Disruptions to the Limb Muscle Core Molecular Clock Coincide with Changes in Mitochondrial Quality Control following Androgen Depletion

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ABSTRACT

Androgen depletion in humans leads to significant atrophy of the limb muscles. However, the pathways by which androgens regulate limb muscle mass are unclear. Our laboratory previously showed that mitochondrial degradation was related to the induction of autophagy and the degree of muscle atrophy following androgen depletion, implying decreased mitochondrial quality contributes to muscle atrophy. To increase our understanding of androgen-sensitive pathways regulating decreased mitochondrial quality, total RNA from the tibialis anterior of sham and castrated mice was subjected to microarray analysis. Using this unbiased approach, we identified significant changes in the expression of genes that comprise the core molecular clock. To assess the extent in which androgen depletion altered the limb muscle clock, the tibialis anterior muscles from sham and castrated mice were harvested every 4 hr throughout a diurnal cycle. The circadian expression patterns of various core clock genes and known clock-controlled genes were disrupted by castration, with most genes exhibiting an overall reduction in phase amplitude. Given that the core clock regulates mitochondrial quality, disruption of the clock coincided with changes in the expression of genes involved with mitochondrial quality control, suggesting a novel mechanism by which androgens may regulate mitochondrial quality. These events coincided with an overall increase in mitochondrial degradation in the muscle of castrated mice, and an increase in markers of global autophagy-mediated protein breakdown. In all, these data are consistent with a novel conceptual model linking androgen depletion induced limb muscle atrophy to reduced mitochondrial quality control via disruption of the molecular clock.

Key Words: Testosterone, Autophagy, Muscle Atrophy

INTRODUCTION

Maintaining skeletal muscle mass is directly linked to a reduced risk of morbidity and mortality (34, 62, 63). In males, the decrease in the production and/or bioavailability of testicular androgens (termed hypogonadism) contributes to the loss of muscle mass during various pathological conditions (6, 9, 21, 22, 66, 72). Though multiple muscle groups are affected by hypogonadism, atrophy of the limb muscles is particularly important as they comprise the large majority of total muscle mass (37), and they are the primary muscles involved with physical function. Whereas limb muscle atrophy is a consequence of hypogonadism, the pathways by which androgens regulate limb muscle mass remain equivocal. This is likely attributed in large part to a lack of information defining the androgen-sensitive pathways in limb muscles. For instance, the levator ani (LA) muscle is commonly used as a model system to study androgen signaling because LA mass is highly sensitive to androgen depletion (46, 59), and use of this model has shown that the canonical androgen receptor (AR) signaling pathway as a predominant pathway regulating muscle mass (51, 59). Pharmacological inhibition of the androgen receptor in primary myocytes and C_2C_{12} cell culture systems yielded similar results as those observed *in vivo* (5, 28). Despite the necessity of AR signaling in those models, deletion of the androgen receptor did not affect mass of the limb muscles (e.g. tibialis anterior (TA)) (2, 15, 51, 52, 67). Rather, it was the presence of androgens themselves that dictated limb muscle mass (67). This dichotomy between model systems is further illustrated by work from our laboratory that showed signaling through the mechanistic target of rapamycin in complex 1 (mTORC1) was dispensable for androgen-mediated growth of the limb skeletal muscles (54), even though mTORC1 was absolutely required for androgen-mediated growth in AR-dependent model

system (1, 5), implying that androgens regulate limb muscle mass through distinct pathways that have yet to be fully defined.

In pursuit of novel androgen-sensitive pathways in limb skeletal muscle, our laboratory recently found that mitochondrial degradation was increased in the atrophied TA of mice subjected to androgen depletion via castration surgery (56). Importantly, the measures of degradation were related to measures of autophagy activation and the degree of muscle atrophy, implying changes in mitochondrial quality control may contribute to autophagy activation and subsequent limb muscle atrophy under androgen deprived conditions (56). With this idea in mind, the present study subjected total RNA from the TA of those sham and castrated mice to microarray analysis in order to define novel androgen-sensitive pathways in the limb skeletal muscle known to regulate mitochondrial quality control. Using this unbiased approach, we identified significant castration-induced changes in the expression of genes that comprise the core molecular clock. The core molecular clock is a transcription-translation feedback system that regulates various metabolic processes, including mitochondrial quality, by mediating diurnal changes in gene expression (3, 8, 29). The positive portion of the core clock is regulated by the dimer between Brain and Muscle Arntl 1 (Bmal1) and Circadian Locomoter Output Cycles Kaput (Clock), which induce transcription of various clock-controlled genes (8). The Period genes (*Per1-3*) and Cryptochrome genes (*Cry1-2*) are amongst those transcribed by the Bmal1-Clock dimer (8, 58), which when translated into proteins, feedback to inhibit the transcriptional activity of BMAL1-Clock, thus creating the negative portion of the core clock (8). Herein we demonstrate that cycling and function of the core molecular clock is disrupted within the limb skeletal muscle by depletion of testicular androgens, and that this disruption coincides with altered expression of genes that regulate mitochondrial quality control. Alterations in

mitochondrial quality control overlapped with increased mitochondrial degradation (i.e. mitophagy) and global autophagy activation, supporting a novel conceptual model linking androgen depletion induced limb muscle atrophy to reduced mitochondrial quality control via disruption of the molecular clock.

MATERIALS and METHODS

Animals, Castration Surgery, and Experimental Design

Microarray Sample Generation: The muscle samples analyzed by microarray were generated from a previous study conducted by our laboratory where the objective was to determine whether androgen depletion altered the molecular response following anabolic stimuli (64). In brief, mice from that study were subjected to a sham or castration surgery (N = 7-8 per group). Seven weeks following surgery, all mice were subjected to an overnight fast beginning at 1700 hr. The next morning, sham and castrated mice were given access to food for 30 min. After the refeeding, mice were fasted for an additional 4.5 hr with unrestricted access to water until sacrifice occurring between 1200-1500 hr. The TA muscles were used for analysis as androgens regulate mass of the TA in an AR-independent manner (67). The Institutional Animal Care and Use Committee at the University of Central Florida approved these procedures and the animal facility.

Circadian Study: Male, C57Bl/6NHsd mice (12 weeks of age) were purchased from Envigo (Indianapolis, IN). Upon arrival, all mice were housed individually for 2 weeks with *ad libitum* access to food (5001 rodent chow (LabDiet, St. Louis, MO) and water prior to being randomized into 2 groups of equal body weight. One group was subjected to a castration surgery to effectively stop testicular androgen production, while the other group was subjected to a sham

surgery in which testicular androgen production was left intact. All mice were given buprenorphine (0.05 mg/kg) immediately following surgery and again 5 hr later to alleviate post-operative pain. Mice recovered for 8 weeks prior to testing. Testing included sacrificing a subset of mice from each group (N=3/group/time point) in alternating fashion (i.e sham/castrated) every 4 hr beginning at the onset of the dark cycle (1900 hr). Food and water were consumed *ad libitum* throughout the data collection. For sacrifice during the dark cycle, the mouse cage was placed into a black Tupperware container under dim red light for transport to a separate surgical suite. In the surgical suite, mice were lightly anesthetized with isoflurane under dim red light, and then mice were euthanized via cervical dislocation. The lights were then turned on and the limb muscles were rapidly extracted, and flash frozen in liquid nitrogen. Preliminary tests using a HOBO light detector (Onset Computer Corp., Bourne, MA) performed prior to the experiment showed that the Tupperware container prevented light exposure inside the container during transport and therefore, light exposure was assumed to be negligible during the actual experiment. The Animal Care and Use Committee at Florida State University approved these procedures and the animal facility.

RNA Extraction, Microarray and Microarray Data Analysis

TA muscles (~20 mg) were homogenized in 600 µl of Zymo Tri Reagent (Irvine, CA), and RNA was isolated using a Zymo RNA Miniprep extraction kit (cat. #R2071) with on column DNase treatment (Irvine, CA). RNA quantity was determined spectrophotometrically by the 260-to-280 nm ratio. The quality of total RNA was assessed by the Agilent Bioanalyzer Nano Chip (Agilent Technologies; Santa Clara, CA), and RNA integrity scores >6 were required for subsequent microarray analysis. Microarray analysis was conducted as previously described (25). The microarray and microarray analysis were performed at the Sanford Burnham Prebys

Medical Discovery Institutes (Orlando, FL and La Jolla, CA, respectively) (N = 3/group). Lists of differentially expressed genes (DEGs) were generated using a flexible *P* value (Fold Change ≥ 1.5 and P-value without False Discovery Rate < 0.05) as this resulted in more than one hundred DEGs (Table S1). A rigorous *P* value was not used for microarray analysis as it resulted in only a handful of differentially expressed genes. The list of DEGs was uploaded into the publicly available Database for Annotation, Visualization and Integrated Discovery (DAVID) software (https://ncifcrf.gov) and analyzed using 2 separate algorithms: 1) Functional Category analysis, and 2) KEGG Pathway analysis (Table S2 & S3). Gene expression data for this study has been made available at GEO (http://www.ncbi.nlm.nih.gov/geo; GSE126965).

cDNA Synthesis and RT-PCR

cDNA was synthesized from 1.5 µg of total RNA using a High Capacity cDNA Reverse Transcription Kit (cat. #4368814; Thermo Fisher Scientific, Waltham, MA). RT-PCR was conducted on either a QuantStudio3 (Thermo Fisher Scientific) or a CFX Connect (Bio-Rad, Hercules, CA) RT-PCR thermal cycler using PowerUp Sybr Green Master Mix (cat. #A2742; Thermo Fisher Scientific) or TaqMan Fast Advanced Master Mix (cat. #4444557; Thermo Fisher Scientific). The conditions for RT-PCR with Sybr Green included an initial 2 min at 50 ºC and 2 min at 95 ºC, followed by 40 cycles which included a 15 sec denature step at 95 ºC, a 15 sec annealing step at 55 ºC, and a 1 min extension step at 72 ºC within each cycle. A melt curve analysis was performed for each primer pair to ensure that a single product was efficiently amplified, and the product sizes for each primer pair were verified via agarose gel electrophoresis prior to experimentation. Measurement of *BNIP3* (assay ID Mm01275600_g1), *Tfam* (assay ID Mm00447485_m1), *Nrf1* (assay ID Mm01135606_m1), *Parkin* (assay ID Mm00450187_m1), *Pink1* (assay ID Mm00550827_m1), *Clock* (assay ID Mm00455950_m1),

LC3B (assay ID Mm00782868_sH), and *MAFbx* (assay ID Mm00499523_m1) were quantified using TaqMan predesigned primer probes according to the manufacturer-recommended conditions for the QuantStudio3. Relative expression levels of all genes were normalized using the delta delta Ct method. *GAPDH* was used as the internal control for validation of the microarray, while Ribosomal Protein Lateral Stalk Subunit P0 (*RPLP0*) was used as the internal control for the circadian study as *RPLP0* expression was not affected by either time or castration (Table S4). Primer sequences for all Sybr Green RT-PCR reactions are listed in Table 1. The mean reaction efficiency for all experimental genes was 86.6% ± a standard deviation of 6.3%. The reaction efficiencies of *RPLP0* and *GAPDH* were 87.3% and 81.3%, respectively.

microRNA Analysis

microRNA expression was determined using TaqMan assay primer probes (Thermo Fisher Scientific) against *miR-181a* (cat. #4427975; assay ID 000480). As per the manufacturer recommendations, 15 ng of total RNA (isolated using Zymo Miniprep extraction kit as described above) was reverse transcribed using a miRNA Reverse Transcription kit (cat. # 4427975; Thermo Fisher Scientific) with microRNA specific primers provided with each TaqMan assay. Relative microRNA expression levels were normalized using the delta delta Ct method. snRNA *U6* (cat. #4427975; assay ID 001973) was used as the internal control as previously recommended (43). Expression of *U6* was not affected by either time or castration (Table S4). Reaction efficiencies for U6 and miR-181a were 95.3% and 96.8%, respectively.

Western blot Analysis

Western blotting was conducted as previously described with slight modifications (64). Whole muscle protein from the TA was extracted by glass on glass homogenization in 10 volumes of buffer (10 µl/mg of muscle) consisting of 50 mM HEPES (pH 7.4), 0.1% Triton-X

100, 4 mM EGTA, 10 mM EDTA, 50 mM $Na_4P_2O_7$, 100 mM β-glycerophosphate, 25 mM NaF, 5 mM Na_3VO_4, and 10 µl/ml protease inhibitor cocktail (cat. #P8340, Sigma-Aldrich). Muscle extract was centrifuged for 10 min at 10,000 *g* at 4°C, and the supernatant fraction was quantified via the Bradford method. After quantification, all samples were diluted to the same concentration in 2X Laemmli buffer. At each circadian time point, an equal amount of protein from the 3 samples within a group (sham or castrated) was pooled together prior to Western blot analysis as a way to identify potential differences between groups in diurnal protein expression patterns (29). Though this method has clear limitations (i.e. inability to estimate variability at each time point), this method was chosen due to limited statistical power at each time point (i.e. N=3/group) and the lack of feasibility for determining relative protein expression by Western blot analysis from a single cohort consisting of 36 samples. Though pooling is not the preferred method for detecting differences as it can mask individual variation, others have shown that pooling samples can be a viable approach to identify potential differences when experimental constraints limit traditional analysis (17, 36). Thus, future work that is sufficiently powered will be needed to verify differences in protein expression within each time point. Once the samples were pooled, 20-60 µg of protein from each group and time point were fractionated on 4%–20% Bio-Rad Tris-Glycine Criterion precast gels (Hercules, CA) and transferred to PVDF membranes. Ponceau-S staining was used to assess effective transfer and equal protein loading. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline + 0.1% Tween20 (Tris-buffered saline-Tween 20). Membranes were then incubated overnight at 4°C with antibodies against BNIP3 (cat. no. 3769), Parkin (cat. no. 2132), LC3B (cat. no. 2775), p62 (cat. no. 5114), COX IV (cat. no. 4844), VDAC (cat. no. 4866), Bcl-xL (cat. no. 2764), GSK3β (Ser9) (cat. no. 5558), total GSK3β (cat. no. 12456), Akt (Ser473) (cat. no. 4060), total Akt (cat. no.

9272), and Sirt1 (cat. no. 2028), which were all obtained from Cell Signaling Technology (Danvers, MA). Antibodies against Per2 were produced in house (14). Antibodies against Bmal1 were obtained from Sigma-Aldrich (cat. no. SAB4300614). Antibodies against 4-HNE were obtained from Alpha Diagnostics (San Antonio, TX; cat. no. HNE 13-M). After incubation with secondary antibodies (Bethyl Laboratories; cat. no. A120–101P or A90–116P; Sigma Aldrich; cat. No. A7289), the antigen-antibody complex was visualized by enhanced chemiluminescence using Clarity reagent (Bio-Rad) on a Bio-Rad ChemiDoc Touch imaging system. The exposure time for all blots occurred within 10 minutes. The pixel density from all blots was quantified as the ratio of total protein to the 45 kD band of the Ponceau-S stain using Image J software (National Institutes of Health, Bethesda, MD) or ImageLab Software (Bio-Rad). We have previously shown that our anti-mouse secondary antibody reacts non-specifically with the presumed endogenous heavy chain (~50kD) and light chain (~25 kD) IgG within our mouse muscle extracts (24). As such, those bands were excluded from the quantification of 4-HNE. The antibodies used in this study have been previously validated by our laboratory (23, 24, 55, 56) or by others (7, 10, 16, 26, 60, 71, 74, 77).

Cytosolic/Nuclear Fraction Separation

Whole gastrocnemius muscle samples were homogenized using glass on glass in 10 volumes (10 µl/mg tissue) of buffer (referred here after as buffer A) consisting of 10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES, 20% glycerol, 0.1% Triton-X 100, 1 mM DTT, and 10 µl/ml protease inhibitor cocktail (Sigma Aldrich cat. #P8340, St. Louis, MO). Samples were centrifuged for 5 min/2,400 *g*/4º C. The supernatant was collected and saved as the cytosolic enriched fraction. This fraction was further centrifuged 3 times, each at 5 min/3,500 *g*/4º C, to pellet and remove any remaining non-cytosolic material. The pellet containing the nuclear-

enriched fraction was then gently washed 3 times in buffer A. Between each wash, the nuclear pellet was centrifuged for 5 min/2,400 *g*/4º C. The final nuclear pellet was then suspended in 400 µl of the protein extraction buffer described in the Western blot analysis section. The sample was then centrifuged for 15 min/21,000 *g*/4º C. The supernatant was collected and saved as the nuclear enriched fraction. The protein content of each fraction was quantified by the Bradford method, and equal quantities of protein were diluted into 2X Laemmli buffer. At each circadian time point, an equal amount of protein from each fraction generated from the 3 samples within a group (sham or castrated) was pooled together prior to Western blot analysis.

Statistical Analysis

Circadian protein expression data from pooled samples are presented as a single value at each time point. All other data are presented as mean ± SEM. Analysis of the microarray was described above. Student's *t*-test was used to compare body and tissue mass between groups, and to validate the microarray. Two-way ANOVA was used to assess changes in mRNA across the sampling period using castration and time as the 2 factors. If an interaction was observed, Fisher's LSD was used post hoc to define specific differences. Otherwise, main effects are shown. Differences in protein expression patterns from ≥3 consecutive time points were initially detected visually. If an expression pattern was visually observed, differences in the mean pixel intensity values obtained from the ≥3 time points were assessed by Student's *t*-test. Given that this study is underpowered to detect differences in protein expression at each time point, future work will need to confirm the specific changes in protein expression at each time point. All analysis was performed using ImageLab Software (Bio-Rad) or GraphPad Prism Software (La Jolla, CA). Significance for all analysis was set at $P \leq 0.05$.

RESULTS

Androgen depletion disrupts core clock phase and function in the limb skeletal muscle

Total RNA from the TA of sham and castrated mice was analyzed by microarray. DAVID bioinformatics software identified significant changes in genes that comprise "Biological Rhythms" and "Circadian Rhythms" using *Functional Category* and *KEGG Pathway* analyses, respectively (Tables 2 & 3). Bmal1, Per2, and Per3 were common genes included in both analyses (Table 4), and RT-PCR validation confirmed that expression of *Bmal1* was decreased, and expression of *Per1-3* was increased in the atrophied TA muscle of castrated mice relative to values in sham mice (Fig. 1A-D). To assess the magnitude by which androgen depletion disrupted the core clock, TA muscles were harvested at 4 hr intervals across a single circadian cycle from mice that were previously subjected to a sham or castration surgery. The muscle and tissue characteristics of those mice are presented in Table 5 and Table 6, respectively. As expected (64, 71), mass of various limb muscles was lower in the castrated mice compared to the sham group without a corresponding change in tibia length or fat pad mass (Table 5 & 6). The efficacy of the castration surgery was also confirmed by changes in the mass of various androgen sensitive tissues, including the seminal vesicle [(55, 64) and Table 6]. Gene expression of *Bmal1* and *Per1-3* in the TA of sham mice exhibited the expected circadian patterns (58), and these patterns were altered in the TA of castrated mice (Fig. 1E, G-I). The diurnal expression pattern of *Clock* and *Cry1* were also disrupted (Fig. 1F & J), while circadian expression of *Cry2* was not different between groups (data not shown).

Previous work has identified various genes in skeletal muscle whose expression is under the control of the core clock (45, 58), including the nuclear receptors RAR-Related Orphan Receptor alpha (*RORα*) and Nuclear Receptor Subfamily 1 group D member 1 alpha (*Rev-Erbα*),

Myogenic Differentiation (*MyoD*), D-Box Binding PAR BZIP Transcription Factor (*Dbp*), and Muscle Atrophy F-Box (*MAFbx*). *Rev-Erbα*, *MyoD*, *Dbp*, and *MAFbx* each exhibited a circadian expression pattern in the TA of sham mice, while *RORα* did not (Fig. 2A-E). Androgen depletion significantly altered the rhythmicity or overall expression of each gene (Fig. 2A-E), suggesting androgens are required for proper core clock function in addition to the normal cycling of core clock genes.

Expression of various mitochondrial quality control genes are disrupted by androgen depletion

The core molecular clock has been implicated in regulating the expression of genes involved with mitochondrial quality control (29). Indeed, disruption of the core clock decreased measures of mitochondrial function in skeletal muscle (3). This is pertinent to androgen depletion as disruption of mitochondrial quality control can induce muscle atrophy (53, 61), and our group previously observed a strong relationship between indices of impaired mitochondrial quality and the degree of muscle atrophy following androgen depletion (56). Moreover, the magnitude of change in expression of core clock genes identified by the microarray were strongly related to both the indices of impaired mitochondrial quality (e.g. $r = 0.94$) and the degree of muscle atrophy (e.g. $r = 0.58$) previously reported in those same samples (56).

Mitochondria are a dynamic organelle that change in number, size, and network complexity in order to handle metabolic demand by the coordinated balance of mitochondrial fission/fusion and mitochondrial biogenesis/degradation (50, 65). Mitofusion 1 & 2 (Mfn1-2) and the Mitochondrial Dynamin Like GTPase (Opa1) promote fusion of mitochondria into larger networks (73), while Mitochondrial fission 1 (Fis1) and Dynamin 1 Like (Drp1) promote network fragmentation (73). Though genes involved with mitochondrial fission (*Drp1* and *Fis1*) changed in expression over time in other tissues (29), expression of these genes did not differ by

time in the present study (Fig. 3A & B). However, castration lead to an overall reduction in the mRNA expression of *Drp1*. Conversely, expression of genes involved with mitochondrial fusion (*Opa1* and *Mfn2*) changed over time, and castration lead to an overall decrease in expression of these genes (Fig. 3C & E). There was a strong trend for expression of *Mfn1* to change over time (P=0.053) with castration causing a significant overall reduction in expression (Fig. 3D).

Mitochondrial biogenesis is mediated in large part by the Peroxisome Proliferator Activated Receptor Gamma Co-activator 1 Alpha (PGC-1α) signaling nexus, which regulates the expression of various mitochondrial associated genes from both nuclear and mitochondrial genomes (44). Expression of *PGC-1α* is a known clock controlled gene in skeletal muscle (58), and as such, *PGC-1α* mRNA exhibited a change in expression over time (Fig. 3F). Consistent with disruption of the core clock, the overall expression of *PGC-1α* was significantly reduced in the TA of castrated mice (Fig. 3F). Expression of Nuclear Respiratory Factor 1 (*Nrf1*), a co-activator of PGC-1α within the biogenesis nexus (20), was largely unaffected by castration (Fig. 3G). However, expression of Transcription Factor A, Mitochondrial (*Tfam*), a target gene of the Nrf1-PGC-1α transcriptional complex (33), exhibited an overall reduction (Fig. 3H), suggesting impaired signaling downstream of the Nrf1-PGC-1α complex.

Mitochondrial degradation is an important quality control mechanism for the removal of old or dysfunctional mitochondria via the lysosomal-mediated process termed mitophagy (40), which occurs through at least two distinct pathways. BCL2/Adenovirus E1B 19kDa Interacting Protein 3 (BNIP3) shuttles mitochondria to the phagophore for disposal into the lysosome, whereas degradation via PTEN Induced Kinase 1 (Pink1) and Parkin RBR E3 Ubiquitin Protein Ligase (Parkin) involves ubiquitylation of mitochondrial proteins and subsequent shuttling to the phagophore by the p62 adaptor protein (32, 49). *BNIP3* was the only mitophagy-related gene to

exhibit a change in expression over time, and this occurred in both groups (Fig. 3I). However, castration lead to an overall reduction in the expression of *BNIP3* as well as an overall increase in *Pink1* (Fig. 3I & J). Parkin mRNA was not affected by castration (Fig. 3K). In all, these data suggest that genes involved with mitochondrial quality control, including those that exhibit changes in expression over time, were disrupted by androgen depletion.

Indices of mitophagy and autophagy activation are enhanced following androgen deprivation, and this coincides with changes in mitochondrial protein expression patterns

Mitochondrial degradation and global autophagy are enhanced when mitochondria quality is impaired in order to protect the cell against adverse events such as apoptosis (12, 13, 47). Our previous work found that BNIP3 protein content was reduced in the TA muscle of castrated mice 4 hr following consumption of a meal, but the change in BNIP3 protein did not coincide with a change in the corresponding BNIP3 transcript (56). Because BNIP3 protein is turned over during the mitophagy process (75), it was concluded that activation of that degradative process was increased. Consistent with those previous data, the BNIP3 protein expression pattern in the muscle of castrated mice exhibited an accelerated decrease from circadian time 12 to 20 corresponding to the period of time when food consumption in mice is elevated (Fig. 4A & H). This pattern was not matched by a change in the corresponding transcript (i.e. Fig. 3I), suggesting enhanced activation of this degradative pathway. In addition to BNIP3, the pattern of Parkin protein was elevated from circadian time 16 to 24 (Fig. 4B & H), implying activation of this degradative pathway as well. Since Parkin-induced mitophagy occurs via p62 (32), and p62 protein is degraded during this process (38), the pattern of p62 protein was decreased in the muscle of castrated mice at those same time points, further supporting activation of this degradative pathway (Fig. 4C & H).

The LC3 II/I ratio is used to represent global autophagy activation since lipidation of the LC3 protein (conversion of LC3 I to LC3 II) is required for closure of the autophagosome and subsequent disposal of components at the lysosome (35). Typically, a decrease in the ratio of LC3 II to LC3 I suggests autophagy inhibition and vice versa (35, 38). In sham mice, the pattern of the LC3 II/I ratio was lowest from circadian time 16 to 24/0 (Fig. 4D & H), consistent with nutrient-mediated inhibition of this degradative process when mice consume the majority of food [i.e. the dark cycle (19, 35)], and this pattern was higher from circadian time 24/0 to 8 when mice are typically fasting. Interestingly, the LC3 II/I ratio pattern was lower in the muscle of castrated mice from circadian time 12 to 24/0 (Fig. 4D & H), which would initially suggest greater autophagy inhibition. However, comparison of the individual changes in the LC3 I and LC3 II patterns suggest that LC3 I lipidation (i.e. conversion to LC3 II) and LC3 II clearance were higher in the muscle of castrated mice during this time, implying enhanced autophagy activation. For instance, the LC3 I pattern increased in the TA of sham mice, but not in the castrated group (Fig. 4E & H), even though *LC3B* mRNA content did not differ between groups (Fig. 4G). This suggests the conversion of LC3 I to II was blunted in the muscle of sham mice, but this conversion was maintained in the muscle of castrated mice. Further, LC3 II is degraded when autophagy is activated (35), and thus, the decrease in the LC3 II pattern that occurred only in the muscle of castrated mice likely resulted from enhanced LC3 II turnover (Fig. 4F & H). The notion that autophagy was increased in the muscle of castrated mice is further supported by the decrease in the p62 expression pattern at these same time points (i.e. Fig. 4C), which serves as a complimentary marker of autophagy activation (38). In all, these data suggest that disruption of mitochondrial quality control gene expression in the limb muscle following androgen depletion

coincides with increased activation of BNIP3 and Pink/Parkin-mediated mitochondrial degradation and global autophagy activation.

Markers of mitochondrial content were assessed to determine whether changes in these markers coincided with the upregulation of mitochondrial degradation. Though COX IV and VDAC expression appeared similar between groups at circadian time 12 to 16, the pattern of COX IV was significantly lower in the muscle of castrated mice from circadian time 20 to 8 and the pattern of VDAC expression was lower at circadian time 20 to 4 (Fig. 5A, B & E), coinciding with the observed increase in markers of mitophagy pathway activation (i.e. Fig. 4). The Bcl-xl expression pattern was also lower in the muscle of castrated mice from circadian time 12 to 20 before returning to sham values for the remainder of the time course (Fig. 5C & E), suggesting a compensatory effect by the muscle to maintain levels of this antiapoptotic protein (30). The pattern of 4-HNE, a free radical byproduct, was higher in the muscle of castrated mice prior to the change in the mitochondrial protein expression pattern, and this elevation persisted throughout the remaining sampling time course (Fig. 5D & E), suggesting production of reactive oxygen species coincided with enhanced turnover of mitochondrial proteins following androgen depletion.

Various regulators of the core clock are altered in the limb muscle following androgen depletion

The most evident change to the core clock following androgen depletion was an overall reduction in the phase amplitude of clock-controlled genes. In addition to regulating *Bmal1* gene transcription, MyoD also feeds back to enhance the transcriptional activity of the Bmal1-Clock dimer in skeletal muscle (27). As such, the reduction in *MyoD* (i.e. Fig. 2) may have contributed to impaired transcription of Bmal1-Clock target genes (i.e. *Dbp*). Despite reduced amplitude of Bmal1-Clock target genes, we observed a contradictory increase in the protein expression pattern

of Bmal1 in the muscle of castrated mice from circadian time 24/0 to 8 (Fig. 6A & E). Further analysis indicates that impairment of Bmal1-Clock transcription was not due to Bmal1 protein export from the nucleus as Bmal1 protein content appeared to be higher in the nuclear-enriched fraction from gastrocnemius muscles of castrated mice from circadian time 24/0 to 8, (long exposure Fig. 6B).

Previous work found that loss of Glycogen Synthase Kinase 3β (GSK3β) function not only impaired Bmal1-Clock transcriptional activity, but it resulted in accumulation of Bmal1 protein (57). Accordingly, the pattern of GSK3β phosphorylation on the inhibitory Ser9 site was higher in the muscle of castrated mice at the circadian times where the Bmal1 protein pattern was also elevated (Fig. 6C & E), suggesting inhibition of GSK3β function may have contributed in part to disruption of the muscle clock and the observed increase in Bmal1 protein expression pattern. Akt phosphorylates GSK3β on Ser9 to inhibit function (11), and we previously found Akt phosphorylation to be increased in the muscle following androgen deprivation (55, 56). Though phosphorylation pattern of Akt (Thr308) did not appear different between groups (Data not shown), the phosphorylation pattern of Akt (Ser473) was higher in the muscle of castrated mice from circadian times 24/0 to 8 (Fig. 6D & E), implying activation of Akt may have also contributed to disruption of the clock via GSK3β.

The Per2-Cry dimer also represses Bmal1-Clock transcriptional activity (8), with changes in expression of Per2 being the primary factor mediating this repression (10). Consistent with reduced amplitude of Bmal1-Clock target genes, the pattern of total Per2 protein was higher in the TA of castrated mice from circadian time 20 to 4 (Fig. 7A & F). Per2 undergoes extensive phosphorylation as a signal to induce degradation (41, 69), and changes in Per2 phosphorylation can be observed by altered migration by SDS-PAGE (41). Consequently, the pattern of Per2 in

413 the faster migrating immunoreactive band was higher in the muscle of castrated mice (lower
414 band Fig. 7B & F), indicating accumulation of Per2 was due at least in part to reduced
415 phosphorylation.

416 Sirtuin 1 (Sirt1) is an NAD(+)-dependent deacetylase whose protein not only exhibits a
417 circadian expression pattern, but also regulates function of the core clock genes by promoting
418 degradation of the Per2 protein (4). Sirt1 protein exhibited the anticipated circadian expression
419 pattern in the TA of sham mice (Fig. 7C & F), but, this pattern was completely lost in the muscle
420 of castrated mice (Fig. 7C & F) as the overall pattern was lower throughout the sampling period.
421 Interestingly, this overall reduction in the Sirt1 protein pattern was independent of changes in the
422 corresponding *Sirt1* transcript (Fig. 7D), implying post transcriptional regulation of the Sirt1
423 protein.

424 MicroRNA 181a (*miR-181a*) not only influenced core clock function in other tissues
425 (39), but it also repressed Sirt1 expression at the level of translation (76), implying a potential
426 role for change in this microRNA in the regulation of Sirt1. *miR-181a* expression oscillated in
427 the muscle of both groups across the sampling period (main effect of time), but *miR-181a* levels
428 were significantly higher in the TA of castrated mice at circadian time 24/0 (Fig. 7E),
429 corresponding to the circadian time where Sirt1 protein expression pattern peaked in sham mice
430 (i.e. Fig. 7C). While this implies a potential repressive role for *miR-181a* at that time point, other
431 factors likely contributed to the overall reduction in the Sirt1 protein pattern at other time points.
432 In all, these data suggest that disruption of the clock following androgen deprivation is likely due
433 to changes to various known clock regulatory components.

434

435 **DISCUSSION**

436 The absence of circulating androgens contributes to atrophy of the limb skeletal muscles
437 (21, 22, 68), however, the underlying mechanisms remain ill-defined. For instance, the
438 canonical AR signaling pathway is not required for maintenance of limb skeletal muscle mass (2,
439 15, 51, 67). Previous work by our laboratory provided initial evidence that altered mitochondrial
440 quality may contribute to limb muscle atrophy as mitochondrial degradation was related to the
441 induction of autophagy markers and the degree of muscle atrophy (56). During our search for
442 regulatory mechanisms that mediate mitochondrial quality, we identified changes in genes
443 associated with the core clock (e.g. *Bmal1*). Since the core clock regulates mitochondrial quality
444 in skeletal muscle (3) and other tissues [i.e. liver (29)], we hypothesized that disruption to the
445 core clock may coincide with impaired mitochondrial quality control following androgen
446 depletion. Consequently, our current findings show that disruption of the core clock following
447 androgen depletion coincides with altered expression of various genes involved with
448 mitochondrial quality control, including those that exhibit a change in expression over time.
449 This disruption overlapped with indices of enhanced mitochondrial degradation (mitophagy),
450 global autophagy activation, and subsequent muscle atrophy. Therefore, we posit a novel
451 conceptual model linking androgen depletion-induced limb muscle atrophy to reduced
452 mitochondrial quality control via disruption of the molecular clock (Fig. 8).

453 The results of the present study provide evidence that atrophy of the limb muscle
454 following androgen depletion may be due in part to changes in core clock-mediated regulation of
455 mitochondrial quality control. Despite this notion, disrupting the core clock in adult skeletal
456 muscle via inducible deletion of *Bmal1* was not sufficient to induce atrophy in previous work
457 (18, 58), questioning this as a mechanism in the regulation of limb muscle mass. One possible
458 explanation is that only some mitochondrial quality control genes exhibited a change in

459 expression over time, implying that disruption of the core clock contributes to, but is not the only
460 cause of, decreased mitochondrial quality control following androgen depletion. Indeed, analysis
461 of publicly available gene arrays from the gastrocnemius of adult mice in which the muscle clock
462 was disrupted via inducible Bmal1 deletion (58) showed that Pink1, which did not exhibit
463 changes in expression over time in our study, was unaffected by deletion of Bmal1. Conversely,
464 *PGC-1α* and *BNIP3*, which did exhibit changes in expression over time in our study, were
465 altered by deleting Bmal1. This suggests that the Bmal1-Clock transcriptional dimer is
466 responsible for expression of some, but not all mitochondrial quality control genes. An
467 alternative explanation is that disruption of Bmal1-Clock in combination with changes in other
468 core clock regulatory factors may contribute to the regulation of mitochondrial quality control
469 and subsequent muscle mass. For instance, maintaining Sirt1 protein expression following
470 nutrient deprivation preserved muscle mass by inhibiting the transcription of genes involved with
471 muscle protein breakdown including *MAFbx* and *BNIP3* (42). That mechanism of Sirt1 action
472 with fasting would not be consistent with findings in the present study as *MAFbx* and *BNIP3*
473 mRNA were suppressed in the muscle under androgen-deprived conditions (i.e. Fig. 2 & 3 and
474 (31, 56)), suggesting an alternative mechanism. A more likely explanation is the decrease in
475 muscle mass was due to the failure of Sirt1 to mediate degradation of Per2, which would disrupt
476 not only the Bmal1-Clock transcriptional activity, but possibly expression of other genes that
477 regulate muscle mass as well. For instance, in addition to mediating Bmal1-Clock function, Per2
478 has also been shown to regulate expression of mitochondrial genes (48) and to regulate growth in
479 ovarian tumors (70). Therefore, the way in which the clock is disrupted under androgen-
480 deprived conditions (i.e. decreased Sirt1 and increased Per2) may have a different effect on
481 muscle mass compared to inhibition of just the Bmal1-Clock transcriptional complex.

It is known that testosterone is released in a circadian manner, but the sensitivity of the limb muscle clock to changes in testosterone concentration remains unknown. Our group showed that castration decreased plasma testosterone in mice by ~70% (56, 64), which is sufficient to disrupt the clock (Fig. 1 & 2). A previous study from our laboratory showed that administering Nandrolone Decanoate (ND) to previously castrated mice restored muscle mass (54). In that study, the TA muscles were harvested 7 days following the final ND injection. Even though muscle mass was completely restored by ND administration (54), testosterone levels at the time of sacrifice were reduced by ~50% compared to sham values (Data not shown). While that study was not properly designed to analyze changes to the core clock, the expression of some core clock genes (e.g. *Per1* and *Per3*) were restored to sham levels by ND administration while others (e.g. *Clock* and *RORα*) were not (Data not shown). This suggests that the sensitivity of the core clock to changes in circulating testosterone may be gene dependent, and will require additional work to assess the sensitivity of the core clock to changes in circulating testosterone.

In humans, androgens have been deemed to regulate muscle mass in large part by blunting muscle protein breakdown during the fasted metabolic state (21, 22). In contrast, our data show that markers of autophagy, oxidative stress, and mitochondrial degradation (e.g. increased Parkin and BNIP3 protein content, decreased p62 protein content) were increased in the muscle of castrated mice during a time when these nocturnal animals consume much of their food (i.e. circadian time 12-24). Given that we previously showed a strong relationship between mitochondrial degradation and autophagy activation (56), it is possible that nutrient consumption initiates mitochondrial stress, leading to the subsequent increase in autophagy-mediated protein breakdown that persists into the post-absorptive state (12, 13). This idea is supported by our

recent finding in that markers of mitophagy were increased in the muscle of castrated mice that were refed following an overnight fast (56), but those same markers of mitophagy were not altered if castrated mice remained fasted despite elevated markers of autophagy [(64) and unpublished observations]. Future work is needed to understand whether increased availability of ATP producing substrates (i.e. fatty acids and/or NADH+) might impose a detrimental stress on the mitochondria in the limb muscle under androgen-deprived conditions, and whether this effect is augmented by disruption of the core clock.

In conclusion, we provide evidence that androgen depletion disrupts the core molecular clock in the limb skeletal muscle, and this disruption coincides with changes in mitochondrial quality control and subsequent muscle atrophy. The change in expression of mitochondrial quality control genes coincides with an increase in mitochondrial degradation pathway activation and subsequent change to the circadian expression pattern of mitochondrial proteins. As mitochondrial health is an emerging and important regulator of skeletal muscle mass, these data support a novel conceptual model linking androgen depletion-induced limb muscle atrophy to reduced mitochondrial quality control via disruption of the molecular clock. Such knowledge will be useful for developing therapies that treat limb muscle atrophy in hypogonadal males that are unable to receive androgen replacement therapy.

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The authors have no disclosures.

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FIGURE LEGENDS

Figure 1: Characterization of changes to the core clock gene expression in the limb skeletal muscle following androgen depletion. (A-D) Microarray analysis was confirmed by measuring the relative mRNA content of *Bmal1*, and *Per1-3* in the TA of sham and castrated mice by RT-PCR (N = 7/8 per group for microarray validation). These samples were collected between 1300-1500 hr. (E-J) The circadian expression patterns of *Bmal1, Clock, Per1-3,* and *Cry1* mRNA were determined in the TA of sham and castrated mice by RT-PCR N = 3/group/time point for circadian measurements. Student's *t*-test was used to confirm microarray analysis. Two-way ANOVA was used to assess changes in circadian expression patterns. ME: Main

Effect. * Significantly different than the mean value in the Sham group, or significantly different than the Sham value at the given circadian time. $P \leq 0.05$ for all other analysis.

Figure 2: Assessment of core clock function in the limb muscle following androgen depletion. (A-E) The circadian expression pattern of various clock-controlled genes including *RORα, Rev-Erbα, MyoD, Dbp,* and *MAFbx* were determined in the TA of sham and castrated mice by RT-PCR. N = 3/group/time point for circadian measurements. Student's *t*-test was used to confirm microarray analysis. Two-way ANOVA was used to assess changes in circadian expression patterns. ME: Main Effect. * Significantly different than the Sham value at the given circadian time. $P \leq 0.05$ for all other analysis.

Figure 3: Assessment of circadian expression patterns for genes involved with mitochondrial quality control in the limb skeletal muscle following androgen depletion. The circadian expression pattern of genes involved with (A-B) mitochondrial fission, (C-E) mitochondrial fusion, (F-H) mitochondrial biogenesis, and (I-K) mitophagy were determined in the TA of sham and castrated mice by RT-PCR. Two-way ANOVA was used to assess circadian expression patterns. N = 3/group/time point for circadian measurements. ME: Main Effect. * Significantly different than Sham value at the given circadian time. $P \leq 0.05$ for all other analysis.

Figure 4: Assessment of mitophagy and autophagy activation patterns in the limb skeletal muscle following androgen depletion. (A-C) The circadian protein expression patterns for mitophagy related proteins were determined in the TA of sham and castrated mice by Western blot analysis. (D-F) The circadian protein expression patterns of the LC3 II/I ratio, LC3 I, and

LC3 II were determined in the TA of sham and castrated mice by Western blot analysis. (G) The circadian expression pattern for *LC3B* mRNA was determined in the TA of sham and castrated mice by RT-PCR. For Western blot analysis, an equal amount of protein from each sample within a group (N=3 sham or castrated) at each time point was pooled together for analysis. If a visual difference in the expression patterns across ≥3 consecutive time points was observed, differences in the mean pixel intensity values obtained from the ≥3 time points for each group (sham or castrated) were assessed statistically. (H) Western blot. Black line on blot is used to visually separate the sham and castrated groups. Two-way ANOVA was used to assess the circadian expression pattern of *LC3B* mRNA. Student's *t*-test was used to assess difference in the pixel intensity of ≥3 consecutive time points of protein in the TA. N = 3/group/time point for circadian measurement of *LC3B* mRNA. ME: Main Effect; CT: Circadian Time. * Significant difference from ≥3 consecutive time points between groups under the solid black line. $P \leq 0.05$ for analysis.

Figure 5: Assessment of mitochondrial protein expression patterns in the limb skeletal muscle following androgen depletion. The circadian expression patterns for (A) COX IV, (B) VDAC, (C) Bcl-xL, and (D) 4-HNE were determined in the TA of sham and castrated mice by Western blot analysis. (E) Western blot. Black line on blot is used to visually separate the sham and castrated groups. For Western blot analysis, an equal amount of protein from each sample within a group (N=3 sham or castrated) at each time point was pooled together for analysis. If a visual difference in the expression patterns across ≥3 consecutive time points was observed, differences in the mean pixel intensity values obtained from the ≥3 time points for each group (sham or castrated) were assessed statistically. Student's *t*-test was used to assess difference in the pixel

intensity of ≥3 consecutive time points of protein in the TA. CT: Circadian Time; NS: Non-Specific. The black arrows indicate non-specific reactivity by the anti-mouse secondary antibody. For illustrative purposes only, the lanes of the 4 HNE blot were made vertical in Adobe Photoshop after quantification. * Significant difference from ≥3 consecutive time points between groups under the solid black line. $P \leq 0.05$ for analysis.

Figure 6: Assessment of Bmal1 regulation in the limb skeletal muscle following androgen depletion. The circadian protein expression pattern of (A) Bmal1 was determined in the TA of sham and castrated mice by Western blot analysis. (B) The enrichment of Bmal1 protein in the cytosolic and nuclear-enriched fractions was determined in the gastrocnemius of sham and castrated mice by Western blot analysis. The circadian patterns for the ratio of phosphorylated to total protein for (C) GSK3β (Ser9) and (D) Akt (Ser473) were determined in the TA of sham and castrated mice by Western blot analysis. (E) Western blot. Black line on blot is used to visually separate the sham and castrated groups. For Western blot analysis, an equal amount of protein from each sample within a group (N=3 sham or castrated) at each time point was pooled together for analysis. If a visual difference in the expression patterns across ≥3 consecutive time points was observed, differences in the mean pixel intensity values obtained from the ≥3 time points for each group (sham or castrated) were assessed statistically. Student's *t*-test was used to assess difference in the pixel intensity of ≥3 consecutive time points of protein in the TA. CT: Circadian Time; Exp: Exposure. * Significant difference from ≥3 consecutive time points between groups under the solid black line. $P \leq 0.05$ for all analysis.

Figure 7: Assessment of Per2 regulation in the limb skeletal muscle following androgen depletion. The circadian protein expression pattern of (A) Per2, (B) Per2 Lower Band, and (C) Sirt1 were assessed by Western blot analysis. For Western blot analysis, an equal amount of protein from each sample within a group (N=3 sham or castrated) at each time point was pooled together for analysis. If a visual difference in the expression patterns across ≥3 consecutive time points was observed, differences in the mean pixel intensity values obtained from the ≥3 time points for each group (sham or castrated) were assessed statistically. Student's *t*-test was used to assess difference in the pixel intensity of ≥3 consecutive time points of protein in the TA. The circadian expression patterns for (D) *Sirt1* mRNA and (E) *miR-181a* were determined in the TA of sham and castrated mice by RT-PCR analysis. N = 3/group/time point. Two-way ANOVA was used to assess circadian expression patterns of each RNA. (F) Western blot. Black line on blot is used to visually separate the sham and castrated groups. ME: Main Effect, CT: Circadian Time. * Significant difference from ≥3 consecutive time points between groups under the solid black line (Western blots) or significantly different than Sham values at the given circadian time (RT-PCR). $P \leq 0.05$ for all analysis.

Figure 8: Theoretical model for the androgen-mediated regulation of limb muscle mass.

Table 1: Primer sequences for RT-PCR using Sybr Green

Gene Symbol	Forward (5'-3')	Reverse (5'-3')	Amplicon Size (bp)
Bmal1	TGGAGGGACTCCAGACATTC	TTGCTGCCTCATCGTTACTG	173
Per1	GTCCCCTGGTCCTCTACACA	GCCCGAGATTCAATGAAGAG	159

Per2	AGCCACCCTGAAAAGGAAGT	GGTGAGGGACACCACACTCT	184
Per3	GTCGAGAGGAGGTGCTGAAG	TCTGTCTTCACAGGCGACAC	173
Rev-Erbα	GGCACCTGCCAACAGTCTA	GCTGAGAAAGGTCACGGAAG	197
RORa	GGAAGAGTTTGTGTTCTATGCACC	TTCCATCTTCTCGGTGGTTC	177
Cry1	TTCACTGCTACTGCCCTGTG	CACTTGGCAACCTTCTGGAT	151
MyoD	TACCCAAGGTGGAGATCCTG	CATCATGCCATCAGAGCAGT	200
DBP	TCTAGGGACACACCCAGTCC	TGGTTGAGGCTTCAGTTCCT	159
RPLP0	CAACCCAGCTCTGGAGAAAC	GTTCTGAGCTGGCACAGTGA	169
Drp1	AGGAACCAACAACAGGCAAC	TCACAATCTCGCTGTTCTCG	190
Fis1	AAGTATGTGCGAGGGCTGTT	ACAGCCAGTCCAATGAGTCC	167
Opa1	GATGACACGCTCTCCAGTGA	TCGGGGCTAACAGTACAACC	177
Mfn1	GCTGTCAGAGCCCATCTTTC	CAGCCCACTGTTTTCCAAAT	195
Mfn2	AGCGCCAGTTTGTGGAATAC	CTTTCTTGTTCATGGCAGCA	177
PGC1-α	AAGACGGATTGCCCTCATTT	AGTGCTAAGACCGCTGCATT	191
Sirt1	AGTTCCAGCCGTCTCTGTGT	CTCCACGAACAGCTTCACAA	198
GAPDH	GTTGTCTCCTGCGACTTCA	TGCTGTAGCCGTATTCATTG	124
ID1	TACGACATGAACGGCTGCTA	GTGGTCCCGACTTCAGACTC	155

1183

1184 Table 2: Top 5 Functional Gene Categories altered by castration.

Functional Category	# of Genes	*P*-Value
Polyamine biosynthesis	3	0.0011
Biological Rhythms	5	0.0019

Receptor	22	0.0021
Olfaction	13	0.0030
Decarboxylase	3	0.0034

Table 3: Top 5 KEGG Pathways altered by castration.

KEGG Pathway	# of Genes	*P*-Value
Glutathione metabolism	4	0.0037
Systemic lupus erythematosus	5	0.0095
Circadian rhythm	3	0.013
Herpes simplex infection	5	0.030
Arginine and proline metabolism	3	0.032

Table 4: RT-PCR confirmation of the molecular clock/circadian genes identified via microarray (N =7-8 per group).

Gene Symbol	Fold Change (Castrated vs. Sham)	*P*-Value	95% Confidence Interval
Bmal1	-0.57351	0.0102	-0.985 to -0.1609
ID1	1.8576	0.0327	1.08249 to 2.633
Per2	3.27	<0.0001	2.598 to 3.937
Per3	3.062	<0.0001	2.467 to 3.658
Ppargc1a	1.8999	0.0269	1.1203 to 2.68

Note: *Ppargc1a* gene expression from this data set has been reported previously (56).

1195 Table 5: Muscle Mass and Tibia Length

	Sham (N = 18)	Castrated (N = 18)
Tibialis Anterior (mg)	50.98 ± 0.82	42.60 ± 1.18*
Gastrocnemius (mg)	140.53 ± 1.57	130.66 ± 1.60*
Plantaris (mg)	22.73 ± 0.52	20.06 ± 0.48*
Soleus (mg)	9.79 ± 0.20	8.58 ± 0.21*
Tibia Length (mm)	17.39 ± 0.10	17.17 ± 0.09

1196

1197 Table 6: Body and Tissue Mass

	Sham (N = 18)	Castrated (N = 18)
Initial Body Weight (g)	26.7 ± 0.40	26.7 ± 0.39
Final Body Weight (g)	32.6 ± 0.61	29.4 ± 0.62*
Heart (mg)	139.29 ± 3.44	121.87 ± 2.54*
Spleen (mg)	64.56 ± 2.39	85.32 ± 1.39*
Epididymal Fat Pad (mg)	1035.64 ± 65.72	1129.28 ± 112.61
Seminal Vesicle (mg)	364.42 ± 14.16	11.03 ± 1.23*

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