, Figure 2C). LLC male ACVR2B mRNA contents were significantly lower by ~48%, ~56%, and ~36% at one, two, and four weeks, respectively, of tumor-bearing compared to PBS control mice ($p=0.0006$, Figure 2G). Despite a significant main effect ($p=0.0429$), no significant pairwise differences between groups were observed in GRB10 mRNA content in tumor-bearing female mice (Figure 2D). In LLC males, GRB10 mRNA content was significantly lower by ~41% after one week of tumor implantation compared to LLC PBS control mice ($p=0.0094$, Figure 2H).

ACVR2B and GRB10 mRNA Content in hindlimb unloading male and female mice

Cage control mice for hindlimb unloading demonstrated no significant differences between biological sexes in either ACVR2B or GRB10 mRNA contents ($p>0.05$, Figure 1A & 1C). ACVR2B mRNA content was not significantly different in hindlimb unloading female mice across cohorts ($p=0.192$, Figure 2A). However, there were significant differences in males

with a ~two-fold lower ACVR2B mRNA content at 3 days compared to cage control mice ($p=0.0233$) and 7 days of unloading ($p=0.015$, Figure 2E). In females, GRB10 mRNA content with hindlimb unloading was not significantly different compared to cage controls ($p>0.05$, Figure 2B) although there were statistical differences observed elsewhere. HU female mice after one day of hindlimb unloading were ~2-fold lower in GRB10 mRNA content compared to 3 ($p=0.0142$) and 7 days ($p=0.0364$) of hindlimb unloading. GRB10 mRNA content in male mice was significantly lower at 2 days and 3 days with ~51% lower content compared to control mice while also ~two-fold lower compared to 7 days of hindlimb unloading mice (Figure 2F).

IV. Discussion

In the present study, I sought to define ACVR2B and GRB10 responses to distinct forms of muscle atrophy, cancer cachexia and disuse, in male compared to female mice to identify potential roles of these muscle mass-associated proteins across muscle wasting. The results demonstrate ACVR2B and GRB10 are differentially regulated between sexes during the development of muscle atrophy induced by cancer cachexia and disuse. In addition, both ACVR2B and GRB10 appear to play a greater role in males during muscle atrophy compared to their female counterparts. Although molecular or phenotypical alterations of skeletal muscle have been well reported in muscle atrophy, to my knowledge, no studies have investigated molecular mechanisms involved in ACVR2B and GRB10 throughout cancer cachexia and disuse-induced wasting, nor while investigating potential sex differences. Therefore, major findings of the current project emphasize the importance of biological sex as well as a necessity for further investigations on ACVR2B and GRB10 in muscle atrophy.

Male and Female ACVR2B Content Response Differences in HU & LLC Mice

The investigation of ACVR2B mRNA content in response to cancer cachexia and disuse-induced muscle atrophy between sexes, to my knowledge, has rarely been explored. In this project, ACVR2B mRNA content showed significant differences across cohorts of LLC cancer cachexia and HU disuse-induced atrophy in a sex-dependent manner. In fact, although we did not find a statistical significance in ACVR2B content in female mice during the development of hindlimb unloading-induced muscle atrophy, that of ACVR2B was significantly lower in LLC-mediated tumor-bearing female mice, indicating ACVR2B may play an important role in development of cancer-induced atrophy. Comparable to these results, a Chopard et al. showed no changes in ACVR2B mRNA content in women after two weeks of human immobilization.[51] Although a direct comparison between humans and rodents is limited, current data suggests ACVR2B may not be associated with disuse-induced muscle atrophy in females.

Differing from females, HU male mice showed lower mRNA content in ACVR2B after 2 and 3 days of unloading with only 3 days being significant. A previous study examined ACVR2B mRNA content in the quadriceps of male participants after 2 weeks of immobilization and 6 weeks of rehabilitation. The ACVR2B content was not influenced by immobilization nor by rehabilitation.[52] Although there were no ACVR2B content changes observed in immobilized humans, the loss seen in HU male mice could be due to the use of different muscles analyzed. After 2 weeks of immobilization, the quadriceps muscle had a ~5% decrease in muscle mass while the gastrocnemius in mice had a ~12% loss potentially indicating that alterations to ACVR2B mRNA content may necessitate a more severe muscle loss.[49, 52]

Unlike HU mice, male and female LLC mice both had significantly reduced ACVR2B mRNA content. Female mice had a lower ACVR2B content in the low-tumor cohort (late-stage

cancer) compared to PBS control whereas ACVR2B content in males was lower in all tumor-implanted mice cohorts. Females have been shown to be more protected from inflammation-induced muscle wasting and to lose muscle mass more gradually during cancer cachexia which may contribute to the differences observed between males and females.[6, 18]

LLC male mice displayed significantly low ACVR2B content (~2 folds) during the development and progression of cancer cachexia. Interestingly, lower ACVR2B content was observed in early-stage cancer, which remained lower until marked muscle wasting occurs (late-stage cancer), indicating lower ACVR2B mRNA content may serve as an early biomarker for muscle wasting in male cancer cachexia. Collectively, altered mRNA content of ACVR2B seems to play an important role in cancer-mediated muscle wasting to a greater extent in male mice.

There has been much investigation into the therapeutic work of soluble ACVR2B in the treatment and prevention of cancer cachexia.[7, 53-55] To my knowledge, no studies have specifically examined the response of ACVR2B during cancer without the administration of soluble ACVR2B or knockout. Therefore, further studies on the ACVR2B mechanism in a cancer-mediated environment are warranted to unravel ambiguous muscle wasting mechanisms during the development of cancer cachexia.

Although ACVR2B has rarely been studied in cancer cachexia, a few interesting findings of ACVR2B in response to different forms of muscle atrophy have been reported. For example, a prior study by Yaden et al. investigated the effects of overexpressing activin A and the direct consequences on muscle tissue.[56] Indeed, overexpression of activin A produced a 52% reduction in muscle mass and produced more pronounced decreases than observed with myostatin overexpression.[56] Differences in muscle mass did not relate to mRNA levels in the current study

and further investigation of protein levels may be warranted. The continuation of ACVR2B's role during muscle atrophy is encouraged to be explored alongside myostatin as differences in myostatin activity between males and females could be related to differences in proteins that process, bind, and signal transduce myostatin.[7]

Male and Female GRB10 Content Response Differences in HU & LLC Mice

Significantly less GRB10 content was observed in male and female hindlimb (HU) mice. While both had significant decreases in GRB10 content during HU, females demonstrated lower GBR10 content within 24 hours with levels returning to baseline, however, in males this reduction was shown after 2 days following HU before returning to baseline. Similar to this pattern, females experiencing disuse have shown significantly lower muscle weights after 1 day of unloading, unlike males where lower muscle mass is seen at 2 days of unloading.[49] Although males and females have similar decreases in cross-sectional area during disuse, research has shown females lose muscle faster with males retaining a greater specific strength.[6, 20] With females experiencing the onset of disuse sooner compared to males while correspondingly displaying an earlier decrease of GRB10 mRNA content, the onset of muscle loss is marked by a loss in GRB10 mRNA content as also seen in males.

Male LLC mice displayed an initial loss in GRB10 mRNA content after one week of tumor implantation while this effect was not observed in females nor beyond one week in males. Changes in muscle mass can significantly affect glucose homeostasis and metabolic health, as seen with muscle atrophy, and is associated with decreased insulin sensitivity.[15, 58] As females have increased insulin receptor gene expression and are more protected against inflammation-

induced muscle atrophy, these factors could support the limited differences observed in GRB10 mRNA content during cancer cachexia in female mice.[6, 7]

GRB10 promotes cell proliferation and cell survival.[57] Overexpression has been observed to result in growth retardation and insulin resistance.[57] Looking at short-term muscle disuse, skeletal muscle has been shown to be insulin resistant with the response differing across subjects.[59] The mechanisms regulating disuse-induced insulin resistance between populations are not yet known[59] but due to GRB10's influence of binding and modulating the insulin receptor, decreases in muscle mass and muscle atrophy could explain decreases in GRB10 content during cancer cachexia and disuse-induced atrophy.[36] Investigation of GRB10 content throughout cancer cachexia and disuse-induced wasting is limited as well as knowledge of its role in muscle outside of the insulin signaling pathway and further exploration of GRB10's influence in muscle atrophy is encouraged.[58]

Species Difference of Humans and Mice

The application of mice modeling for human health studies has been widely and extensively used for cancer and disuse studies.[60] While mice models have ethical and practical advantages, there are fundamental differences between species in regard to human disease phenotypes.[60, 61] Although limitations exist, cross-species comparisons at the molecular level are feasible and insights on the degree of conservation between humans and mice have further developed.[62]

There are many biological processes and genetic elements conserved between humans and mice.[62] Mice models can replicate processes within a disease but may not account for all the physiological changes to occur throughout humans.[63] Divergences and phenotypic differences

between species may result in weakly correlated responses between the two species possibly accounting for the differences of the result observed.[62] While mice studies are extremely beneficial, it is important to consider the species differences that may occur on the molecular and transcriptional level when applying to human comparisons.

Vastus Lateralis and Gastrocnemius Fiber Type

It is essential to consider what particular mouse muscles most similarly represent human muscle wasting myopathies as skeletal muscles are dissimilarly affected by varying wasting pathologies.[61] Human and mouse skeletal muscle profiles vary by muscle type with the soleus muscle of the mouse being identified to most closely resemble the molecular expression of human skeletal muscle for both control and disease human tissue.[60, 61] For this thesis, the gastrocnemius was the sole muscle studied. Had the same muscle been studied for humans and mice, results may have been different. Previous studies have displayed similar atrophic changes of the gastrocnemius and vastus lateralis muscle of humans during sarcopenia but comparisons for mice have not been reported.[64]

The vastus lateralis is a well-studied muscle in human studies due to its mixed fiber type composition and accessibility.[65] For both males and females, the vastus lateralis contains approximately 42% type I fibers, 35% type IIA fibers, and 23% type IIB fibers.[65] Prior research has also reported the vastus lateralis encompassing 32% slow-twitch fibers.[66] Sex differences have been observed for fiber size with males having larger cross-sectional areas compared to females by 14%, 28%, and 56% for type I, IIA, and IIB fibers, respectively.[65] The gastrocnemius muscle of C57BL6J mice are predominantly type II fibers.[67] In humans, the gastrocnemius has been described as containing 50% slow-twitch fibers.[66] Due to the influence

of fiber type composition on the wasting characteristics of cancer cachexia and disuse-induced wasting in males and females, it is important to take into consideration the difference between the vastus lateralis and gastrocnemius muscle.

While the vastus lateralis and gastrocnemius muscle are both characterized as predominantly fast-twitch fibers, the use of different muscle groups could account for the lack of basal sexual dimorphic expression observed. Others who are interested in the potential sexual dimorphism of ACVR2B and GRB10 throughout cancer cachexia and disuse-induced wasting are encouraged to examine and compare further muscle groups.

V. Conclusion

In this study, ACVR2B and GRB10 mRNA content in male and female mice experiencing cancer cachexia and disuse-induced muscle atrophy were assessed and ACVR2B and GRB10 are dimorphically regulated between sexes during the progression of muscle atrophy by induced cancer cachexia and disuse. Based on my data, ACVR2B content was less during cancer cachexia for both male and female mice while having a larger influence and effect in male mice. Current data suggests ACVR2B may not be associated with disuse-induced muscle atrophy in females but displays a significant decrease in males after 3 days of unloading. Females are also shown to have stable GRB10 mRNA content during cancer cachexia with males displaying an initial loss after 1 week with a return to baseline. Male and female mice both experienced a loss in GBR10 mRNA content during disuse with females displaying a faster initial loss than compared to males. This data provides information to help combat cancer cachexia and disuse-induced muscle atrophy. With the limited knowledge surrounding ACVR2B and GRB10 content

responses throughout cancer cachexia and disuse-induced wasting, further investigation can aid in improved treatments and understanding of these associated pathways during muscle atrophy.

VI. References

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VII. Appendix

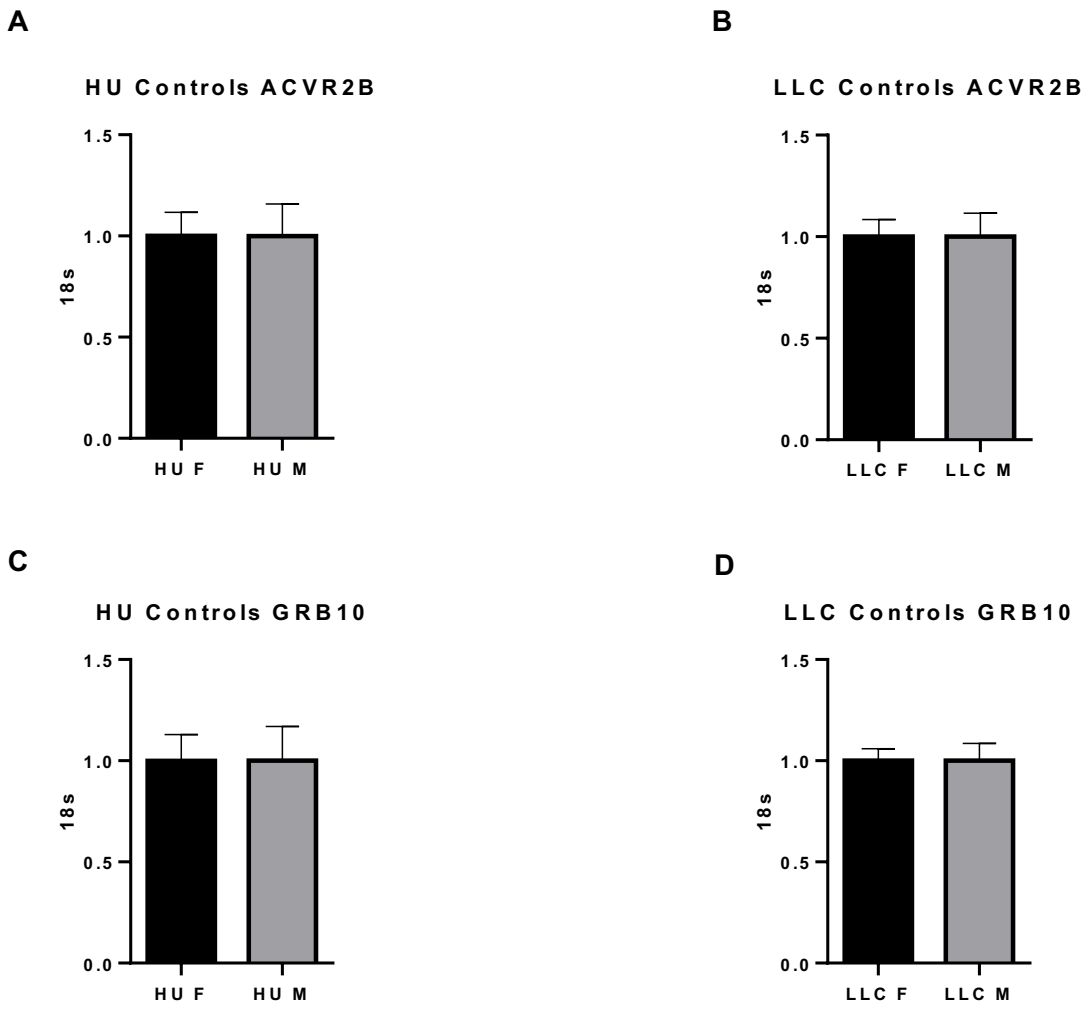

Figure 1. Baseline control comparison of ACVR2B and GRB10 mRNA content between males and females. (A) ACVR2B in HU controls, (B) ACVR2B in LLC controls, (C) GRB10 in HU controls, (D) GRB10 in LLC controls.

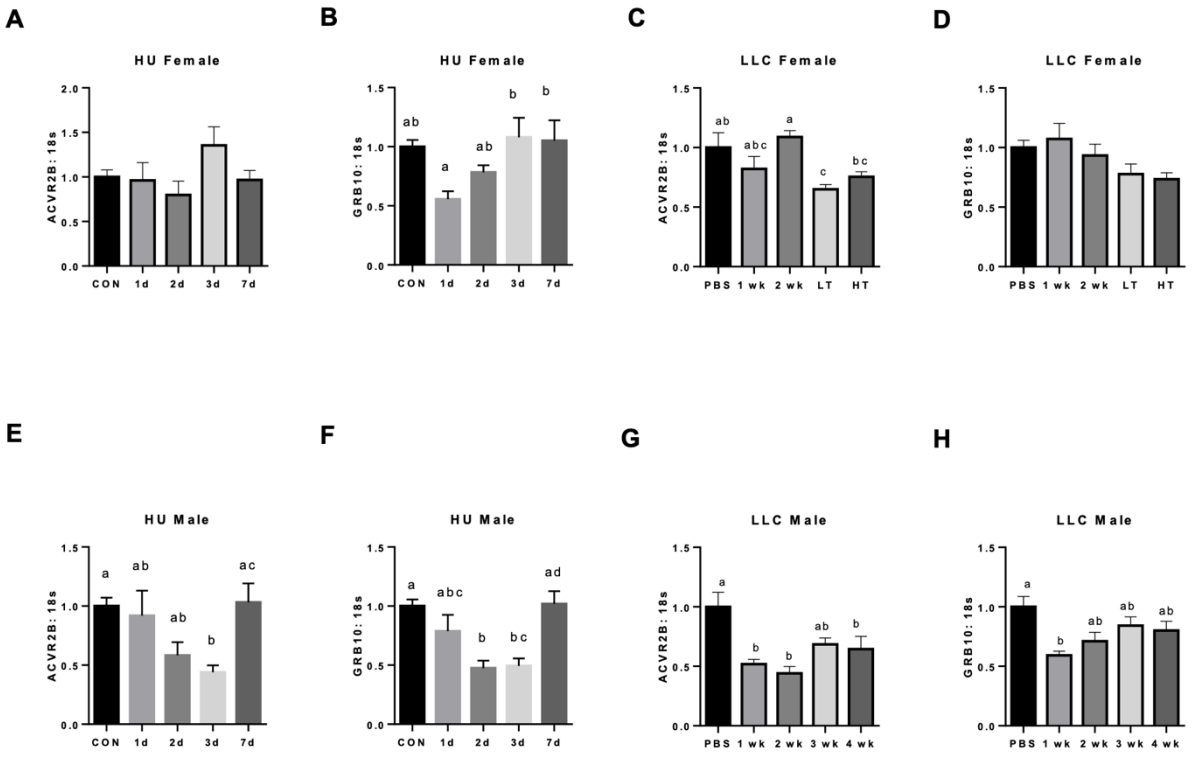

Figure 2. Comparison of mRNA content of ACVR2B and GRB10 during the progression of hindlimb unloading (HU) and cancer cachexia (LLC) of the gastrocnemius muscle in male and female mice. (A) ACVR2B in HU females, (B) GRB10 in HU females, (C) ACVR2B in LLC females, (D) GRB10 in LLC females, (E) ACVR2B in HU males, (F) GRB10 in HU males (G) ACVR2B in LLC males, (H) GRB10 in LLC Male. Different lettering denotes statistical significance between groups, alpha set at $p<0.05$.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Greene

FROM: Craig N. Coon, Chairman

DATE: 7/13/15

SUBJECT: IACUC Approval

Expiration Date: Jan 1, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 15065: "Mitochondrial Degeneration in the Onset of Cancer-Cachexia Induced Muscle Atrophy" you may begin work immediately

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Jan 1, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Nicholas Greene
Fr:	Craig Coon
Date:	April 12th, 2018
Subject:	IACUC Approval
Expiration Date:	April 5th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18111:** *Mitochondrial degeneration in the development of cancer-induced muscle loss in female mice*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 5th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Nicholas Greene, Tyrone Washington, Megan Rosa, and Wesley Haynie. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18111