Castration induces satellite cell activation that contributes to skeletal muscle maintenance

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Abstract

Aims
Sarcopenia, the age-related loss of skeletal muscle, is a side effect of androgen deprivation therapy (ADT) for prostate cancer patients. Resident stem cells of skeletal muscle, satellite cells (SCs), are an essential source of progenitors for the growth and regeneration of skeletal muscle. Decreased androgen signaling and deficits in the number and function of SCs are features of aging. Although androgen signaling is known to regulate skeletal muscle, the cellular basis for ADT-induced exacerbation of sarcopenia is unknown. Furthermore, the consequences of androgen deprivation on SC fate in adult skeletal muscle remain largely unexplored.

Methods and results
We examined SC fate in an androgen-deprived environment using immunofluorescence and fluorescence-activated cell sorting (FACS) with SC-specific markers in young castrated mice. To study the effects of androgen deprivation on SC function and skeletal muscle regenerative capacity, young castrated mice were subjected to experimental regenerative paradigms. SC-derived-cell contributions to skeletal muscle maintenance were examined in castrated Pax7CreER/+; ROSA26RmTmG/ mice. SCs were depleted in Pax7CreER/+; ROSA26DTR/+ mice to ascertain the consequences of SC ablation in sham and castrated skeletal muscles. Confocal immunofluorescence analysis of neuromuscular junctions (NMJs), and assessment of skeletal muscle physiology, contractile properties, and integrity were conducted. We found castration led to SC activation, however this did not result in a decline in SC function or skeletal muscle regenerative capacity. Surprisingly, castration induced SC-dependent maintenance of young skeletal muscle. The functional independence of skeletal muscles on SCs in young castrated mice was demonstrated by an increase in SC-derived-cell fusion within skeletal muscle fibers. SC depletion was associated with further atrophy and functional decline, as well as the induction of partial innervation and the loss of NMJ-associated myonuclei in skeletal muscles from castrated mice.

Conclusion
The maintenance of skeletal muscles in young castrated mice relies on the cellular contributions of SCs. Considering the well-described age-related decline in SCs, the results in this study highlight the need to devise strategies that promote SC maintenance and activity to attenuate or reverse the progression of sarcopenia in elderly androgen-deprived individuals.

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Introduction
Skeletal muscle possesses a remarkable capacity for regeneration, which is mediated by adult resident stem/progenitor cell populations that contribute to repair and maintenance [1-4]. Resident Pax7-expressing muscle stem cells (satellite cells (SCs)) function as an essential source of myonuclei for the early growth, regeneration and lifelong maintenance of skeletal muscle [2-5]. In adults,
most SCs reside in a quiescent state at the interface between the muscle fiber (myofiber) and basal lamina [6,7]. In response to a degenerative injury, SCs activate and divide to produce myogenic progenitors for myofiber regeneration [2,8,9]. Effective maintenance and responsiveness of quiescent SCs at homeostasis is required for the preservation of skeletal muscle integrity and regenerative potential [4,10]. Disruption of quiescence, and the resulting inappropriate cell cycle entry, has been associated with depletion of the adult SC pool and loss of stem cell regenerative potential [6,10]. Specific loss of the Notch effector RBPjk in SCs leads to SC depletion stemming from spontaneous activation, terminal differentiation, and failure to self-renew [11,12]. Similarly, ablation of the miRNA processing enzyme Dicer and knockdown of miRNA-489 leads to SC activation, diminution of the SC-pool, and impaired skeletal muscle regeneration [10]. In normal aging, alterations in intrinsic and extrinsic signaling combine with disruptions in SC quiescence to culminate in SC dysfunction and depletion. [6,13]. In contrast to the dysfunction of SC pool maintenance seen in aging or the loss of Notch signaling, other systemic signals can prime quiescent SCs to a poised state that enables a more efficient regenerative response [14].

Among the systemic signals that can influence SC fate are sex hormones such as androgens. Consistent with the well-described role for androgens in skeletal muscle maintenance, a significant reduction in skeletal muscle integrity is a known consequence of castrate levels of testosterone in hypogonadal men, elderly prostate cancer patients undergoing androgen deprivation therapy, and castrated mice [15-23]. Furthermore, a decline in circulating androgen levels is a feature of age-related skeletal muscle loss in rodents and humans [24]. In humans, androgens are known to induce skeletal muscle hypertrophy through anabolism and the addition of myonuclei to myofibers (myonuclear accretion) [25,26]. When androgens are depleted by castration in mice, a reduced number of proliferating myogenic progenitors are observed at early stages of recovery following muscle injury [27]. This numeric deficiency of myogenic progenitors early in regeneration can be reversed by testosterone administration. Additionally, castration of mice prior to the onset of puberty results in a failure to establish the adult quiescent SC pool, in part through loss of Notch signaling [28]. Although castration prior to puberty leads to a deficit in the establishment of adult SCs, the consequence of adult castration on SC quiescence, derived myogenic progenitor fate, and skeletal muscle maintenance remains largely unexplored.

Here we examined the fate of SCs in castrated adult mouse skeletal muscles at homeostasis and during regeneration. We observed that castration was sufficient to activate SCs at homeostasis in uninjured muscle. Surprisingly, this disruption in quiescence was not associated with decreased SC number or loss of renewal potential. Further assessment of SC fate revealed that SC-derived cells contribute to castrated myofibers. Disruption of this contribution through castration and SC depletion resulted in degenerated NMJ morphology and smaller post-synaptic myonuclear cluster size. Furthermore, SC depletion exacerbated the castration-induced decline of skeletal muscle integrity and force generation. Collectively, our findings reveal a dynamic role for SCs in the maintenance of castrated skeletal muscle.

Materials and Methods

Animals

All procedures involving mice were done in accordance with guidelines set by the Animal Care and Use Committee at the University of Rochester Medical Center. Mice were housed in the animal facility with free access to standard rodent chow and water. C57BL/6, Pax7CreERT2 (017763) Rosa26SmTmG (007576) and Rosa26DTA (009669) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Rosa26SmTmG or Rosa26DTA mice were crossed with Pax7CreERT2 mice to generate Pax7CreER+/; Rosa26SmTmG/+ (P7mTmG) or Pax7CreER+/; Rosa26DTA/+ (P7DTA) mice and control CreER negative (Ctrl) littermates. PCR genotyping was performed using protocols described by Quanta, with primer sequences and annealing temps provided by JAX. To induce recombination, mice were administered intraperitoneal (IP) injections of 100uL Tamoxifen (Sigma-Aldrich, St. Louis, MO, T5648, 20mg/ml, 90% corn oil/10% EtOH) for 5 consecutive days. Mice were used at 3-6 months of age.

Castration Surgery

Mice were anesthetized with an IP injection of ketamine (110 mg/kg) and xylazine (10 mg/kg), and received a subcutaneous dose of buprenorphine (0.6mg/kg) prior to surgery. Sham Surgery: An incision was made in the scrotum and was closed with 1-2 staples. The testis, vas deferens, and attached testicular fat pad were left intact. Castration Surgery: The blood vessels supplying the testis were ligated with silk suture (SP 118, Look Surgical Specialties). The testis, vas deferens and fatty tissue were severed just below the site of the ligation. The scrotum incision was closed with 1-2 staples [23].

Cell proliferation analysis.

To assess cell proliferation during homeostasis, BrdU (5-bromo-2'-deoxyuridine, Sigma, 0.5 mg ml⁻¹ supplemented with 2.5% sucrose) was administered via drinking water to sham and castrated mice 8 weeks post-
surgery for 28 days[3,6]. BrdU incorporation was then assayed by immunostaining for Pax7, BrdU, and Laminin.

Skeletal muscle injury and regeneration

Mice were first anesthetized with an i.p. injection of ketamine (110 mg/kg) and xylazine (10 mg/kg). The skin overlaying the tibialis anterior (TA) muscle was then carefully shaved. The TA was directly injected with a 1.2% solution of BaCl2 in normal saline[2]. At 28 days post-injury, injured and contralateral (uninjured) TAs were collected. TAs used for histology or immunofluorescence were incubated at 4°C overnight in 30% sucrose solution before being embedded in OCT and flash-frozen in dry-ice-cooled isopentane.

Fluorescence-activated cell sorting (FACS)

To obtain highly purified SCs, primary cells were isolated from uninjured muscles as described previously[2,6]. Hind limb muscles were isolated and myofiber fragments were obtained by collagenase type II digestion, trituration, and multiple sedimentation. Mononucleated cells were then liberated by further dispase and collagenase type II digestion, trituration, sedimentation and filtration. Cells were stained with CD31, Sca1, CD45 (BD Biosciences, San Jose, CA, #561410, #562058, Bolegend, San Diego, CA, #103132), and Integrin α7 (AbLab, Vancouver, Canada, clone R2F2) fluorescent-conjugated antibodies. Cells were sorted with FACS Aria-II (BD Biosciences). SCs were collected using forward and side scatter profiles, negative selection for DAPI, CD31/45, and Sca1, and positive selection for Integrin α7.

FACS-purified SC cultures

To assay SC fate, 4,000 sorted SCs were seeded per well in eight-well Permanox chamber slides (Nunc) coated with ECM (Sigma-Aldrich) and cultured for 5 days in plating media (10% Horse Serum, 1% Pen/Strep, 1% 1M Hepes, 6ng/ml FGF2, DMEM)[2]. Culture purity was characterized by immunofluorescence using primary antibodies for renewal potential (Pax7) or myogenic differentiation (Myogenin). To assess proliferative capacity, SCs were plated at a density of 30 events per well in 96 well plates coated with ECM and cultured for 7 days in plating media. The number of Crystal Violet-stained cells present in each individual well was determined after seven days in culture.

Immunofluorescence

For immunofluorescence, flash-frozen muscles were cross-sectioned at 10 μm (transverse) or 30 μm (longitudinal) and stored at -80°C. Muscle sections were fixed for 3 minutes in 4% paraformaldehyde (PFA) (no PFA fixation for MyHC antibodies), and if needed, subjected to antigen retrieval: heating slides in citrate buffer (10 mM sodium citrate in H2O, pH 6.0) in a steamer (Oster #5712) for 15 min after 10 min preheating of buffer[2,3]. Tissue sections and cells were permeabilized with 0.2% Triton X-100 for 10 minutes, then blocked in 10% normal goat serum (NGS; Jackson Immuno Research) for 30 minutes at room temperature. If necessary (when mouse primary antibodies were used), sections were blocked in 3% affinitypure Fab fragment goat anti-mouse IgG(H+L) (Jackson Immuno Research) with 2% NGS in PBS at room temperature for 1 hour. Primary antibody incubation was carried out in 2% NGS/PBS at 4°C overnight or 2 hours at room temperature, and secondary antibody incubation was carried out for 1 hour at room temperature. DAPI staining was used to mark nuclei. All slides were mounted with Fluoromount-G (SouthernBiotech). Immunofluorescent images were taken with a Zeiss Axio Observer A1 microscope and analyzed using ImageJ software.

Histology and bright-field microscopy

For Sirius Red staining, a Picosirisiriv Red stain kit (Polysciences) was used. Briefly flash-frozen sections were fixed for 1 hour at 56°C in Bouin's fixative, washed in water, stained for 1 hour in Picosirisiriv Red, washed in 1M HCl, dehydrated, equilibrated with xylene and mounted using Cytoseal 60 (Richard-Allan Scientific). Bright-field images were collected with a Zeiss Axioskop 40 microscope. Olympus VS110 virtual microscopy system was utilized for whole-slide scanning. Automatic quantification of inter-myofiber collagen content was accomplished via VisioPharm software.

Confocal IF microscopy and analysis

Longitudinal muscle sections were stained with SV2, Znp-1, 2H3, Btx and DAPI as described above and viewed with an Olympus Fluoview 1000 confocal microscope with 40X objective at a 0.47 μm step size. Amira software was used to analyze 3-D reconstructed NMJs for innervation analysis and to identify and enumerate post-synaptic myonuclei. Max-projection z-stack images of NMJs were generated with ImageJ software. The post-synaptic side was identified based on the entry of the terminal axon and as the concave side of the NMJ [3,4].

Single myofiber analysis

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For assessing single myofiber size and myonuclear number, whole lower limbs were fixed in 4% PFA for 48 hours prior to EDL dissection and NaOH mediated digestion [3,4]. Fixed muscles were incubated in 40% NaOH for 2 hours. Dissociated fibers were then washed in PBS and processed for DAPI staining.

**Ex vivo muscle contraction assay**

Whole EDL muscle contractility and force generation were analyzed using an ASI muscle contraction system (Aurora Scientific, Aurora, Canada) as described previously [3,4]. Briefly, mice were anaesthetized and TA muscles removed. EDLs were then carefully isolated, adjusted to optimal length and stimulated at various frequencies to obtain absolute force values. To obtain specific force values, absolute force was normalized to pennation angle and cross-sectional area (determined by EDL weight and length). Muscle force was recorded and analyzed using Dynamic Muscle Control, Clamp fit and Graph Pad Prism software.

**RNA isolation and RT-qPCR**

RNA was isolated from sorted SCs or whole triceps brachii muscle using phase separation in Trizol (Invitrogen, Carlsbad, CA) followed by cleanup with the RNeasy Plus Minikit (Qiagen, Germany), according to manufacturer protocols. To prepare sorted cell RNA for RT-qPCR, first-strand complementary DNA was synthesized from ~50 ng of sorted SC RNA and ~500ng whole muscle RNA using the SuperScript First-Strand cDNA Synthesis Kit (Invitrogen). RT-qPCR was performed on a Step One Plus Real Time PCR machine (Applied Biosystems, Carlsbad, CA) using PerfeCTa SYBR Green FastMix with ROX (Quanta). Experiments were standardized to Gapdh. All reactions for RT-qPCR were performed using the following thermal cycler conditions: 50°C for 2 min, 95°C for 2 min, 40 cycles of a two-step reaction, denaturation at 95°C for 15 s, annealing at 60°C for 30 s.

**Antibodies**

The following antibodies were used: Pax7 (mouse IgG1, 1:100, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), Myogenin (rabbit, 1:500, Santa Cruz sc-576), Ki67 (rat, 1:250, BioLegend 652401), BrdU (rat, 1:250, Abcam ab6326, Cambridge, UK), GFP (rabbit, 1:400, Millipore AB3030P, Billerica, MA), Fibronection (Rb, 1:400, Millipore AB2033), Laminin (rat or rabbit, 1:1500, Sigma-Aldrich L0663 or L9393), A4.840 (mouse IgM, 1:40, DSHB), SC-71 (mouse IgG1, 1:40, DSHB), 6H1 (mouse IgM, 1:40, DSHB), BF-35 (mouse IgG1, 1:40, DSHB), BF-F3 (mouse IgG1, 1:40, DSHB), SV2 (synaptic vesicle protein-2, mouse IgG1, 1:100, DSHB), Znp-1 (synaptotagmin-2, mouse IgG1, 1:200, DSHB) and 2H3 (neurofilament, mouse IgG1, 1:200, DSHB), AlexaFluor 488-conjugated α-Bungarotoxin (1:1000, Life Technologies B-13422, Grand Island, NY), AlexaFluor 647-conjugated α-Bungarotoxin (1:1000, Life Technologies B-35450), AlexaFluor 594-conjugated goat anti-mouse IgG (1:1500), Life Technologies A-11032, AlexaFluor 594-conjugated goat anti-mouse IgG1 (1:1500, Life Technologies A-21125), AlexaFluor 488-conjugated goat anti-mouse IgM (1:1500, Life Technologies A-21042), AlexaFluor 488-conjugated goat anti-rat IgG (1:1500, Life Technologies A-11006), AlexaFluor 647-conjugated goat anti-rabbit (1:1500, Life Technologies A-21244). DyLight 405 Goat anti Mouse IgG2b (1:2000, Jackson ImmunoResearch 115-477-187), DAPI (1:3000, Life Technologies D-3571).

**Data analysis**

The average values were further evaluated in Graph Pad Prism software. Results were presented as mean ± SEM. Statistical significance was determined by Student’s t-tests for simple comparison, or one-way ANOVA with Bonferroni post-hoc and Fisher’s LSD post-hoc. P<0.05 was considered as statistically significant.

**Results**

Castration leads to disruption in SC quiescence.

Failure to maintain SC quiescence, SC loss, and reduced androgen levels are all features of aging [4,6,24]. To assess the consequences of androgen deprivation on SC fate in a young environment, we employed a BrdU (5-bromo-2’-deoxyuridine) incorporation assay in 12-week old castrated or sham mice. Eight weeks after surgery, mice were continuously fed the thymidine analog BrdU in drinking water for 28 days (Figure 1A). Indicative of androgen depletion, we observed severe atrophy of the androgen-sensitive levator ani muscle in castrated animals (supplementary material, Figure S1) [29]. As expected, there was limited SC activation observed in young tibialis anterior (TA) muscles. Only a small fraction, ~5%, of Pax7+ SCs from sham skeletal muscles demonstrated BrdU incorporation (Figure 1B, D). Castration led to a 4-fold increase in the percentage of BrdU+ Pax7+ SCs (Figure 1B, D). We also found that a higher proportion of SCs from castrated skeletal muscles were Ki67+, a marker of non-quiescent or activated cells (Figure 1C, E). In aged muscles, disruption in SC quiescence is associated with elevated levels of FGF2 in the niche and reduced levels of the RTK/FGF feedback regulator Sprouty1 (Spry1) in SCs [6]. FGF2 expression was significantly elevated in whole muscle.
from castrated mice compared to sham, while levels of FGF6 and Myostatin did not change (supplementary material, Figure S2). Additionally, the expression of Spry1 was significantly lower in SCs purified from castrated mouse muscle, whereas Hes1 expression, a downstream target of Notch signaling, was not altered (supplementary material, Figure S2).

Disruption of SC quiescence is often associated with Pax7+ SC loss or dysfunction [6,10,13,30]. Surprisingly, we did not observe significant SC loss 12 weeks after castration based on enumeration of Pax7+ SC number in transverse tibialis anterior (TA) sections and the proportion of SCs recovered by FACS (Figure 1B, C, F and supplementary material, Figure S3). In vitro fate analysis of sorted sham or castrated SCs confirmed their purity and capacity for myogenic differentiation (supplementary material, Figure S3). To examine growth capacity, FACS-purified SCs from castrated and sham muscles were plated at low density (30 cells per well) in a clonal growth assay. Cultures of 100 FACS-purified SCs or less can identify a deficit in the clonal growth capacity of aged SCs [2,6,31]. We found no significant difference in clonal growth between SCs derived from sham or castrated skeletal muscles (Figure 2A, B). Finally, we examined the regenerative capacity of castrated skeletal muscles. Twelve weeks after sham or castration surgery, TA muscles were injected intramuscularly with a 1.2% BaCl2 solution to induce a degenerative injury and allowed to recover for 28 days (Figure 2C). Enumeration of Pax7+ SC numbers 28 days after injury did not reveal any difference in castrated skeletal muscle regeneration or Pax7+ SC pool renewal (Figure 2D, E).

Figure 1 Castration induces disruptions in SC quiescence. (A) Strategy for labeling SCs with BrdU in adult mice 8 weeks after sham or castration surgery. (B) Representative TA sections stained with anti-BrdU (green), anti-Pax7 (red), and anti-Laminin (white). White arrowheads indicate BrdU+/Pax7+ cell; yellow arrowhead indicates BrdU+/Pax7+ cell; green arrowheads indicate BrdU+/Pax7+ cell; brown arrowheads indicate BrdU+/Pax7+ cell; scale bar = 50um. (C) Quantification of Ki67+/SC percentage. (D) Quantification of BrdU+/SC/mm² percentage. N = 4 mice. *p < 0.05, t-tests.
Castration does not compromise SC function or skeletal muscle regeneration. (A) Representative images of Crystal Violet stained FACs-sorted adult sham or castrated myogenic cells cultured for 7 days at clonal density, Scale = 200 um. (B) Quantification of cell growth. N = 96 cultures, *p<0.05 t-test. (C) Strategy to examine skeletal muscle regeneration 12 weeks after sham or castration surgery. (D) Representative images of Pax7 (red), DAPI (blue) and Laminin (white) immunofluorescence in adult 28dpi sham and castrated TA muscle sections. White arrowheads indicate Pax7+ cell. (E) Quantification of SCs/mm² in adult 28dpi sham and castrated TA muscle sections.

Castration leads to SC contribution to myofibers necessary for the maintenance of neuromuscular junctions (NMJs).

To examine if castration-associated SC activation leads to the generation of fusion-competent myogenic progenitors, we utilized an established SC-specific Pax7CreER/⁺; Rosa26mTmG/⁺ (P7mTmG) transgenic mouse line [3,4,32]. The P7mTmG mouse ubiquitously expresses a loxP-flanked membrane tomato red fluorescence reporter (RFP). Upon tamoxifen (Tmx) treatment, the reporter undergoes Cre-mediated excision to indelibly label Pax7+ SCs and derived cells with membrane GFP. Initial Tmx administration restricts GFP to Pax7+ SCs, but GFP labeling in myofibers stemming from SC-derived progenitor fusion is observed in response to injury or during postnatal growth and maintenance [3,4,32-34]. Castration and sham surgeries were performed one week after Tmx administration and animals were sacrificed 4 weeks later (Figure 3A). We detected an increased proportion of GFP+ myofibers in transverse sections from the mid-belly of castrated TA muscles, indicative of fusion of SC-derived myogenic progenitors (Figure 3B, C).

To examine the relevance of SC contribution to castrated skeletal muscles, we employed a mouse model of SC ablation: Pax7CreER/⁺; Rosa26DTA/⁺ (P7DTA) and Pax7⁻/⁻; Rosa26DTA/⁺ (Ctl) mice. These mice enable Tmx-mediated expression of diphtheria toxin-A (DTA) to deplete Pax7+ SCs to levels that prevent regeneration, and accelerate age-related atrophy of skeletal muscle and NMJ degeneration [3-5,35]. After Tmx administration, extensive depletion of Pax7+ SCs occurred regardless of sham or castration surgery (supplementary material, Figure S4). To test the requirement for a SC-derived contribution to the NMJ we examined P7DTA and Ctl muscle NMJ integrity after sham or castration surgery [3,4]. The NMJ is a specialized region composed of a pre-synaptic motor axon terminal, post-synaptic AChRs, and a cluster of post-synaptic myonuclei [3]. Assessment of the overlay between pre-synaptic markers and post-synaptic AChRs (innervation) as well as the number of post-synaptic myonuclei provides a measure of NMJ degeneration [3,4,36]. Previously, this evaluation has been used to show that loss of SC contribution to myofibers in the context of aging and denervation-related atrophy leads to degenerated NMJ morphology [3,4]. Initially, we examined the extent of NMJ innervation. All NMJs, regardless of genotype or procedure, were innervated (Figure 4A). An NMJ is considered to be partially innervated if > 5 um length of an AChR-enriched branch within the post-synaptic apparatus is not covered by pre-synaptic terminal markers [3,4]. No increase in partially innervated NMJs in Ctl TA muscles was observed after castration. However, examination of P7DTA NMJs following castration revealed that SC depletion was associated with a significantly higher proportion of partially innervated NMJs (Figure 4B).

Enriched at most adult NMJs is a cluster of specialized post-synaptic myonuclei [37-40]. A reduction in the number of myonuclei within a post-synaptic myonuclear cluster is significantly correlated with NMJ degeneration during aging and denervation atrophy [3,4]. To examine if loss of SC contributions to castrated...
myofibers can alter post-synaptic myonuclear cluster size, we quantified the number of Ctrl and P7DTA post-synaptic myonuclei (>25% DAPI covered by the Btx+ post-synaptic apparatus) [3,4,38,39,41]. Assessment of Ctrl and P7DTA NMJs after sham surgery did not reveal any significant alteration in the distribution of NMJs based on post-synaptic myonuclear cluster size (Figure 4C). Also, no significant reduction in post-synaptic myonuclear cluster size was found at Ctrl NMJs 12 weeks after castration (Figure 4C). However, a significant shift towards a smaller post-synaptic myonuclear cluster size was observed at P7DTA NMJs after castration, suggesting that there are an increased proportion of degenerated NMJs (Figure 4C) [3,4]. Specifically, we found that the percentage of NMJs with ≥5 postsynaptic myonuclei was ~20% less in castrated DTA muscle (Figure 4C). Collectively, these data indicate that loss of the SC-derived cellular contribution to castrated myofibers is associated with NMJ deterioration.

Figure 3 Castration stimulates SC contribution to myofibers. (A) Schematic demonstrating time of tamoxifen treatment, surgery, and harvest of tissue. (B) Representative images of GFP labeling of myofibers in P7mTmG TA muscles 4 weeks after sham and castration surgery. (C) Quantification of the percentage of GFP+ myofibers from the midsection of TA muscles 4 weeks after sham or castration surgery. Scale bar = 50 μm. N = 3 mice, 3 sections/mouse, 6 fields/section. *p < 0.05, t-tests.

Figure 4 Reductions in NMJ innervation and post-synaptic myonuclei in castrated SC depleted skeletal muscle. (A) Representative confocal IF images and 3-D Amira based reconstructions of control (Ctl) and P7DTA NMJs 12 weeks after sham or castration surgery, stained for post-synaptic (AChRs labeled with Btx, green), pre-synaptic markers (SV2, Syt-2, neurofilament, red) and myonuclei (DAPI, blue). Post-synaptic myonuclei are indicated with asterisks. Pre (pre-synaptic view) and Post (post-synaptic view). (B) Quantification of % partial innervation. (C) Distribution (% total) of NMJs based on number of post-synaptic myonuclei. Scale bar = 10 μm. N = 3 mice, 20 NMJs/mouse. *p < 0.05 compared to Ctrl-sham, P7DTA-sham and Ctrl-cast, ANOVA/Bonferroni multiple comparisons test.
SC depletion exacerbates castration-induced myofiber atrophy and force generation deficits.

Castration was sufficient to induce SC activation, division, and derived progenitor contribution to myofibers. Therefore, we sought to determine the consequences of SC depletion on castrated skeletal muscle integrity. We first examined the time required for a given muscle to reach peak tension (speed of contraction) upon stimulation at 150 Hz, a frequency that elicits peak tetanic contractile force. No alterations in time to peak tension (TTP) were observed regardless of genotype or procedure (supplementary material, Figure 5A). In line with unaltered contractile speed, no changes in myofiber type proportions (myosin heavy chain (MyHC) isoform-specific labeling) were observed as a result of castration or SC depletion (supplementary material, Figure S5B). We next measured ex vivo tetanic force generation ofCtl and P7DTA extensor digitorum longus (EDL) muscles 12 weeks after sham or castration surgery. We found a significant decline in the peak absolute tetanic force generated by castrated muscles (~12.5% less than sham), which was exacerbated by SC depletion (~25% less than sham) (Figure 5A, B). To account for muscle girth, force measurements were normalized to EDL physiological cross-sectional area (CSA) and are reported as specific force. We observed no significant decline in specific force in castrated Ctl EDLs (Figure 5C, filled columns), indicating that loss of skeletal muscle size was the principal determinant of the force generation deficit. In contrast, we observed a ~25% lower specific force generation capacity in SC-depleted castrated EDLs (Figure 5C, outlined columns).

During aging, denervation atrophy, or muscle overload by surgical manipulation SC depletion is associated with exacerbated myofiber atrophy, loss of myonuclei, and/or increased inter-myofiber connective tissue content (indicative of fibrosis) [3,4,42,43]. To assess CSA and myonuclear number, we examined individual myofibers derived from EDLs fixed prior to dissection [3,4,44]. As suggested by the observed absolute force decline (Fig. 5B), EDL myofiber CSA was ~30% less in castrated Ctl mice and ~50% less in castrated P7DTA mice compared to sham controls (Figure 6A, B). However, a significant but modest decline in myonuclear number (<10 myonuclei/500 μm) was only observed in SC-depleted castrated mice (Figure 6C). Finally, we performed Picosirius Red staining to examine inter-myofiber collagen content in order to assess fibrosis. Although castration increased inter-myofiber collagen content, no further elevation was observed with SC depletion (supplementary material, Figure S6). Therefore, specific force declines in P7DTA muscles following castration were not associated with increased fibrosis.

Figure 5 SC depletion leads to further declines in force generation of skeletal muscles after castration. (A) Representative traces for specific force from in vitro muscle contraction measurements in EDL muscles stimulated at 150Hz. (B) Absolute and (C) Specific force frequency values at 150Hz for Ctl and P7DTA EDL muscles 12 weeks after sham or castration surgery. N=3-6 mice, *p < 0.05 compared to sham Ctl and sham P7DTA. **p < 0.05 compared to sham Ctl, sham P7DTA, and cast Ctl.
Discussion

In this study, we have characterized the effects of castration on the function of adult SCs and skeletal muscle. Unexpectedly, we found that castration leads to a disruption in SC quiescence that is associated with SC-derived fusion to myofibers, but does not lead to SC depletion. Inducible depletion experiments demonstrated the importance of Pax7+ SCs for the maintenance of NMJs in skeletal muscle of castrated mice. Force generation decline and atrophy of skeletal muscle from castrated mice were exacerbated by the induction of SC depletion. Collectively, these observations indicate that SC-derived cell fusion contributes to the maintenance of castrated skeletal muscles, and reveal that the absence of SCs is sufficient to worsen castration-induced skeletal muscle decline.

There is evidence suggesting that sex hormones play a role in establishing SC quiescence at puberty via Notch signaling. For adult SC quiescence, this sex hormone-Notch signaling axis was determined to be dispensable based on the maintenance of Pax7+ SCs following castration of adult mice [28]. Although we found no reduction in the size of the adult Pax7+ SC pool 12 weeks after castration, we did demonstrate that SCs activate, divide, and contribute to myofibers in sedentary castrated adult mice. Even though androgen deprivation led to a disruption in SC quiescence, there was no alteration in the expression of Hes1, a Notch downstream target, in SCs purified from castrated mouse muscle. Specific ablation of Notch signaling through RBPjk loss in SCs leads to disruption in quiescence, with subsequent depletion of Pax7+ SCs within 3 weeks, and the complete failure of skeletal muscle regeneration [12]. Consistent with the maintenance of functional Notch signaling, there was no significant reduction in the size of the SC pool following castration, and skeletal muscle regenerative capacity was preserved. Disruption of aged SC quiescence is associated with reduction in Spry1 expression in SCs and elevated FGF2 levels in skeletal muscle [6]. After castration, we
detected a similar reduction of Spry1 transcripts in SCs and an elevation of FG2 in skeletal muscle. Although Spry1 loss and FG2 gain is associated with loss of SCs in aged skeletal muscle, these modifications do not lead to diminution of the SC pool in young uninjured muscle [6,45]. Other factors such as disruption in metabolism, cellular debris clearance, or low-grade inflammation have been shown to contribute to decline in the size and function of the aged muscle SC pool [30,46]. Therefore, it will be of interest to determine how these factors, combined with sex hormone loss and disruption in FGF signaling, contribute to age-related SC decline and skeletal muscle regenerative impairments.

Castration has been reported to reduce the size of the Pax7+ SC pool in regenerated mouse skeletal muscles 21 days post-injury [28]. We did not observe any such reduction of the Pax7+ SC pool in 28 day-regenerated castrated muscle using similar protocols. A possible explanation for this discrepancy is that reduced myogenic progenitor and SC number characterize early stages of castrated skeletal muscle regeneration with eventual recovery at later stages [27]. Therefore, differences in SC renewal post-injury in castrated skeletal muscle could reflect the regenerative stage examined. In addition, even if the number of SCs is conserved, whether or not the renewed SC pool is functional after regeneration in a castrated environment is unknown.

We have previously shown that SCs activate and SC-derived cells fuse to myofibers during denervation atrophy [3]. Similarly, we observed castration-induced SC-derived cell fusion to myofibers, however here we found that all NMJs were innervated regardless of SC status or procedure ( sham vs castrated, Figure 4). A significant increase in the proportion of partially innervated NMJs and reduction in post-synaptic myonuclear cluster size occurred only in SC-depleted muscle from castrated mice. This result is consistent with previous reports demonstrating that castration alone is insufficient to alter NMJ morphology in lower limb muscles [29,47]. One mechanism whereby SC depletion could lead to morphological alterations at the site of castrated NMJs is through exacerbation of denervation-reinnervation-induced NMJ deterioration [3]. However, castration alone did not lead to inductions of partial innervation or significant modifications in myofiber type—hallmark features of denervation-reinnervation-induced NMJ degeneration [3,48]. NMJ degeneration could potentially be a consequence of myofiber deficiencies caused by SC loss. It has been shown that degeneration or a change in myofiber size is sufficient to stimulate modification of NMJ morphology [47,49]. Thus NMJ degeneration could potentially be a consequence of myofiber deficiencies caused by SC loss. Furthermore, we found that SC depletion after castration led to further declines in myofiber size, myonuclear number, and force production—phenotypes often associated with NMJ deterioration [3,4].

An unresolved question is how SC depletion could be triggering skeletal muscle deficits. Based on the deterioration of castrated NMJs upon SC depletion, one possibility is that there is a loss of neurotransmission. However, the examination of post-synaptic endplate potentials has not shown a correlation between degenerated NMJ morphology and neurotransmission efficacy in aged or dystrophic muscles [50,51]. In addition, we did not observe obvious signs of neurotransmission failure such as tremor or paralysis in castrated PD-TA mice. Regular SC-derived cellular turnover at the NMJ could be required for local maintenance to withstand intense intracellular ion changes required for action potential stimulation and propagation [4,52]. In models of neuromuscular disease, sciatic nerve stimulation has been shown to promote NMJ degeneration [53]. It would be interesting to determine if such stimulation could promote a further decline in aged or SC-depleted skeletal muscles post-castration.

There is a known correlation between muscle force production, myofiber size and myonuclear number [54,55]. We observed lower absolute force production and smaller myofiber size in androgen-deprived skeletal muscle; these deficits were exacerbated when SCs were depleted prior to castration. Interestingly, reduced specific force in castrated skeletal muscle was only detected with SC depletion, and not with castration alone. Specific force, the absolute force normalized to muscle girth, serves as a read-out of intrinsic myofiber characteristics that could contribute to functional decline apart from size, myofiber number, or mass. Factors that accompany reduced specific force include myofiber type transitions, increased myonuclear domain (MND, the myofiber region that an individual myonucleus regulates), and fibrosis [3,54,55]. We did not observe significant myofiber type transitions based on MyHC isoform analysis. Although both SC depletion and castration led to a reduction in myonuclear number, this was associated with significantly more myofiber atrophy than castration alone. This coupling of more severe atrophy with loss of myonuclei runs counter to the MND expansion model as an explanation for the specific force deficit. In the contexts of denervation atrophy and functional overload, SC depletion leads to increased fibrosis [3,42,43]. Although castration was associated with a modest increase in fibrosis, no additional increase was observed following SC depletion. Therefore, the cause of specific force decline following castration of SC-depleted skeletal muscle remains to be determined. Additional mediators to be investigated include, but are not limited to, disruption in sarcomere integrity, calcium handling, and/or mitochondrial function [54,55].

An estimated 50% of men diagnosed with prostate cancer will at some point receive androgen deprivation
therapy (ADT) to stem the reoccurrence of tumors [56].
Alarming, the lean body mass (LBM) of prostate cancer patients receiving ADT for as little as 6-12 months has been reported to decrease by ~3% [16,18-20]. This accelerated loss in LBM correlates with significant declines in muscular strength, performance, and overall quality of life [15-17].
The only treatment currently available for ADT-induced muscle wasting is exercise-based intervention [17,57,58]. However, the intensity of exercise heightens the susceptibility of prostate cancer patients to fractures, which emphasizes the need for other therapies [16-18,59-61]. The results from this study suggest that age-related SC loss could be a significant contributor to ADT-induced exacerbation of sarcopenia. Thus, strategies targeting stem cells could attenuate muscle loss in elderly prostate cancer patients undergoing androgen deprivation therapy.

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Conflict of Interest
AK, WL, NP, SF, JJK, KLN, and JVC, declare no conflict of interest. The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia and Muscle – Rapid Communications (von Haehling S, Ebner N, Morley JE, Coats AJS, Anker SD. Ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle – Rapid Communications. J Cachexia Sarcopenia Muscle Rapid Communications 2017; 1:e44:1-2.)

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SUPPLEMENTARY FIGURES

Figure S1. Representative images of sham and castrated levator ani muscle. (A) sham, (B) castrated levator ani.

A

Sham

B

Cast

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Castration induces satellite cell activation that contributes to skeletal muscle maintenance

Figure S2. Castration induces FGF2 in skeletal muscle and reduces sprouty1 in SCs. Quantification of (A) muscle FGF2, FGF6, and myostatin (MSTN) mRNA levels relative to sham (B) SC Spry1 and Hes1 mRNA levels relative to sham. *p < 0.05 compared to sham t-tests.

Figure S3. Castration does not alter SC proportion by FACS. (A) Representative FACS plots of sorted SCs. (B) Quantification of percent SC recovered by FACS. (C) Fate analysis 5-day culture, IF for Pax7 (red), Myogenin (MyoG, green) and DAPI (blue). Scale bar = 200 μm.
Castration induces satellite cell activation that contributes to skeletal muscle maintenance

Figure S4. Depletion of Pax7+ SCs in P7DTA skeletal muscles. (A) Scheme demonstrating time of Tmx treatment, surgery, and harvest of tissue. (B) Quantification of Pax7+ satellite cell (SC) number from Ctl and P7DTA TA muscles 12 weeks after sham or castration surgery. N = 3 mice, 3 sections/mouse, 6 fields/section. *p < 0.05 compared to Ctl, ANOVA/Bonferroni multiple comparisons test.

Figure S5. Castration is not associated with myofiber type transitions connected to neuromuscular disruption. (A) Average time to peak tension (TTP) during 150 Hz stimulation in EDL muscles. (B) Quantification of type IIA, IIX, and IIB myofiber type percentage. N = 4 mice, 3 sections/mouse, 3 fields/section.
Castration induces satellite cell activation that contributes to skeletal muscle maintenance.

Figure S6. Castration induces connective tissue accumulation in skeletal muscles. (A) Representative images of TA sections stained Picrosirius Red and pseudocolor images generated by VisioPharm software; numbers indicate myofiber connective tissue (MCT) (red) content in each representative image. (B) Quantification of fibrosis index (MCT content) in TA muscles. N = 4 mice. *p < 0.05 compared to Ctrl-sham and P7DTA-sham and Ctrl-SNT, ANOVA/Bonferroni multiple comparisons test.