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RNA Sequencing in the Development of Cancer-Cachexia

Thomas Allen Blackwell
University of Arkansas, Fayetteville

RNA Sequencing in the Development of Cancer-Cachexia

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_____
Dr. Nicholas Greene
Thesis Director

_____
Dr. Tyrone Washington
Committee Member

_____
Dr. Walter Bottje
Committee Member

Abstract

Introduction: Cancer is a major public health problem in the U.S. and the world. In 2013 there were an estimated 1,660,290 new cases of cancer in the U.S. Cancer-Cachexia (CC) is a common effect of many cancers, and is directly responsible for 20-40% of cancer-related deaths. The mechanisms that control the development of CC are not well understood. Most investigations of CC focus on the post-cachectic state and do not examine the progression of the condition. The purpose of this study was to utilize RNA sequencing to analyze transcriptomic alterations throughout the progression of CC. **Methods:** Lewis Lung Carcinoma cells (LLC) or Phosphate Buffered Saline (PBS, control) were injected into the hind-flank of wildtype C57BL6/J mice at 8 wks of age, and tumor allowed to develop for 1, 2, 3, or 4 wks before euthanasia. RNA was isolated from the gastrocnemius and RNA sequencing performed. **Results:** RNA sequencing showed widespread alterations in LLC when compared to PBS animals with largest differences seen in 4 wk LLC compared to other conditions. Commonly altered pathways included: Oxidative Phosphorylation, Mitochondrial Dysfunction, and Protein Ubiquitination. **Discussion:** We demonstrated that alterations in the muscle belly likely occur in phases beginning with early mitochondrial degenerations, which now appear to lead to large transcriptomic shifts concurrent to the onset of muscle atrophy. In agreement with previous work, we observed multiple aspects related to degeneration of mitochondria and oxidative metabolism. The early onset of these alterations shows a need for early interventions in order to effectively ameliorate the effects of CC.

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Dedication

I would like to dedicate this thesis to my wife, Mrs. Meredith Blackwell. Thank you for being an endless source of support and encouragement.

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Cancer-Cachexia Overview

Approximately 39.6% of men and women will receive a cancer diagnosis during their lifetime, with an estimated 1,685,210 new cancer diagnoses in the United States in 2016 alone, of which 595,690 will eventually die as a result of their cancer diagnosis (1). On a global scale, it is anticipated that annual new cancer diagnoses will surpass 20 million as soon as 2025 (2). In a 2011 statement from the World Health Organization, cancer is now recognized as one of the leading causes of death within the Western World, recently surpassing coronary heart disease and all stroke-related mortality (2). Since 1970 mortality rates for other common life threatening diseases have declined significantly (3). The most significant of which were heart disease and cerebrovascular disease, with a 62% and 73% decline in mortality respectively (3). In this same timeline, mortality rates due to cancer have only declined 12% (3). This shows us that there is a need for new evidences to enhance the treatment of cancer. One of the biggest issues facing the treatment of cancer is the vast number of types and causes of cancer as well as the difficulty in treating such a multi-faceted disease.

One significant aspect of these cancer diagnoses is that up to 80% of individuals will experience varying degrees of Cancer-Cachexia (CC) (4). CC is defined as the ongoing loss of muscle mass, which may be accompanied by wasting of other tissues, that cannot be fully reversed by nutritional interventions alone (5, 6). CC is estimated to be responsible for 20-40% of cancer-related deaths, depending on the type and severity of cancer present, along with how soon preventative measures are taken (7). Currently, the exact mechanisms responsible for the development of CC are not well understood, but it is clear that CC is a serious health care concern that lacks adequate interventions.

CC is a multifaceted, complex phenomenon. By definition nutritional changes alone cannot reverse the effects of CC, which in turn suggests that the muscle wasting is largely due to altered metabolism both systemically and intramuscularly (7). In order for any form of skeletal muscle atrophy to occur the rate of protein degradation must surpass the rate of protein synthesis, leading to a net loss in total muscle protein. This effect has been seen and exhaustively analyzed in other cases of atrophy, notably: denervation (8), sarcopenia (9), and morbid obesity (10). In the case of CC, however, the specific metabolic dysfunctions are not fully understood. Most current investigations of CC primarily focus on comparisons between the pre- and post-cachectic state, and offer little insight into the development of the condition. When facing a disease with such a high mortality rate, it is essential to understand how to effectively prevent CC from developing in the first place. This review of literature will outline known mechanisms in CC and the likely utility of –omics based approaches to further understand the development of CC.

Known Pathways of Muscular Atrophy in Cancer-Cachexia

An in-depth understanding of CC is essential for understanding the progression of this disease and any potential interventions to truly reverse or reduce progression. In order to impact CC before there is significant muscle wasting, we must develop an understanding of altered cellular mechanisms at early stages in this complex disease. There are numerous mechanisms and alterations that contribute to dysregulation of muscle protein turnover in CC (11, 12). These include, but are not limited to, protein synthetic, myogenic, autophagic, proteasomal, inflammatory, and mitochondrial pathways (11-14). The following section will analyze current research in these areas.

Protein Synthetic Pathways

In CC, rates of protein synthesis are initially decreased ~19% and progress toward ~50% when compared to healthy controls throughout the progression of CC (15). Some factors that influence signal transduction pathways regulating protein synthesis are mechanical stimuli, anabolic and catabolic hormones, and energy balance (16). The regulation of protein synthesis is largely accomplished via mTOR-related signal transduction pathways (16). By far the most predominant signaling regulator of translation initiation is the AKT/mTOR pathway, of which insulin and IGF-1 are major activators (17). In CC, protein synthesis is down regulated in a number of ways. Both serum IGF-1 and the synthesis of IGF-1 from skeletal muscle are impaired in CC (18). Also, a decrease in mTOR activation is noticeable in early and late stage CC in mice following MAC16 tumor implantation (19). Though many theories related to decreased mTOR activation have been proposed, the two most promising appear to work through AMPK and systemic inflammation (20). AMPK inhibits mTOR by preventing the interaction with both p70S6K and 4E-BP1 (21). Inflammatory proteins, such as IL-6, also reduce the activation of mTOR directly and indirectly through phosphorylation of AMPK, among other impacts of IL-6 and inflammatory cytokines (20). Both of these potential mechanisms of protein synthesis reduction via the mTOR pathway have been observed in models of CC (19, 22).

Myogenic Pathways

In CC, there is significant damage to the sarcolemma. This becomes problematic whenever combined with decreased capacity for myogenesis, which has been linked to dysregulated myogenesis and muscle loss (23). It has been suggested that this is due to an increase in compensatory protein synthesis via other pathways, which are not present in CC (24). Satellite cell function is likely reduced through the NF-κB pathway (25). NF-κB activation leads

to sustained Pax7 activation, which in turn likely propagates a self-renewal signal in myogenic cells, rendering them unable to progress through differentiation (25). Despite these findings, Satellite Cell depletion alone does not appear to induce muscle atrophy but at the least may in part explain inabilities to reverse cachexia (24). Furthermore, NF-κB mediated Pax7 activation has also been shown to inhibit MyoD synthesis (25) Myogenic regulatory factors MyoD, Myogenin, Myf5, and Myf6 demonstrate some interplay with IGF-1 (26). Since reduced IGF-1 signaling has been observed in CC (18), it is likely impairment of these processes is interrelated. Another potential area of myogenesis dysregulation has been examined in denervation models; it has been shown that denervation results in an increase in Myogenin production, which seems to lead to the activation of E3 ubiquitin ligases MuRF1 and Atrogin which would ultimately promote protein degradation (8).

Protein Degradation

Autophagy is often described as a process that is inversely related to protein synthesis (27). It has been shown that autophagy is dysregulated in CC (13). Autophagy is a process of lysosomal degradation whereby aggregated protein and damaged organelles are engulfed by an autophagosome prior to ultimate joining with lysosome and degradation. Increased autophagy in CC is evidenced by an increased LC3 II content, which implies increased autophagasome activity (13). Lysosomal clearance may also be negatively impacted, as evidenced by increased p62 levels (13). Another likely method for increased muscle loss is the combination of mTOR inhibition and enhanced AMPK phosphorylation, which increases autophagy signaling via increased Beclin activation (28-30). Though it has not been adequately examined, there is potential for increased Reactive Oxygen Species (ROS) production in skeletal muscle, which may promote autophagy (28, 29). While there are signs of impaired autophagy in CC, the

inconsistent nature of these findings leads us to conclude that there are likely more significant mechanisms of muscular atrophy at play, such as proteolysis.

Proteasome-induced muscular atrophy has been observed in CC (31). The proteasome is essential for cellular function, as demonstrated by extreme oxidative stress and cell death following the administration of proteasome inhibitors (32). Though the proteasome is essential for cellular health, over-activation of this pathway leads to muscular atrophy as evidenced in high-fat diet in mice (33). This over-activation of proteolysis has been observed in CC and is highly implicated as a primary pathway of protein degradation through which muscle wasting occurs in this disease, despite adequate mechanistic understanding (34). Due to the complex nature of proteolysis and the negative implications of proteasome inhibitors on cellular health, it is imperative that future literature investigates the signaling pathways that cause the observed over-activation of proteolysis in CC.

In skeletal muscle there are two major muscle specific E3 ligases that are particularly involved in the induction of skeletal muscle wasting: Atrogin-1 and MuRF-1 (35). Under normal conditions, Atrogin-1 and MuRF-1 are scarcely expressed (36). Atrogin-1 and MuRF-1 facilitate the ubiquitination of many proteins involved in hypertrophic signaling, including proteins involved in: ATP generation, protein synthesis, myogenic regulatory factors, and myofibrillar proteins (36). Both Atrogin-1 and MuRF-1 are regulated by the transcription factors FOX-O 1 and FOX-O 3 in skeletal muscle (37). FOX-O 1/3 signaling appears to modify protein synthetic signaling by via negative regulation of AKT phosphorylation (38). In addition to modifying protein synthesis, FOX-O 1/3 signaling also upregulates autophagic processes (39). FOX-O 1/3 signaling seems to be promoted by cellular stresses, which supports theories that FOX-O 1/3

signaling is modulated in CC (39). Clearly, there is a dysregulation in muscle protein balance in CC; however, the instigating signals leading to this imbalance are not well understood.

Inflammatory Pathways

CC is a systemic disease that affects the muscle, fat, brain, liver, heart, blood, spleen, and likely numerous other tissues (40). This systemic response is largely due to circulating inflammatory cytokines that create a proinflammatory environment, which has been demonstrated in models of CC (41). Specific inflammatory cytokines that have been linked to the progression of CC are TNF-α, STAT3, C-Reactive Proteins, MCP-1, IL-6, and IL-1 beta (42-44). In this review of literature we will focus on the roles of IL-6 and TNF-α, as they are the most thoroughly investigated inflammatory factors known to be modified in CC.

IL-6 is one of the most heavily examined inflammatory cytokines in CC literature to date. Exogenous IL-6 and related cytokines alone can induce most symptoms of CC, including muscle and fat wasting, whereas conversely the inhibition of IL-6 reduces muscle loss in CC (45). Following tumor growth in ApcMin/+ mice dramatically increased plasma IL-6 levels (46). Mice lacking gp130, the cellular cytokine receptor for IL-6, and therefore unreceptive to IL-6 exhibit diminished symptoms of CC as well as pathological phosphorylation of p38, MAPK, STAT3, and FOXO3 (45). Chronic phosphorylation of p38α/β MAPK can lead to impairments in the ability of skeletal muscle to regenerate (47). IL-6 (via the IL-6->JAK->STAT3 pathway) is causally linked to many of the effects of CC, notable skeletal muscle atrophy, cardiac dysfunction, and hypothalamic inflammation (20, 30, 40). Furthermore, chronic IL-6 exposure decreases protein synthesis via inhibition of mTOR and induction of proteolysis (20, 48). Combined these series of evidences strongly indicate a significant role for IL-6 in mediating muscle wasting phenotypes such as in CC.

TNF-α is an inflammatory cytokine present in the tumor microenvironment that has been connected with carcinogenesis, specifically in production of reactive oxygen and nitrogen species, and is involved with mechanisms responsible for epithelial mesenchymal transition, angiogenesis, and metastasis (49). TNF-α has also been implicated in the reduction of protein, lipid, and glycogen synthesis (50). TNF-α is further a promoter of apoptopic signaling in Type II muscle fibers, which alone can induce muscle atrophy (51). TNF-α expression can also be responsible for increased production of ubiquitin and other key E3 ubiquitin ligases, notably Atrogin and MuRF-1 (52). This can lead to a buildup of ubiquitinated proteins which promote protein degradation and shift net protein balance toward a loss, leading to reduced muscle protein and wasting (53). The role of this mechanism in CC is further supported by work from Zhang et al., who implanted mice with tumor and treated them with MG132, a proteasome inhibitor that specifically reduces the breakdown of ubiquitin-conjugated proteins (54). Treatment with MG132 significantly alleviated CC related muscle atrophy (54). It appears increased TNF-α levels significantly contribute to the muscle wasting seen in CC by increasing protein degradation, decreasing protein synthesis, and negatively impacting energy balance.

Mitochondrial Modulation

Mitochondrial function is another recently examined aspect of CC, which demonstrates a fiber-type specific effect: specifically that glycolytic muscle fibers are much more susceptible to wasting (55). It has been demonstrated that mitochondrial dysfunction can cause muscle wasting, via pathways that are observed in CC (55). One possible explanation for this relationship may be reduced ATP output from dysfunctional mitochondria. If there is insufficient ATP for protein synthesis, then rates of protein synthesis may decrease as rates of protein degradation increase as the cell attempts to produce ATP via alternative pathways. Secondly, increased ROS production

from dysfunctional mitochondria has been causally implicated in inducing atrophy (56). FOXO3 is activated by the presence ROS, and increases autophagy in order to reduce ROS production (57). Interestingly, work by White et al. has demonstrated reductions in muscle oxidative phenotype and mitochondrial content concomitant with dysregulated mitochondrial dynamics in CC (15). These findings may suggest a prominent role for mitochondria in the development of this condition.

Current Pharmacological Interventions in Cancer-Cachexia

The current definition of CC states that it is primarily a muscle atrophy that cannot be reversed by nutritional interventions alone (5). However, most current pharmaceutical interventions focus on increasing the appetite of patients, increasing the protein-synthetic impact of the foods ingested, or pharmacologically increasing anabolic hormone signaling within the (12). It is of the utmost importance to understand the methodology as well as the successes or failures of interventions focused on addressing hypophagia, or decreased energy intake and eating behavior, in order to understand the need and direction for future research into the mechanistic aspects of CC.

The most common treatment methods of CC are focused on increasing appetite. Megestrol Acetate administration has demonstrated improvements in appetite, fatigue, and general well being (58). With Megestrol Acetate, there is a dose-dependent increase in appetite within one week and weight gain that begins after several weeks; however this effect is seen in less than 25% of patients suffering from CC (58). Another medical intervention aimed at increasing appetite is Medroxyprogesterone Acetate. Medroxyprogesterone Acetate use in patients with severe CC has been shown to be effective at increasing appetite within 6 weeks as well as lead to significant increases in total body mass (+0.6 kg +/- 4.4 kg) when compared to the

placebo group, who lost weight throughout the course of this experiment (-1.4 kg +/- 4.6 kg) (59). Another medication being used in the treatment of CC is Dronabinol, a synthetic cannabinoid. Despite successes in treating wasting associated with Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome, it appears to have limited efficacy with CC (60). Another cannabinoid, Thalidomide, is showing some promise in the treatment of CC. A recent randomized, placebo controlled trial showed that Thalidomide treatment attenuated lean body mass loss in patients with advanced CC despite no difference in appetite between treatment and control groups (61). While treatments aimed at increasing energy intake by increasing appetite have shown some promise, the results are highly variable and have limited success rates. By the very definition of CC, these interventions focused on increasing nutritional intake would appear to be unlikely to exhibit high efficacy in the reversal of muscular atrophy.

Utilization of testosterone derivatives to increase lean mass has also been examined. Nandrolone Propionate administration to MCG 101 sarcoma bearing mice (C57BL/6J) showed an increase in body weight, but this was attributed mostly to fluid retention with little to no increase in muscle wet weight (62). One of the most promising therapeutic treatments so far has been combination treatment with Oxandrolone, another Testosterone derivative, and Megestrol Acetate. Treatment with Oxandrolone alone showed significant increases in lean body mass and a decrease in fat mass (63). These improvements have shown potential as a pharmaceutical intervention to offset the effects of CC. These interventions have shown some promise in treating CC, but still do not address the underlying mechanistic causes. In order to make a significant impact on CC mortality rates, a better understanding of its causes and mechanisms is required in order to stop the disease before it causes significant muscle wasting.

Another direction of research has been through inhibition of IL-6. In AMin/+ mice, administration of PDTC (an anti-inflammatory and antioxidant that inhibits STAT3 and NF-kB signaling in mice) effectively attenuated the muscle loss in CC (55) A review of six clinical trials administering various monoclonal IL-6 antibodies has shown that there is a significant reduction in cancer-related symptoms including CC, fever, and pain in humans (64). These investigations into the inhibition of inflammatory cytokines show an increased need to understand the underlying cell-processes that contribute to CC. However, the overall lack of efficacy in treatments for CC emphasizes the need to reconsider our approach to treatment.

Summary of Known Mechanisms and Treatments for Cancer-Cachexia

When examining the mechanisms of CC, we are limited due to most studies taking place in the post-cachectic state. Nonetheless, this research offers valuable insight into modulated pathways that affect those suffering from CC. We have been able to see, through various models and methodologies, that there is a significant reduction in protein synthesis via inhibition of the AKT/mTOR pathway. Protein degradation is significantly increased in CC, both through ubiquitination and proteolysis. We have also examined that there is significant reduction in myogenic capacity within muscle, largely as a result of the NF-kB pathway. The common thread of Atrogin-1 and MuRF-1 increasing degradation, combined with increased Myogenin levels and altered myogenesis imply that the findings of Olsen et al. may also be present in models of CC (8). The chronic increase in inflammatory cytokines IL-6 and TNF-a are implicated to contribute to dysregulation in protein synthesis, myogenesis, and protein degradation in models of CC.

The current lack of efficacious treatments for CC now requires the utilization of new approaches to the study and identification of underlying mechanisms. Specifically, recent literature calls for a treatment focus on prevention, rather than reversal, of CC (65). Of note,

however, is a relative dearth of data examining the progression of CC. To best develop preventive measures it becomes critical to understand the process of cachectic development rather than a sole focus on the intracellular modulation in the cachectic state itself. Some prior work has inferred progression of this condition by examining varying degrees of cachexia in spontaneous colorectal cancer, but to our knowledge no prior efforts have been put forward to examine the direct time course of progression of CC.

To this end, Fearon et al. has recently called for the use of –omics approaches in order to examine intracellular alterations in this disease (66). These approaches allow for assessments to consider the complex and multifaceted nature of CC to best determine affected processes that may promote this muscle wasting. As Fearon et al. have stated, the end result is more than the sum of its parts (66). Consideration of –omics approaches and early stage developments may hold the key to identify novel mechanisms and pathway interactions key to the development of new and efficacious therapies.

Purpose

Therefore, the purpose of this thesis will be to examine the progression of CC over time course utilizing transcriptomic methodologies to identify significantly modulated pathways. By doing so I will fill major knowledge gaps in by examining both the timecourse progression and utilizing RNA sequencing methods to identify novel altered transcripts and affected pathways across the development of CC.

Objectives for Data Analysis

In order to further elucidate the mechanisms behind CC, this investigation will: 1) describe phenotypic development of CC, including body weight, tissue weight, and tumor weight 2) determine the number of significant differentially expressed genes in the progression of CC in

the gastrocnemius muscle 3) determine the number and indentity of significant genes during early stage CC prior to development of the cachectic condition 4) determine the top canonical pathways impacted by identified differentially expressed genes in comparison of control and cachectic groups.

Materials and Methods

Animals and Interventions

All methods were be approved by the Institutional Animal Care and Use Committee of the University of Arkansas. A cohort of C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME) from a larger study (Brown et al.; manuscript in review) was randomly selected, and phenotypic statistics were assessed to ensure representation of the larger cohort. Mice were housed in a temperature-controlled room on a 12:12h light-dark cycle with food and water provided *ad libitum*. Lewis Lung Carcinoma (LLC) cells were injected subcutaneously into the hind flank of C57BL6/J mice at 1 x 10^6 cells in 100 μL PBS as previously described (45, 67). Control mice received a similar injection of 100μL PBS. To examine progression of CC over time course, tumors were allowed to grow for 1, 2, 3, or 4 weeks; mice injected with PBS were euthanized at 12 weeks of age in order to age match Week 4 LLC mice. At the appropriate time point above mice were anesthetized under isoflurane anesthesia, and gastrocnemius muscles were removed and snap-frozen before storage at -80°C prior to euthanization. To control for alterations in total body size between groups, tibia lengths were assessed as a surrogate of body size independent of total body weight. No differences were observed in tibia length among groups, therefore descriptive data are presented as raw wet weights.

RNA Isolation and Quality

RNA isolation was performed as previously described (68). Whole gastrocnemius muscle form each animal was pulverized at the temperature of liquid nitrogen allowing a uniform mixture of the full muscle to be represented in all experiments. Approximately 120 mg of pulverized muscle was then suspended in 1 mL of Trizol (Invitrogen cat # 10296-028). Samples were then homogenized using Polytron for approximately 5 seconds before being transferred into

1.5 mL cryotube and placed on ice. After 15 minutes, samples were placed in centrifuge for 10 minutes and the clear supernatant placed into a new tube. 200 μl of 100% chloroform was then added, and the sample centrifuged for 25 minutes. The clear solution was removed from the top and placed in a new, sterile tube. An equal amount to the sample of 70% DEPC EtOH was added, and the sample loaded in to an RNeasy column. RNA isolation was then performed using an Ambion RNA Isolation Kit (Life Technologies, Carlsbad, CA). RNA quality and concentration were determined using Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Mean RIN^e scores from TapeStation analysis for submitted samples were 8.56 ± 0.06 (mean ± SEM).

RNA Sequencing and Data Analysis

RNA sequencing was completed at the Genomics Core at Michigan State University (East Lansing, MI). Libraries were prepared using the Illumina TruSeq Stranded mRNA Library Preparation Kit. The pool was loaded onto Illumina HiSeq 2500 High Output flow cell (v4) and sequenced in a 1x50bp single read format using HiSeq v4 SBS reagents. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.64 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.8.4. Quality control of raw reads was determined using FastQC tool kit (Babraham Bioinformatics, http://bioinformatics.babraham.ac.uk/projects/fastqc) The reads were then aligned with reference genome of *Mus musculus* (GRCm38.p5) downloaded from NCBI using Hisat2 tool (69) and reads aligning to gene exons were counted using HTSeq framework (70). List of differentially expressed (DE) genes between individual experimental groups was obtained by analyzing HTSeq counts using DESeq2 package by applying regularized log transformation (71). DE genes were identified as surpassing threshold levels of FDR = 0.05 and $Log_2FC = 0.6$. Ingenuity Pathway Analysis (IPA;

Qiagen, Valencia, CA; http://www.ingenuity.com) software was used for canonical pathway analysis and functional annotation. Prediction of upstream transcription factor signatures for DE genes was performed using Dire tool (dire.dcode.org). Visualization of intersections of genes between experimental groups was performed using UpSetR package (72) and subsequent functional clustering of individual sets was based on Gene Ontology and performed using AmiGO tool (73). Two separate investigators analyzed RNA Sequencing data (I.C. and B.K.) with strong agreement between analyses.

Comparison of RNA Sequencing to the MitoCarta

Identified DE genes were compared to the mouse MitoCarta 2.0 utilizing custom computer software. Identified genes that were identified by RNA Sequencing as DE and appeared in the MitoCarta 2.0 were tallied (74).

Statistical Analysis

A One-Way ANOVA was employed for the global analysis for all variables of interest. Descriptive data including body, tumor and tissue weights were analyzed using SAS version 9.4 (SAS, Cary, NC, USA). Data from RNA sequencing analysis was analyzed using JMP Genomics. The comparison-wise error rate, α, was set at 0.05 for all statistical tests. For descriptive data, when significant F-ratios were found, multiple comparisons were made among all groups using Student-Newman-Keuls post hoc analysis. For sequencing analysis, when significant F-ratios were found, multiple comparisons were made among all groups with p-value correction through FDR calculation. DE genes were identified as surpassing threshold levels of FDR = 0.05 and $Log_2FC = 0.6$.

Results

Phenotypic description of LLC-induced muscle atrophy across time course

These analyses were performed on a cohort of animals from a larger study (Brown et al.; manuscript in review) representing 8 mice per condition. For the current cohort, body weights without tumor were not different between experimental conditions across the 4 weeks cancer development despite significantly lower (~10-18%) muscle weights of the Gastrocenmius, Tibialis Anterior, Extensor Digitorum Longus, and Plantaris in Week 4 LLC animals (Table 1). Epididymal fat mass also was significantly lower (~33%) in Week 3 and Week 4 LLC animals compared to PBS animals (Table 1). Spleen mass was ~300% greater in Week 3 LLC, and further enlarged ~500% in Week 4 LLC compared to PBS animals (Table 1).

RNA Sequencing effectively discriminates cachectic vs. non-cachectic animals

Approximately 25,000 total genes were identified in RNA Sequencing. Two separate laboratory groups performed RNA sequencing analysis with a high level of agreement (data not shown). Following FDR correction most statistically significant comparison-to-comparison differences are seen when examining Week 4 LLC mice compared to all other groups (Figure 1A). Heirarchical clustering, PCA analysis and sample-to-sample distances demonstrate good discrimination between experimental conditions (Figure 1B-D). Heatmap of hierarchical clustering of the top 1,000 most highly expressed genes showed grouping of Week 4 LLC mice with significant differences in gene expressions compared to animals from all other experimental conditions (Figure 1B).

Differentially expressed genes appeared primarily in comparisons to Week 4 LLC mice

Volcano plots of DE genes in each individual comparison are shown in Figure 2. These plots show that the highest alterations in gene expression are seen when comparing Week 4 LLC animals to other groups. The number of DE genes was tallied and revealed 932 down-regulated and 993 up-regulated genes in 4 wk LLC compared to PBS control (Table 2).

Pathway analysis of differentially expressed genes for all comparisons

IPA pathway analysis was performed for all comparisons with sufficient number of DE genes (Figure 3). Commonly impacted pathways across individual comparison groups in descending order of frequency included granulocyte adhesion and diapedesis, protein ubiquitination, TH1 and TH2 activation, Fcy-receptor mediated phagocytosis in macrophages and monocytes, leukocyte extravasation, aryl-hydrocarbon receptor signaling, PPARα and RXRα activation, TH2 pathway, agranulocyte adhesion and diapedesis, oxidative phosphorylation, mitochondrial dysfunction, TCA Cycle II (Eukaryotic), LXR/RXR activation, and B cell receptor signaling (Figure 3). Venn diagram for commonalities among multiple comparisons elucidates a clear relationship between Week 4 LLC animals and altered gene expression (Figure 4). When comparing Week 4 LLC to PBS, Week 1 LLC, and Week 2 LLC, we observe alterations in ATP synthesis coupled electron transport, oxidative phosphorylation, regulation of tumor necrosis factor production, ubiquitin-dependent protein catabolic processes, and cellular protein catabolic processes (Figure 4). Considering the common occurrence of processes including mitochondrial dysfunction and electron transport along with our prior finding (Brown et al.; manuscript in review), we performed IPA analysis of mitochondrial dysfunction between Week 4 LLC and PBS control groups showing 93 of 171 known genes in mitochondrial dysfunction affected

(Figure 5). Furthermore, DE genes in each comparison were matched against the MitoCarta 2.0 (Table 3). Most notably we observed: 255 DE genes in the comparison of Week 4 LLC to Week 2 LLC , 213 DE genes in the Week 4 LLC to Week 1 LLC comparison, and 340 similar genes in the Week 4 LLC to PBS comparison with matches to the MitoCarta.

Analysis of transcription factors common to DE genes among comparisons

Subsequently we analyzed DE genes in each comparison for transcription factors likely mediating the alterations observed. Transcription factors and families of transcription factors that appeared among multiple comparisons included: OCT1, SRY, Myogenin, DR3, ZIC2, TBX5, SREBP1, STAT, PU1, T3R, TAL1BETAITF2, HSF, LEF1, S8, ETS, and SOX9_BP1. Furthermore, multiple transcriptional factors with specific influence over muscle processes (i.e., myogenesis) were observed including: Myogenin, FOXO3, NF-kB p65, and PAX (Figure 6).

Discussion

We are the first to combine RNA sequencing techniques with a time-course design to gain insight into the transcriptomic alterations in the development of muscle wasting in CC. We recently demonstrated early onset mitochondrial derangements well prior to development of muscle wasting in CC (Brown et al.; manuscript in review), this investigation has furthered that work by clearly demonstrated potential pathways involved in the pathology of CC induced muscle atrophy. The utilization of the time course design in conjunction with RNA sequencing allowed us to explore transciptomic shifts through the development of CC in tumor-bearing animals. Our analyses demonstrated clear differentiation between experimental groups. This effect can be clearly seen in Figure 2 as the number of DE genes massively increases, both positively and negatively, when comparing Week 4 LLC animals to all other groups suggesting a clear point at which large scale transcriptomic shifts occur in conjunction with the onset of muscle wasting. We can also see distinct modulation in critical pathways, namely autoimmune function, protein ubiquitination, and oxidative metabolism, as muscle wasting develops. In conjunction with our prior report, we now see that mitochondrial impacts appear central to development of muscle wasting in CC, and that CC develops in a clear progression from early onset metabolic derangements to large transcriptomic shifts at the onset of wasting itself. This study will help to create a basis for future research by identifying future pathways for analysis.

Our experimental model was successful as per the utilized definition of CC: the ongoing loss of muscle mass, which may be accompanied by wasting of other tissues, which cannot be fully reversed by nutritional interventions alone (5). Our assays were able to effectively differentiate between pre- and post- cachectic conditions, seen in Figure 1. Phenotypic analysis of muscle wet-weights showed significant decrease in muscle mass and increases in spleen

weights beginning in Week 3 LLC animals, consistent with previously reports using LLC implantation models of CC (75, 76). Upon examination of Figure 2, a clear increase in DE genes is seen when comparing Week 4 LLC animals to other groups (both LLC and PBS groups). Phenotypic analysis of Week 4 LLC animals shows ~10-18% reduction in muscle wet weights along with a ~500% enlargement of the spleen. Hierarchical clustering analysis (Figure 1B) showed grouping of Week 4 LLC animals when compared to PBS controls and other developmental stages of LLC implanted mice.

When examining the shift in transcriptomic signaling, large shifts do not occur until stages where significant muscle wasting is evident (Figure 2 and Table 2). In contrast, we have recently observed impairment in functional mitochondrial health preceding decreases in muscle wet weight (Brown et al.; manuscript in review) which were observed as early as 1 week following tumor implantation through a doubling in mitochondrial ROS emission. It is then of significant interest that large transcriptomic shifts do not occur until 4 weeks following tumor implantation. Thus the data presented here, combined with our early findings, suggest that alterations within the muscle belly occur first through functional changes in metabolic parameters and ultimately leads to large transcriptomic shifts concurrent to measurable muscle wasting. These combined findings suggest that intramuscular alterations occur via a pair of trigger points: one early leading to functional metabolic derangements and a second wherein muscle wasting and transcriptomic shifts occur.

Prior literature has tied CC to alterations in muscle protein degradation, protein synthesis and inflammatory signaling, among others (11-14). Here we have built upon this by performing IPA analysis of transcriptomic data across development of CC to provide a more clear understanding of the totality of cellular processes impacted in this condition. Consistent with that

prior work we have identified alterations in inflammatory, immune, protein ubiquitination, and protein synthetic pathways. We further identified altered pathways throughout development of the condition seeing alterations in such pathways as circadian rhythm signaling, among others. When examining the pathways that are specifically modulated in post-cachectic groups when compared to pre-cachectic groups and PBS controls we see negative regulation of several important pathways, namely: actin filament depolymerization, cell morphogenesis, supramolecular fiber organization, cytoskeleton organization, and cell adhesion. These combined pathways suggest that tumor-bearing animals display impaired capabilities to maintain cellular morphology and structure, which will ultimately lead to impaired motor function. We also see a large increase in pathways associated with DNA maintenance, transcription, and translation, namely; histone acetylation, chromatin assembly and disassembly, translation, RNA splicing, DNA repair, and intracellular protein transport. Thus the cells ability to encode and provide functional protein becomes diminished. Therefore, in conjunction with some classic pathways altered in the wasting condition of CC, we see alterations in many additional pathways, which are critical for maintenance of total cellular health. The alteration of these pathways, combined with the large increase in DE genes, imply that in Week 4 LLC animals the tumor is beginning to breakdown essential functions within the animal.

Considering our prior work demonstrating functional mitochondrial degeneration early in development of CC, it is most interesting that many pathways involved in oxidative metabolism were identified. Such identified pathways included: ATP synthesis coupled electron transport, oxidative phosphorylation, mitochondrial dysfunction, and mitochondrial organization. In addition to these specific aspects of mitochondrial energy metabolism we note impacts in signaling through AMPK and PPARα, suggesting that both direct functional components as well

as mitochondrial regulatory pathways are impacted. That so many of the affected pathways identified are vital either to direct mitochondrial quality or to the regulation therein is consistent with our recent findings of early onset functional mitochondrial degenerations in these animals. These combined findings are strongly indicative of a critical role for mitochondrial maintenance in development of muscular atrophy in CC. This would lead to a decreased capacity for ATP production, which could create chronic low-energy conditions within the muscle. This effect can be seen in increased AMPK signaling when comparing Week 4 LLC to PBS groups in Figure 3. Models of CC also demonstrate a decrease in oxidative capacity and an increase in mitochondrial ROS production (Brown et al.; manuscript in review). We also noted increases in NO and ROS in macrophages when comparing Week 4 LLC to Week 1 LLC animals, consistent with findings by Brown et al (manuscript in review).

Next, to provide insight to the means by which identified DE genes were altered in progression of CC, we performed transcription factor analyses to identify likely regulations of these genes. Interestingly, we identified transcription factors commonly associated with processes such as myogenesis, tumor necrotic factors, sterol biosynthesis, immunosuppression, and increased angiogenesis (Figure 6). The alterations in these potential transcription factors, combined with the large quantity of DE genes over the course of CC progression, further support that the tumor is systemically breaking down essential functions within the animal.

Summary

Our experiment was able to effectively demonstrate large-scale transcriptomic alterations throughout the timecourse of CC progression. The largest difference in expression was observed when comparing Week 4 LLC animals, which demonstrated the cachectic phenotype, compared

to all other conditions. Combined with our prior work (Brown et al.; manuscript in review) we now see that alterations in the muscle belly likely occur in phases beginning with early mitochondrial degenerations, which now appear to lead to large transcriptomic shifts concurrent to the onset of muscle atrophy. We were then able to identify multiple pathways, both in classic protein turnover and inflammatory pathways. We also identified multiple other cell functions contributing to the development of the cachectic phenotype, which included aspects of maintenance of cell structure and DNA repair/transcription. Furthermore, in agreement with our prior work (Brown et al.; manuscript in review), we observed multiple aspects related to degeneration of mitochondria and oxidative metabolism. The data presented herein has provided novel evidence for altered genes and pathways throughout the progression of CC in tumor bearing mice. Future studies should utilize these data to identify which pathways may be critical to allowing the development of muscle atrophy in CC.

Objectives for Data Analysis

Relative to my experimental objectives, the cachectic phenotype presented at 4 weeks following tumor implantation, as seen in Table 1 (Objective 1). We observed that 5,538 of genes were DE in tumor-implanted mice when compared to PBS controls, as seen in Table 2 (Objective 2). We further observed that, of the 5,538 DE genes, 140 genes were DE prior to the development of the cachectic phenotype, as seen in Figures 2 and 3 (Objective 3). When comparing PBS controls to Week 4 LLC animals, the top identified canonical pathways (As seen in Figure 3) were: Oxidative Phosphorylation, Mitochondrial Dysfunction, Protein Ubiquitination, and TCA Cycle II (Objective 4).

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Figures and Tables

Legends:

Table 1: Phenotypic Data of Cohort. All values are represented as "mean ± SEM". * signifies significant difference ($p \leq 0.05$) from PBS. # signifies significant difference ($p \leq 0.05$) from 1 wk.

Figure 1: A) Summary table of the number of up and down regulated genes in individual group comparisons. Red signifies down-regulated genes; green signifies up-regulated genes. B) Hierarchical Clustering Heatmap of 1000 most expressed genes. Red signifies increased gene expression, while blue signifies decreased gene expression. C) Principle Component Analysis (PCA) analysis of individual samples. D) Heatmap of sample-to-sample distances. Darker colors indicate greater differences in expression.

Figure 2: Volcano plots of differentially expressed genes in individual comparisons. Blue signifies a gene that reached an FDR< 0.05, but $\log_2$(Fold Change) was not >|0.6|. Red signifies a gene that reached a FDR < 0.05 and $\log_2$(Fold Change) > |0.6|.

Table 2: Summary table of the number of up and down regulated genes in individual group comparisons that were differentially expressed (FDR<0.05, $\log_2$(Fold Change) > |0.6|). Red signifies down-regulated DE genes; green signifies up-regulated DE genes.

Figure 3: IPA analysis of comparisons with sufficient number of DE genes. Graphs identify 10 most highly affected pathways with the highest significance. If applicable, z-score indicates whether pathway was up- or down- regulated.

Supplemental Figure 1: IPA analysis of comparisons with sufficient number of DE genes. Graphs demonstrate significantly altered biological processes and diseases that show similar modulation.

Figure 4: Venn diagram of the number of genes that were common or exclusive between comparison groups. Illustrations below show gene ontology clustering of subsets that contain sufficient amount of differentially expressed genes.

Figure 5: DE genes in Mitochondrial Dysfunction. All colored in genes were DE when comparing Week 4 LLC to PBS controls.

Table 3: Portion of genes with a positive match on the MitoCarta 2.0

Figure 6: IPA analysis of comparisons with sufficient number of DE genes. Illustrations show prediction of common regulatory elements for transcription factors in identified up- and down-regulated genes (dire.dcode.org).

Table 1:

	PBS	1 wk	2 wk	3 wk	4 wk
Body Weight (with Tumor) (g)	n/a	23.3 ± .4	23.1 ± .6	23.2 ± 1.0	26.6 ± 0.6 #
Tumor Weight (g)	n/a	0.03 ± 0.01	0.14 ± 0.04	0.66 ± 0.15	3.56 ± 0.45 #
Body Weight (without Tumor) (g)	24.3 ± 0.5	23.2 ± 0.4	23.0 ± 0.6	22.5 ± 0.8	23.1 ± 0.3
Gastrocnemius (mg)	134.0 ± 3.7	122.6 ± 3.1	125.7 ± 4.3	119.3 ± 3.5	111.6 ± 3.6 *#
Tibialis Anterior (mg)	45.2 ± 1.6	41.2 ± 1.3	43.2 ± 1.3	40.6 ± 1.8 *	36.0 ± .9 *#
EDL (mg)	9.1 ± 0.6	9.5 ± 0.3	9.9 ± 0.4	9.6 ± 0.5	7.9 ± 0.4 *
Soleus (mg)	8.7 ± 0.4	8.1 ± 0.3	7.9 ± 0.3	7.7 ± 0.4	7.4 ± 0.3
Plantaris (mg)	18.4 ± 0.5	17.7 ± 0.4	17.9 ± 0.6	16.4 ± 0.7	15.3 ± 0.6 *
Spleen (mg)	81.1 ± 6.1	76.6 ± 2.9	79.6 ± 3.5	238.7 ± 42.9 *#	391.5 ± 37.4 *#
Epi Fat (mg)	360.3 ± 19.4	368.2 ± 39.2	348.7 ± 15.7	237.6 ± 42.1 *#	252.3 ± 28.9 *#

Figure 1:

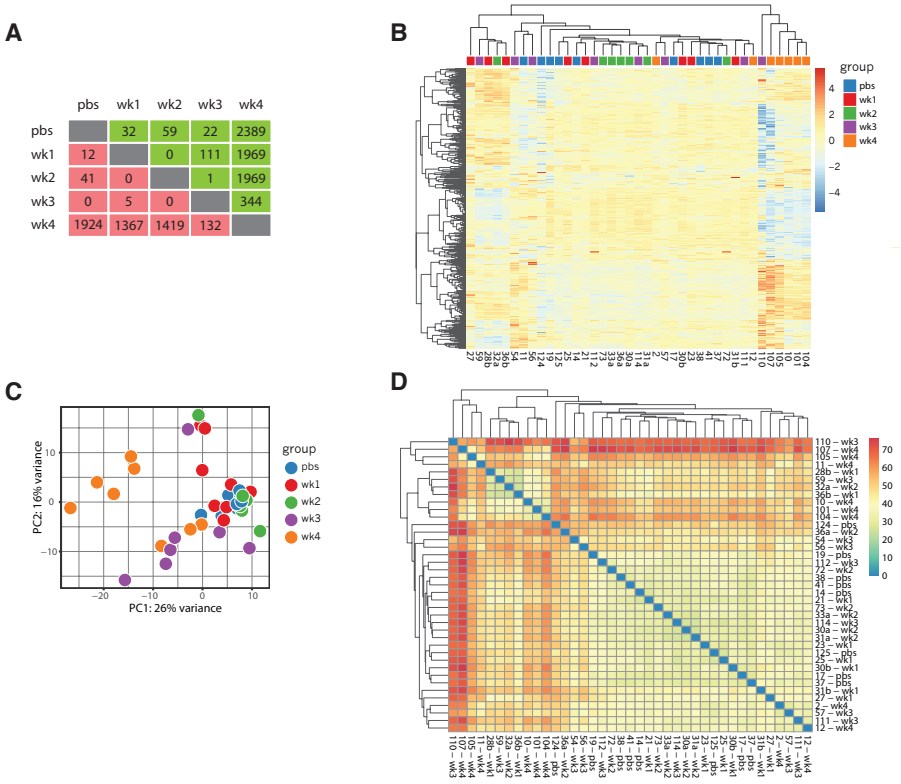


Figure 2:

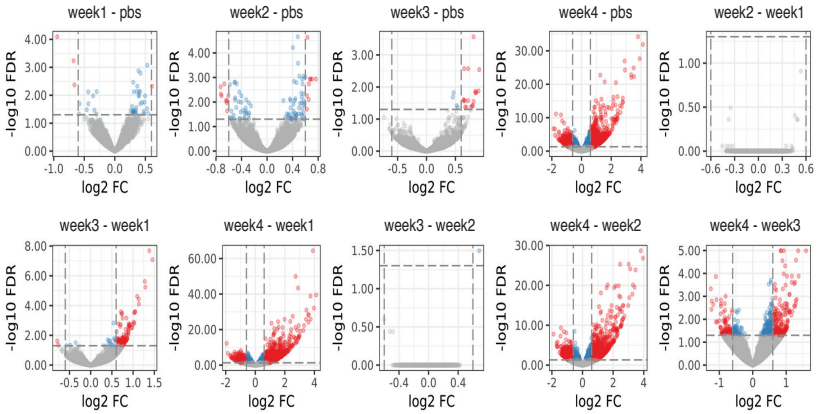
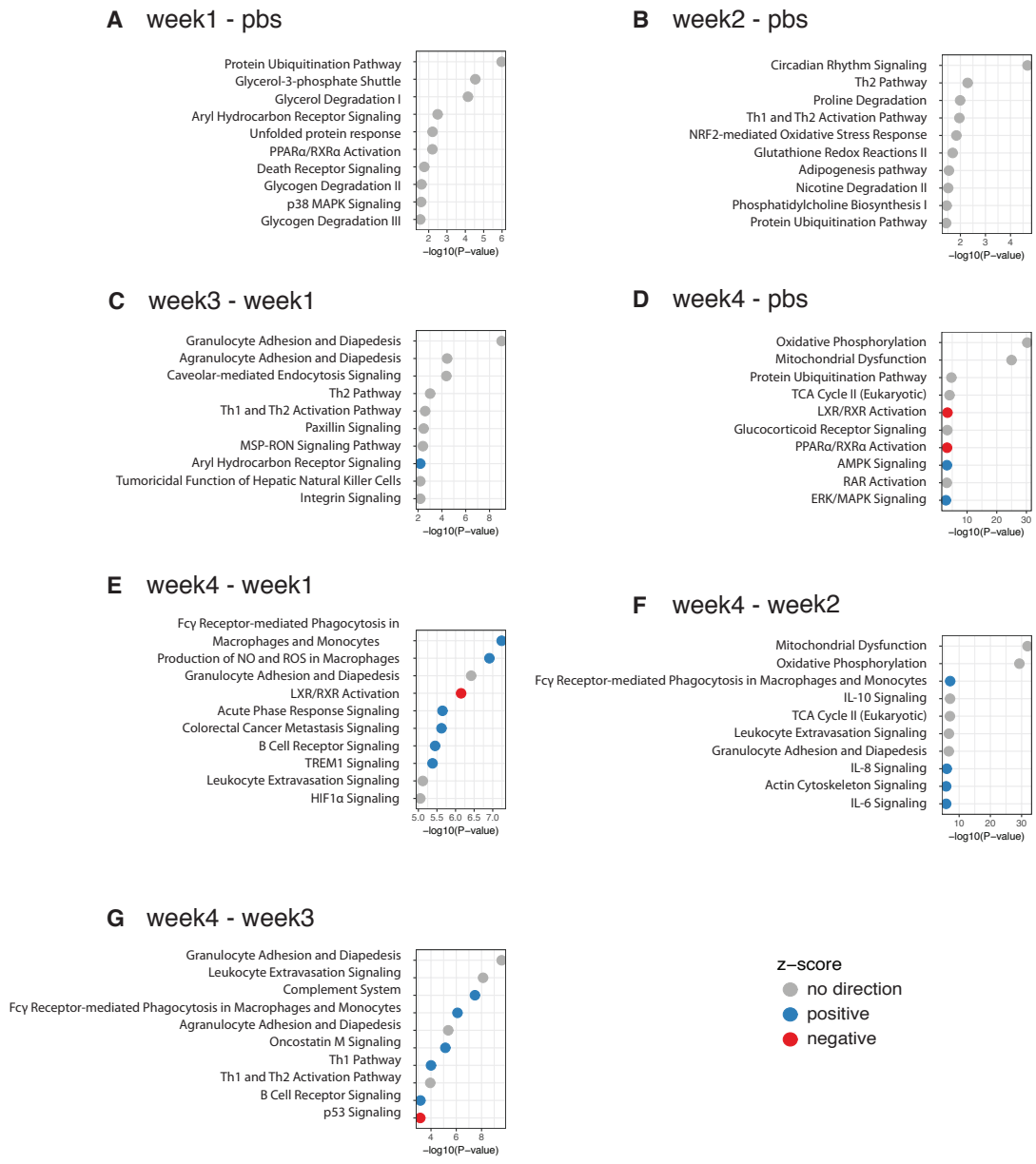


Table 2:

	pbs	wk1	wk2	wk3	wk4
pbs		1	9	18	993
wk1	3		0	99	1024
wk2	9	0		1	961
wk3	0	2	0		178
wk4	932	568	720	80	

Figure 3:


A week1 - pbs
Protein Ubiquitination Pathway
Glycerol-3-phosphate Shuttle
Glycerol Degradation I
Aryl Hydrocarbon Receptor Signaling
Unfolded protein response
PPARα/RXRα Activation
Death Receptor Signaling
Glycogen Degradation II
p38 MAPK Signaling
Glycogen Degradation III
2 3 4 5 6
−log10(P−value)
B week2 - pbs
Circadian Rhythm Signaling
Th2 Pathway
Proline Degradation
Th1 and Th2 Activation Pathway
NRF2-mediated Oxidative Stress Response
Glutathione Redox Reactions II
Adipogenesis pathway
Nicotine Degradation II
Phosphatidylcholine Biosynthesis I
Protein Ubiquitination Pathway
2 3 4
−log10(P−value)
C week3 - week1
Granulocyte Adhesion and Diapedesis
Agranulocyte Adhesion and Diapedesis
Caveolar-mediated Endocytosis Signaling
Th2 Pathway
Th1 and Th2 Activation Pathway
Paxillin Signaling
MSP-RON Signaling Pathway
Aryl Hydrocarbon Receptor Signaling
Tumoricidal Function of Hepatic Natural Killer Cells
Integrin Signaling
2 4 6 8
−log10(P−value)
D week4 - pbs
Oxidative Phosphorylation
Mitochondrial Dysfunction
Protein Ubiquitination Pathway
TCA Cycle II (Eukaryotic)
LXR/RXR Activation
Glucocorticoid Receptor Signaling
PPARα/RXRα Activation
AMPK Signaling
RAR Activation
ERK/MAPK Signaling
10 20 30
−log10(P−value)
E week4 - week1
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes
Production of NO and ROS in Macrophages
Granulocyte Adhesion and Diapedesis
LXR/RXR Activation
Acute Phase Response Signaling
Colorectal Cancer Metastasis Signaling
B Cell Receptor Signaling
TREM1 Signaling
Leukocyte Extravasation Signaling
HIF1α Signaling
5.0 5.5 6.0 6.5 7.0
−log10(P−value)
F week4 - week2
Mitochondrial Dysfunction
Oxidative Phosphorylation
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes
IL-10 Signaling
TCA Cycle II (Eukaryotic)
Leukocyte Extravasation Signaling
Granulocyte Adhesion and Diapedesis
IL-8 Signaling
Actin Cytoskeleton Signaling
IL-6 Signaling
10 20 30
−log10(P−value)
G week4 - week3
Granulocyte Adhesion and Diapedesis
Leukocyte Extravasation Signaling
Complement System
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes
Agranulocyte Adhesion and Diapedesis
Oncostatin M Signaling
Th1 Pathway
Th1 and Th2 Activation Pathway
B Cell Receptor Signaling
p53 Signaling
4 6 8
−log10(P−value)
z−score
no direction
positive
negative

Supplemental Figure 1:

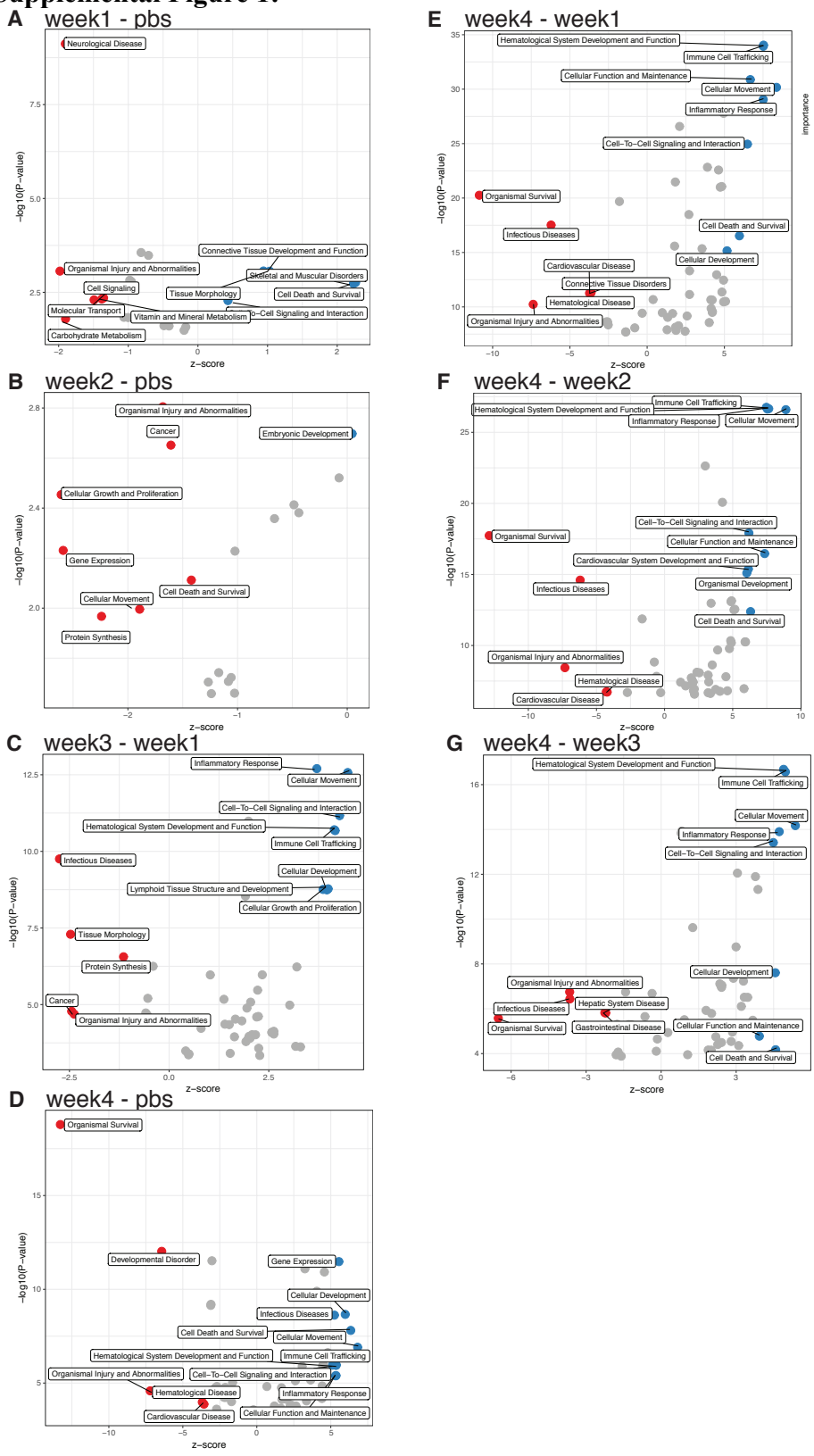


Figure 4:

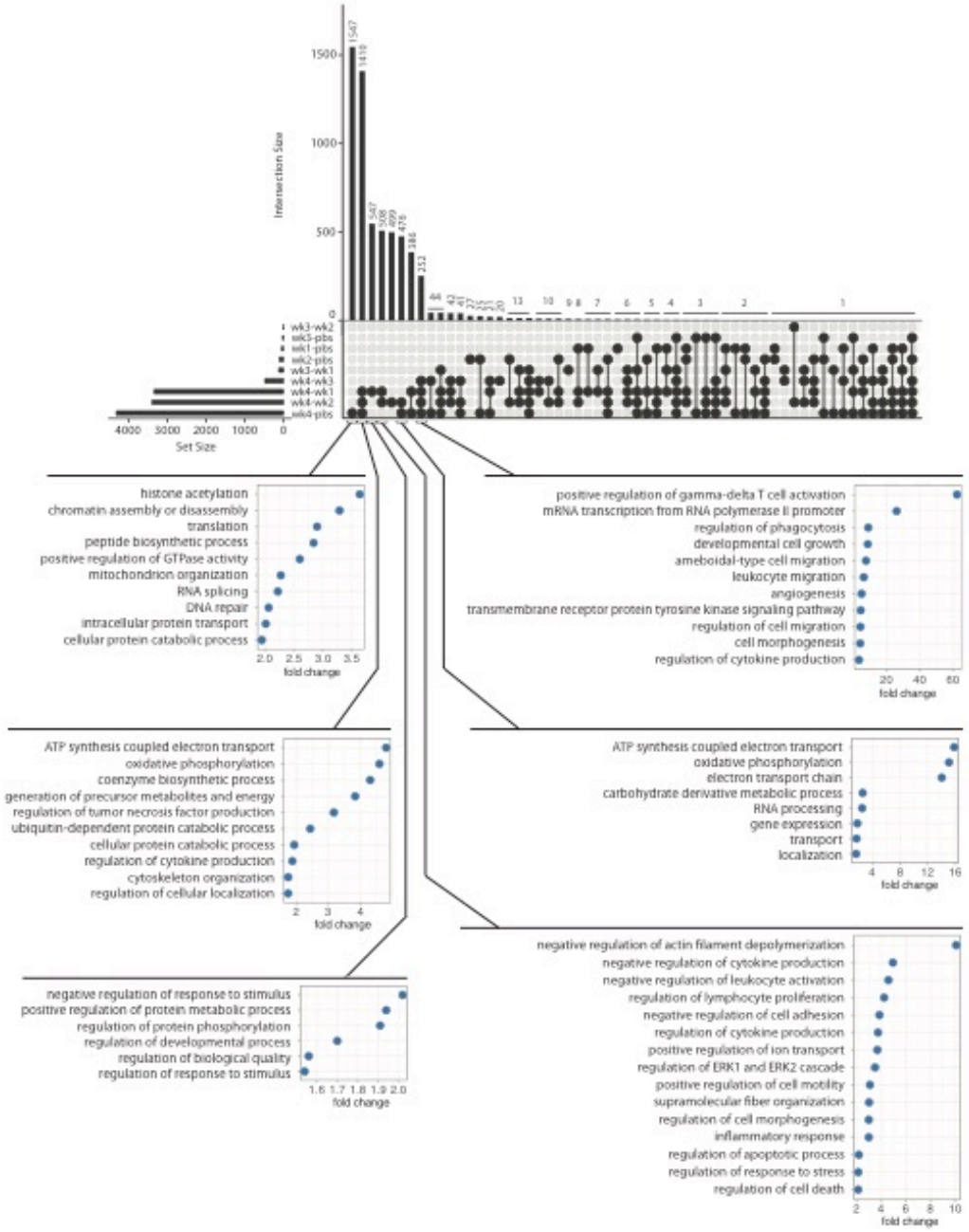

Intersection Size
1500
1000
500
0
1547
1416
547
508
499
476
386
252
44
42
27
20
13
10
9
8
7
6
5
4
3
2
1
wk3-wk2
wk3-pbs
wk1-pbs
wk2-pbs
wk3-wk1
wk4-wk3
wk4-wk1
wk4-wk2
wk4-pbs
4000 3000 2000 1000 0
Set Size
histone acetylation
chromatin assembly or disassembly
translation
peptide biosynthetic process
positive regulation of GTPase activity
mitochondrion organization
RNA splicing
DNA repair
intracellular protein transport
cellular protein catabolic process
2.0 2.5 3.0 3.5
fold change
positive regulation of gamma-delta T cell activation
mRNA transcription from RNA polymerase II promoter
regulation of phagocytosis
developmental cell growth
ameboidal-type cell migration
leukocyte migration
angiogenesis
transmembrane receptor protein tyrosine kinase signaling pathway
regulation of cell migration
cell morphogenesis
regulation of cytokine production
20 40 60
fold change
ATP synthesis coupled electron transport
oxidative phosphorylation
coenzyme biosynthetic process
generation of precursor metabolites and energy
regulation of tumor necrosis factor production
ubiquitin-dependent protein catabolic process
cellular protein catabolic process
regulation of cytokine production
cytoskeleton organization
regulation of cellular localization
2 3 4
fold change
ATP synthesis coupled electron transport
oxidative phosphorylation
electron transport chain
carbohydrate derivative metabolic process
RNA processing
gene expression
transport
localization
4 8 12 16
fold change
negative regulation of response to stimulus
positive regulation of protein metabolic process
regulation of protein phosphorylation
regulation of developmental process
regulation of biological quality
regulation of response to stimulus
1.6 1.7 1.8 1.9 2.0
fold change
negative regulation of actin filament depolymerization
negative regulation of cytokine production
negative regulation of leukocyte activation
regulation of lymphocyte proliferation
negative regulation of cell adhesion
regulation of cytokine production
positive regulation of ion transport
regulation of ERK1 and ERK2 cascade
positive regulation of cell motility
supramolecular fiber organization
regulation of cell morphogenesis
inflammatory response
regulation of apoptotic process
regulation of response to stress
regulation of cell death
2 4 6 8 10
fold change

Figure 5:

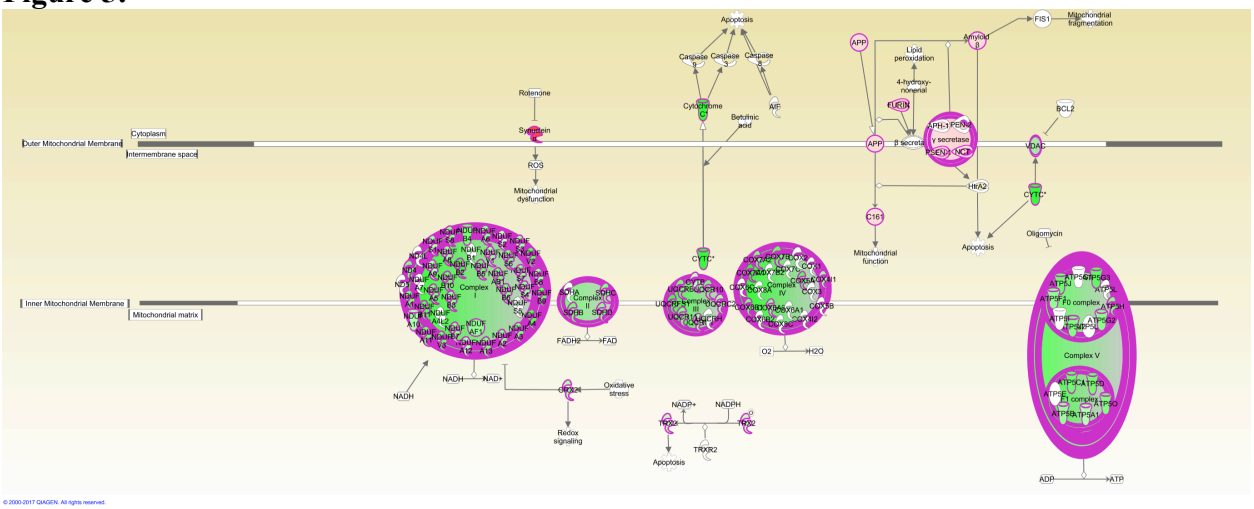
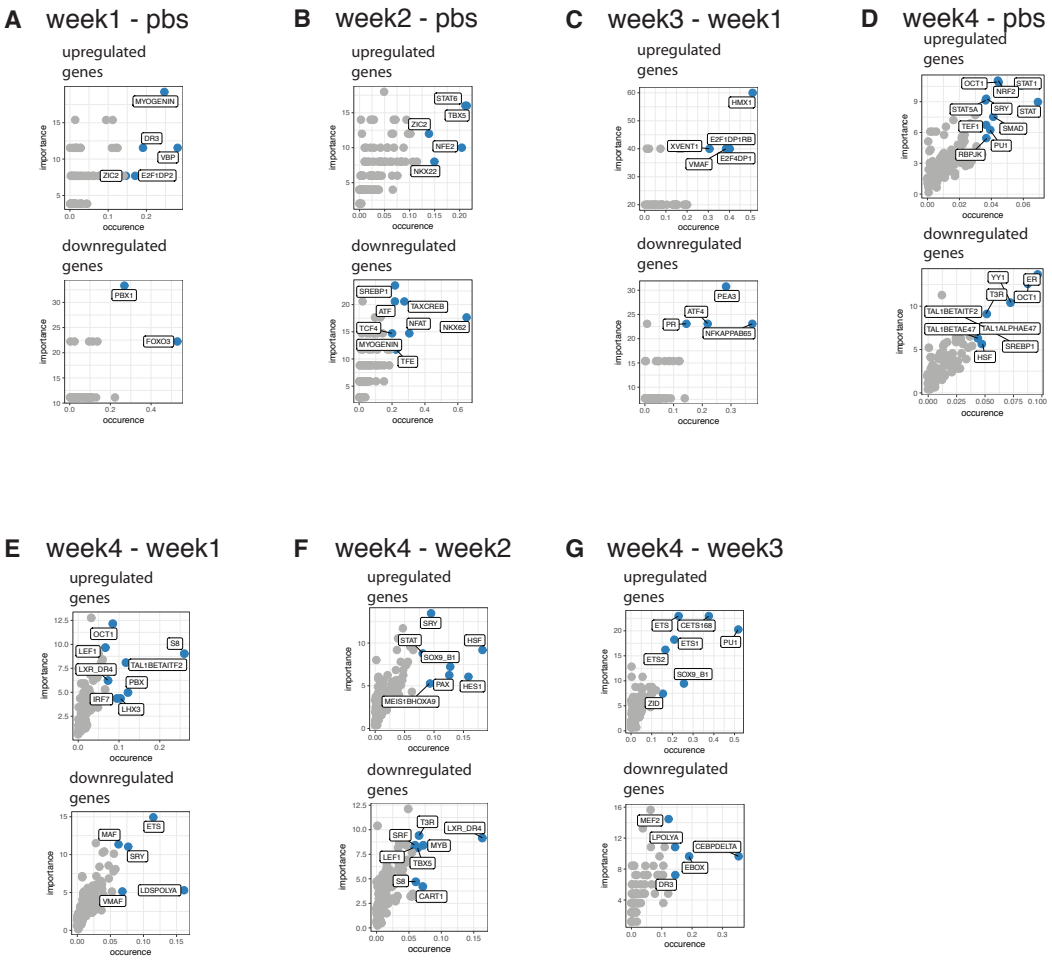


Table 3:

Group	Identified Genes	% of MitoCarta
Wk 1 to PBS	0	0%
Wk 2 to PBS	4	0.3%
Wk 3 to PBS	0	0%
Wk 4 to PBS	340	29.3%
Wk 2 to Wk 1	0	0%
Wk 3 to Wk 2	0	0%
Wk 3 to Wk 1	3	0.3%
Wk 4 to Wk 1	213	18.4%
Wk 4 to Wk 2	255	22.0%
Wk 4 to Wk 3	20	1.7%

Figure 6:


A week1 - pbs
upregulated genes
MYOGENIN
DR3
VBP
ZIC2
E2F1DP2
downregulated genes
PBX1
FOXO3
B week2 - pbs
upregulated genes
STAT6
TBX5
ZIC2
NFE2
NKX22
downregulated genes
SREBP1
ATF
TAXCREB
TCF4
NFAT
NKX62
MYOGENIN
TFE
C week3 - week1
upregulated genes
HMX1
XVENT1
E2F1DP1RB
E2F4DP1
VMAF
downregulated genes
PEA3
ATF4
PR
NFKAPPAB65
D week4 - pbs
upregulated genes
OCT1
STAT1
NRF2
STAT5A
SRY
STAT
TEF1
SMAD
PU1
RBPJK
downregulated genes
YY1
ER
T3R
OCT1
TAL1BETAITF2
TAL1BETAE47
TAL1ALPHAE47
SREBP1
HSF
E week4 - week1
upregulated genes
OCT1
S8
LEF1
LXR_DR4
TAL1BETAITF2
PBX
IRF7
LHX3
downregulated genes
MAF
ETS
SRY
VMAF
LDSPOLYA
F week4 - week2
upregulated genes
SRY
STAT
HSF
SOX9_B1
PAX
HES1
MEIS1BHOXA9
downregulated genes
T3R
LXR_DR4
SRF
MYB
LEF1
TBX5
S8
CART1
G week4 - week3
upregulated genes
ETS
CETS168
ETS1
PU1
ETS2
SOX9_B1
ZID
downregulated genes
MEF2
LPOLYA
CEBPDELTA
EBOX
DR3
importance
occurence

Appendix
Institutional Animal Care and Use Committee Approval

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

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

BioSafety Committee Approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

July 09, 2015

MEMORANDUM

TO: Dr. Nicholas Greene

FROM: Dr. Ines Pinto
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 15025

Protocol Title: "Mitochondrial Degeneration in the Onset of Cancer-Cachexia Induced Muscle Atrophy"

Approved Project Period: Start Date: July 9, 2015
Expiration Date: July 8, 2018

The Institutional Biosafety Committee (IBC) has approved Protocol 15025, "Mitochondrial Degeneration in the Onset of Cancer-Cachexia Induced Muscle Atrophy" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

109 MLKG • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572 • Fax: 479-575-6527 • http://rscp.uark.edu