DYSREGULATION OF THE MUSCLE CLOCK DURING DEVELOPMENT OF CANCER CACHEXIA

Madeline Amos[1], Will Deaver[1], Seongkyun Lim[1], Tyrone A. Washington[1], Nicholas P. Greene, FACSM[1]

[1]Cancer Cachexia Research Laboratory, Department of Health, Human Performance and Recreation, University of Arkansas, Fayetteville,

Abstract

INTRODUCTION: Cancer continues to be one of the leading causes of death across the world.[1, 2] Among many conditions that accompany cancer, cachexia is one that has long been identified as an unfortunate consequence.[3] Cachexia is a skeletal muscle-wasting syndrome that ultimately leads to deterioration in the body's functional ability and is considered to be irreversible solely by nutritional means.[4] Researchers have yet to identify the precise mechanisms responsible for its development[4, 5] and current treatments remain inadequate.[6] **PURPOSE:** The purpose of this study was to confirm the dysregulation of Clock gene expression in male and female tumor-bearing mice, whether such effects can be recapitulated in an in vitro model of cancer cachexia. **METHODS:** RNA Isolation, cDNA synthesis, and Real-Time Polymerase Chain Reaction (RT-PCR) was used to confirm the dysregulation of the muscle Clock during the development of cancer cachexia in male and female mice. The gastrocnemius muscle of male and female mice was used from a previous study due to the mixed fiber type characteristics of the muscle. RT-PCR was conducted for four of the core Clock genes: BMAL1, Clock, PER2, and PER3, thus, eliciting a more sensitive assay clearly defining the dysregulation of the muscle Clock in cancer cachexia. Cachexia was mimicked in tissue culture by treating C2C12 mouse muscle cells with conditioned media from Lewis Lung Carcinoma cells (LCM), an established model of cancer cachexia in cell culture.[7-11] Myotube diameter was analyzed for both experimental media providing an indication of cachectic severity induced. **RESULTS:** Lewis lung carcinoma induced significant muscle atrophy in male and female mice. In males, lowered gene content of Clock, BMAL1, and Per3 (approximately 50%) were seen as early as 1-week post tumor implantation prior to onset of muscle loss ($p<0.05$). In females, lower gene content was solely observed in Clock, and only seen in high humor-bearing (HT) animals ($p<0.05$). Yet, neither males nor females presented significantly lower gene content for PER2. In C2C12 myotubes, LCM sufficiently induced myotube atrophy as defined by the quantification of lower myotube diameter (approximately 25%), however, no effects of LCM were seen for Clock gene expression. **DISCUSSION:** Our findings confirm the early onset dysregulation of Clock gene expression in male tumor-bearing mice and establishes the muscle Clock as a mechanism preceding development of cachexia in male mice. It is worth noting gene expression was significantly lower in both females and males for Clock, as well as BMAL1 and Per3 for males. Interestingly, time of day was not specifically controlled for in tissue collection, yet significantly lower gene content persisted, indicating cancer's robust ability to disrupt muscle circadian rhythm.

Background

- Cancer continues to be one of the leading causes of death in the world (Ferlay, et al., 2015 & Fitzmaurice, et al., 2015).
- Cachexia is a skeletal muscle wasting-syndrome that ultimately leads to deterioration in the body's functional ability and is considered to be irreversible solely by nutritional means (Fearon, et al., 2012)
- Among many conditions that accompany cancer, cachexia is one that has long identified as an unfortunate consequence (Fearon, et al., 2011).
- Researchers have yet to identify the precise mechanisms responsible for the development of cancer cachexia (Fearon, et al., 2012 & Lee, et al., 2017).
- Current treatments of cancer cachexia remain inadequate.
- The circadian rhythm is an internal biological clock that is regulated the suprachiasmatic nuclei
- Circadian rhythm is regulated by environmental factors, such as light and functions in the regulation of different ranges of gene expression and ultimately, the sleep/wake cycle.
- Circadian rhythm genes Clock, BMAL1, PER2 and PER3

Purpose

To investigate confirm the dysregulation of Clock gene expression in male and female tumor-bearing mice, whether such effects can be recapitulated in an in vitro model of cancer cachexia.

Animal Protocol

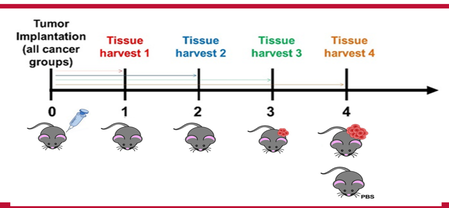

Figure 1

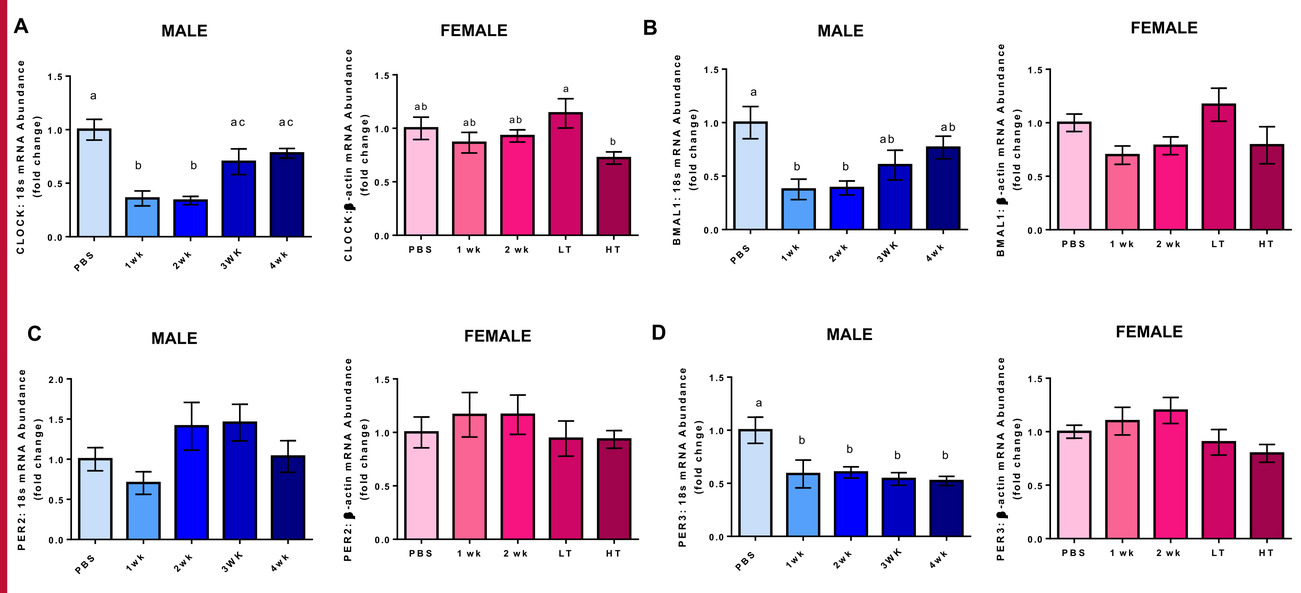

Figure 1: Circadian rhythm gene expression in 4 genes in male and female mice: Clock, BMAL1, PER2 and PER3 in male animals compared to female. Data are presented at Mean ± SEM. Different letters of the same color indicate statistical differences within sexes at $p<0.05$.

Figure 2

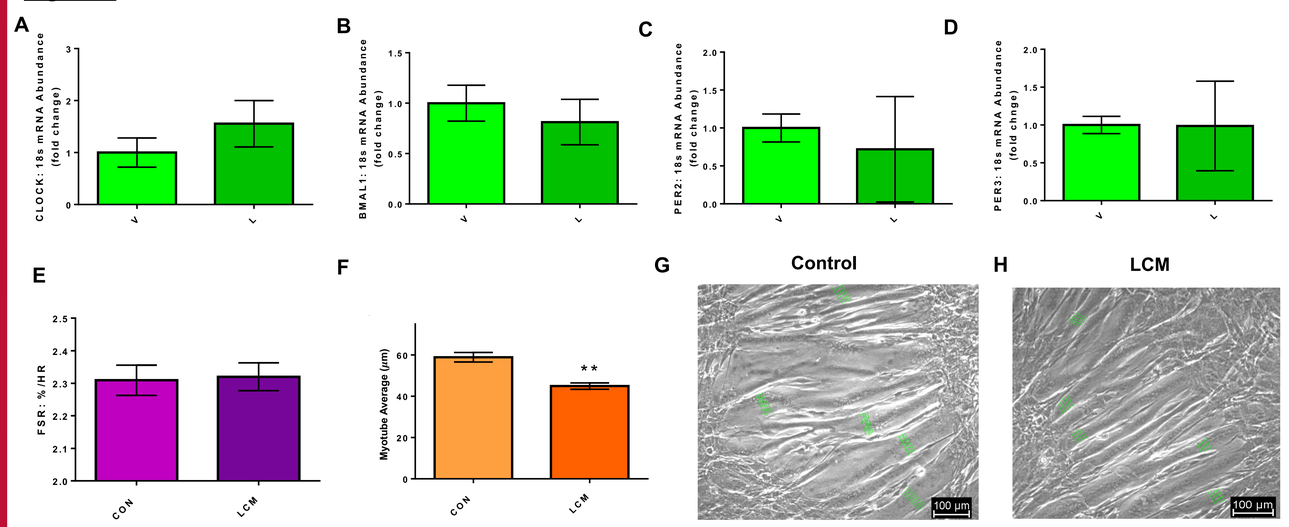

Figure 2: Histograms of core clock genes: Clock, BMAL1, PER2, PER3 (graph A, B, C, D) for Vehicle (V) and LCM (L) groups. FSR comparing deuterium oxide in plasma of LCM and Control (graph E). Myotube diameter averages for CON and LCM (graph F). Representative images of measurements for myotube diameter taken at 10x magnification. Scale bar is 100 μm in length (G and H). ** denotes statistical significance at $p<0.01$.

Methods

- RNA isolation, cDNA synthesis, and Real-Time Polymerase Chain Reaction (RT-PCR) was used to confirm dysregulation of the muscle Clock during the development of cancer cachexia in male and female mice.
- The gastrocnemius muscle of male and female mice was used from a previous study due the the fiber type characteristics of the muscle.
- RT-PCR was conducted for four of the core Clock genes: BMAL1, Clock, PER2, and PER3, thus eliciting a more sensitive assay clearly defining dysregulation of the muscle Clock in cancer cachexia
- Cachexia was mimicked in tissue culture by treating C2C12 mouse muscle cells with conditioned media from Lewis Lung Carcinoma cells (LCM), an established model of cancer cachexia in cell culture (Puppa, et al., 2014, Brown, et al., 2017, Gao, et al., 2016, Lee, et al., 2016, Brown, et al., 2018).
- Myotube diameter was analyzed for both experimental media providing an indication of cachectic severity induced.
- Myotube images were taken with a 10x objective with 5 images per well and 5 myotube measures examined per well.
- Quantification of the average myotube diameter was analyzed by measuring 5 myotubes per image, where 5 lines were then drawn across each of the myotube diameters.
- C2C12 protein Fractional Synthetic Rate (FSR) will determine using by measuring deuterated alanine incorporation into protein.

Statistical Analysis

- Data were analyzed using a one-way ANOVA, a significant difference was denoted at $p<0.05$.
- When significant F ratios were found, a Tukey-Kramer post hoc analysis was used to distinguish differences among means.

Results

- Lewis lung carcinoma induced significant muscle atrophy in male and female mice.
- In males, lowered gene content of Clock, BMAL1, and Per3 (approximately 50%) were seen as early as 1-week post tumor implantation prior to onset of muscle loss ($p<0.05$).
- In females, lower gene content was solely observed in Clock, and only seen in high tumor-bearing (HT) animals ($p<0.05$).
- Neither males nor females presented significantly lower gene content for PER2.
- LCM sufficiently induced myotube atrophy as defined by the quantification of lower myotube diameter (approximately 25%) compared to Control; however, no effects of LCM were seen for Clock gene content nor FSR.

Discussion

Our findings confirm the early onset dysregulation of Clock gene expression in male tumor-bearing mice and establishes the muscle Clock as a mechanism preceding development of cachexia in male mice. Results indicate a clear difference in degraded Clock gene expression experienced between biological sexes with tumor-bearing states. Although, it is worth noting gene expression was significantly lower in both females and males for Clock, as well as BMAL1 and Per3 for males. Interestingly, time of day was not specifically controlled for in tissue collection, yet significantly lower gene content persisted. Although atrophy was seen in C2C12 myotube diameter, further experimentation is necessary due to FSR results depicting a lack of variation in gene expression. These findings indicate cancer's robust ability to disrupt muscle circadian rhythm.

Acknowledgements

This study was funded by the Honors College Research Grant to MGA, and National Institutes of Health (NIH), Award numbers: 1R15 AR069913 and 1R01AR0757794 to NPG. Thank you to the University of Arkansas and the Human Performance Laboratory for their generous support of this project.