

Testosterone is essential for skeletal muscle growth in aged mice in a heterochronic parabiosis model

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Abstract As humans age, they lose both muscle mass and strength (sarcopenia). Testosterone, a circulating hormone, progressively declines in aging and is associated with loss of muscle mass and strength. The surgical joining of a young and old mouse (heterochronic parabiosis) activates Notch signaling and restores muscle regenerative potential in aged mice. We hypothesize that testosterone is at least one of the factors required for the improvement seen in muscles in old mice in heterochronic parabiosis with young mice. To test this hypothesis, we established the following heterochronic parabioses between young (Y; 5 months old) and old (O; 22–23 months old) C57BL6 male mice: (1) Y:O; (2) castrated Y:O (ØY:O); (3) castrated + testosterone-treated Y:O (ØY + T:O). A group

of normal young mice received empty implants, and old mice were used as controls. Parabiotic pairings were maintained for 4 weeks prior to analysis. Serum testosterone levels were three-fold higher in young than in old mice. The ØY + T:O pairing demonstrated significantly elevated levels of serum testosterone and an improvement in gastrocnemius muscle weight, muscle ultrastructure, muscle fiber cross-sectional area, and Notch-1 expression in old mice. These changes were not present in aged mice in the ØY:O pairing. These data indicate that testosterone has a critical role in mediating the improved muscle mass and ultrastructure seen in an experimental model of heterochronic parabiosis.

Keywords Testosterone · Parabiosis · Muscle growth · Aging · Mouse

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Introduction

Sarcopenia is defined as an age-related loss of skeletal muscle mass and strength. Many studies have demonstrated that it is both a widespread and a severely debilitating condition (Baumgartner et al. 1998; Roubenoff and Hughes 2000; Marzetti and Leeuwenburgh 2006). Such age-related loss of muscle mass has a profound effect on the elderly population, manifested by decreased healing after injury, impaired physical function, and increased risk of falls, fractures, dependency, and death (Glass and Roubenoff 2010). Despite its prevalence, mechanisms underlying this age-related loss of skeletal muscle mass remain poorly understood.

Adult skeletal muscles robustly regenerate throughout an organism's life, but as the muscle ages, its ability to repair diminishes and eventually fails. This diminished regenerative potential of aged muscle is largely attributable to a decline in Notch signaling, which is essential for the activation, proliferation, and myogenic progression of satellite cells (Conboy

et al. 2003, 2005; Shadrach and Wagers 2011). Heterochronic parabioses, in which two mice of different ages are surgically joined such that they develop a shared blood circulation without immune rejection, is a powerful model for determining whether circulating factors can influence tissue function in aging and longevity (Conboy et al. 2005, 2013; Shadrach and Wagers 2011; Brack et al. 2007). Heterochronic parabioses with young mice restore the regenerative capacity of aged satellite cells, through the activation of Notch signaling, and promote successful muscle repair after injury (Conboy et al. 2005). Conversely, the exposure of a young mouse to an old systemic environment inhibits myogenesis (Brack et al. 2007). Importantly, these studies implicate a factor(s) that is found in the systemic circulation and that regulates skeletal muscle stem cell niche and regeneration.

The testosterone level progressively declines in aging and is associated with a loss of muscle mass and strength (Sinha-Hikim et al. 2006; Cunningham and Toma 2011). Using a mouse model, we have previously demonstrated that testosterone supplementation prevents the loss of muscle mass in aging through the stimulation of both Notch and Akt signaling in old mice (Kovacheva et al. 2010). Here, by using a heterochronic parabiosis model, we provide preliminary evidence that testosterone is obligatory for the restoration of the systemic environment that supports muscle growth and improves muscle pathology in aging.

Materials and methods

Heterochronic parabiosis

C57Bl-6 J male mice were purchased from Harlan Laboratories (Indianapolis, Ind., USA) and aged in a standard animal facility at Charles R. Drew University of Medicine and Science under a controlled temperature (22°C) and photoperiod (12-h light, 12-h dark cycle) with food and water ad libitum. Young mice of 5 months of age and aged mice of 22–23 months of age were utilized. Young mice were treated as follows: (1) control (sham-operated), (2) castrated, and (3) castrated but having received 1.0-cm testosterone implants. Orchiectomy and sub-dermal testosterone implantation were performed under anesthesia. Testosterone-filled implants were prepared from polydimethylsiloxane tubing (outer diameter, 1.96 mm; inner diameter, 1.47 mm; Dow-Corning). The 1.0-cm testosterone implant size was based on the results of our earlier study (Kovacheva et al. 2010), which showed that this dose of testosterone fully reversed the age-related decline in muscle mass and muscle fiber cross-sectional area (CSA) and suppressed the age-specific increase in muscle cell apoptosis and oxidative stress. It also restored age-specific decreases in Akt and Notch signaling. Heterochronic parabiotic pairings ($n=5$ pairs per group) were created between young and old mice

as previously described (Wagers et al. 2002). In brief, the right side of the normal, castrated, or testosterone-supplemented castrated young mouse and the left side of the old mouse were shaved and sterilized. Matching skin incisions were made from the olecranon to the knee joint of each mouse. The subcutaneous tissue was next dissected to create a 0.5-cm free skin flap, both dorsally and ventrally. The elbows and knees of the parabiotic pair were attached by a single 5–0 coated vicryl suture, and the dorsal and ventral skin flaps were each secured by a running 5–0 vicryl suture. These mice were maintained for 4 weeks following parabiosis. The following parabiotic pairings were established between young (Y) and old (O) mice: (1) Y:O, (2) orchiectomized Y (ØY):O, and (3) orchiectomized plus 1.0 cm testosterone-treated Y (ØY + T):O. A group of normal young mice received empty implants, and old mice were used as controls. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Charles R. Drew University School of Medicine and Science Animal Care and Use Review committee.

Blood collection and tissue preparation

All mice were killed with a lethal intraperitoneal injection of sodium pentobarbital (200 mg/kg body weight). Blood samples were collected from each animal by cardiac puncture immediately after death, and serum was separated and stored at -20°C for subsequent testosterone assay. The gastrocnemius muscles from each mouse were removed and weighed. Portions of the tissues were snap-frozen in liquid N_2 and stored frozen for subsequent analysis by Western blotting. Additional portions from each mouse were either fixed in 2.5 % glutaraldehyde for electron microscopy or 4 % formalin for histological and immunohistochemical studies. Portions of glutaraldehyde-fixed muscles were further diced into small pieces, post-fixed in 1 % osmium tetroxide, and embedded in Epon 812 as described previously (Sinha-Hikim et al. 2007). Thin sections from selected tissue blocks were cut with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Hitachi 600 electron microscope (Hitachi, Indianapolis, Ind., USA). The rationale for using gastrocnemius muscles was based on the results of several earlier studies showing that this muscle exhibited a substantial decline in mass with age (Martin et al. 2007; Braga et al. 2008; Kovacheva et al. 2010; Sinha-Hikim et al. 2013).

Testosterone assay

Serum testosterone levels were measured by a previously reported radioimmunoassay (Sinha-Hikim et al. 2007; Kovacheva et al. 2010). The minimal detection limit in the assay was 0.6 ng/dl. The intra-assay and inter-assay coefficient of variations were 8.2 % and 13.2 %, respectively.

Muscle fiber CSA

Muscle fiber CSA was determined in 5- μm -thick paraffin-embedded, hematoxylin and eosin (H&E)-stained sections of gastrocnemius muscles by using ImagePro Plus, version 5.1 software (Media Cybernetics, Silver Spring, Md., USA) coupled to an Olympus BHS microscope equipped with a VCC video camera (Braga et al. 2008; Kovacheva et al. 2010). At least 50 fibers were measured for each animal.

Assessment of muscle pathology

Muscle pathology was evaluated by using conventional histological analysis of H&E-stained sections. Further evaluation of pathology was achieved by transmission electron microscopy (TEM). An observer who was unaware of the treatment assignment took and analyzed the electron micrographs. We also performed morphometric analysis to estimate the volume density (V_v), i.e., the volume of a given cellular component per unit myofibrillar volume. From each treatment group, 80 micrographs (20 micrographs/mouse) were selected for ultrastructural analysis. The point-counting method (Cruz-Orive and Weibel 1990; Mahapatra et al. 2005) was used to estimate the V_v of various myofibrillar components by superimposing a transparent overlay bearing a double-lattice grid on electron micrographs of muscles. The V_v was obtained by dividing the points on a given cellular organelle by the total number of points counted over the muscle fiber. Values were expressed as percentages of the myofibrillar volume ($V_v\%$), obtained by multiplying volume densities by 100.

Western blotting

Western blotting was performed by using muscle lysates as described previously (Braga et al. 2008; Kovacheva et al. 2010; Sinha-Hikim et al. 2013). In brief, proteins (50–80 μg) were separated on a 4–12 % SDS-polyacrylamide gel with MES or MOPS buffer purchased from Invitrogen (Carlsbad, Calif., USA) at 200 V. Gel was transferred onto an Immuno-blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif., USA) overnight at 4°C. Membranes were blocked in blocking solution (0.3 % Tween 20 in TRIS-buffered saline [TBS-T] and 10 % nonfat dry milk) for 1 h at room temperature and then probed with a rabbit polyclonal Notch 1 antibody (1:200) and a proliferating cell nuclear antigen (PCNA) antibody (1:200) for 1 h at room temperature or overnight at 4°C with constant shaking. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Following three 10-min washes in TBS-T buffer, membranes were then incubated in secondary antibody, namely anti-rabbit IgG (Amersham Biosciences, Piscataway, N.J., USA) conjugated to horseradish peroxidase, at a 1:2000 dilution. All antibodies were diluted in blocking buffer. For

immunodetection, membranes were washed three times in TBS-T wash buffer, incubated with enhanced chemiluminescence (ECL) solutions as suggested in the manufacturer's specifications (Amersham Biosciences), and exposed to Hyper film ECL. The membranes were stripped and re-probed with a rabbit polyclonal D-glyceraldehyde-3-phosphate dehydrogenase antibody (1:2000) for normalization of the loading. Band intensities were determined by using Quantity One software from Bio-Rad (Hercules).

Statistical analysis

Statistical analyses were performed by using the SigmaStat 2.0 Program (Jandel, San Rafael, Calif., USA). Data are presented as means \pm SEM. We used one-way analysis of variance (ANOVA) to compare group differences. If overall ANOVA revealed significant differences, post hoc (pairwise) comparisons were performed by using the Student-Newman-Keuls method. Differences were considered significant when $P < 0.05$. Pearson product moment correlation coefficients were computed to reveal relationships between variables.

Results

Testosterone levels, muscle weight, and muscle fiber CSA

Serum testosterone levels were three-fold higher in young mice than compared with their aged counterparts (Fig. 1a). Compared with the $\emptyset\text{Y}:\text{O}$ pairing, old mice in the $\emptyset\text{Y} + \text{T}:\text{O}$ joining had significantly ($P < 0.01$) elevated levels of serum testosterone (Fig. 1a). Gastrocnemius muscle mass was significantly lower by 15.4 % in old mice in comparison to young mice ($P < 0.05$). Notably, gastrocnemius muscle mass in the old parabiont was increased in the $\emptyset\text{Y} + \text{T}:\text{O}$ pairing (153 ± 8 mg) but not in the $\emptyset\text{Y}:\text{O}$ (127 ± 2 mg) pairing (Fig. 1b). Changes in gastrocnemius muscle mass were significantly ($r = 0.92$; $P < 0.02$) and positively correlated with changes in testosterone levels.

H&E-stained muscle sections revealed smaller muscle fibers in old mice (Fig. 1d) compared with those of young males (Fig. 1c). Muscle fiber histology in old mice in the $\text{Y}:\text{O}$ (Fig. 1e) or $\emptyset\text{Y}:\text{O}$ (Fig. 1f) pairings was indistinguishable from that of old controls (Fig. 1d). Notably, the $\emptyset\text{Y} + \text{T}:\text{O}$ pairing resulted in an increase in muscle fiber size in old mice (Fig. 1g) resembling the phenotype observed in young controls (Fig. 1c). As summarized in Fig. 1h, a significant ($P < 0.05$) reduction occurred in mean fiber CSA in old mice ($980 \pm 25 \mu\text{m}^2$) compared with that of young males ($1346 \pm 27 \mu\text{m}^2$). No significant differences in muscle fiber CSA were noted between old mice ($980 \pm 25 \mu\text{m}^2$) and old mice in the $\text{Y}:\text{O}$ ($978 \pm 21 \mu\text{m}^2$) or $\emptyset\text{Y}:\text{O}$ ($929 \pm 20 \mu\text{m}^2$) pairings. However,

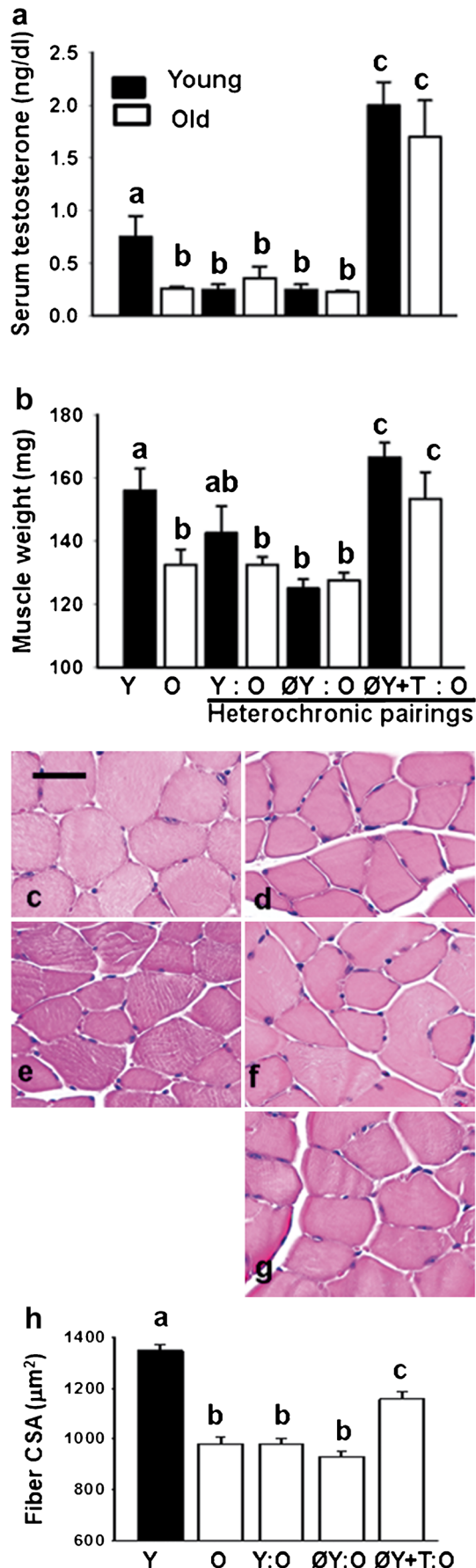


Fig. 1 Testosterone levels, muscle weights, muscle histology, and muscle fiber cross-sectional area (CSA). **a** Serum testosterone levels were three-fold higher in young (Y) than in old (O) mice. Compared with the young to old (Y:O) or castrated young to old (ØY:O) pairings, old mice in pairings with the young castrated but having received 1.0-cm testosterone implants (ØY + T:O) had significantly elevated levels of serum testosterone. Values are given as means ± SEM ($n=5$). Means with *unlike superscripts* are significantly different. **b** The weight of the gastrocnemius muscles was decreased significantly in the old animals compared with young mice. Notably, the ØY + T:O, but not the ØY:O, pairing fully restored gastrocnemius muscle weight in aged mice to levels seen in young controls. Values are given as means ± SEM ($n=5$). Means with *unlike superscripts* are significantly different. Representative gastrocnemius muscle sections (stained with hematoxylin and eosin) from Y (**c**), O (**d**), and old mice in Y:O (**e**), ØY:O (**f**), or ØY + T:O (**g**) pairings reveal that only the ØY + T:O pairing results in an apparent increase in muscle fiber size in old mice (**g**), similar to the phenotype observed in young mice (**c**). *Bar* 25 µm. **h** Quantitative analysis of muscle fiber CSA in various parabionts. Values are given as means ± SEM ($n=5$). Means with *unlike superscripts* are significantly different

the ØY + T:O pairing resulted in a significant ($P<0.05$) increase in muscle fiber CSA ($1158\pm30\ \mu\text{m}^2$) in aged mice.

ØY + T:O joining improves myofibrillar ultrastructure in old mice

We next performed TEM to evaluate myofibrillar architecture in various parabiotic pairings (Fig. 2). Gastrocnemius muscle from young mice exhibited normal architecture (Fig. 2a) with abundant normal-looking mitochondria, no intramyofibrillar lipid (IML) accumulation, and no tubular aggregation (TA). In contrast, variable degrees of abnormalities were noted in aged muscle, including mitochondrial swelling with broken cristae, mitochondrial vacuolization, increased IML accumulation, and the presence of TA (Fig. 2b). The Y:O pairing reversed some of these changes in old mice, as the old parabionts exhibited decreased IML accumulation, fewer mitochondrial abnormalities, and smaller areas of TA (Fig. 2c). In contrast, muscles from old partners in the ØY:O pairing failed to show a reversal of these age-associated changes (Fig. 2d-f). Instead, typical of old muscle, muscle from these mice demonstrated larger areas of TA (Fig. 2d), vacuolated (Fig. 2e) and swollen mitochondria with broken cristae (Fig. 2f), and increased IML accumulation. The ØY + T:O pairing, however, showed remarkable improvement in muscle ultrastructure (Fig. 2g). The ultrastructural appearance of muscle in these old parabionts was similar to that seen in young mice (Fig. 2a).

The ultrastructural analysis of the volumetric composition of myofibrillar organelles is summarized in Table 1. Compared with muscle from young mice, a significant ($P<0.05$) decrease in the V_v % of normal-looking mitochondria together with increases in the V_v % of vacuolated mitochondria, IML accumulation, and TA were noted in muscle from old mice. The Y:O pairing fully attenuated age-related decline in V_v % of normal looking mitochondria. Significant ($P<0.05$)

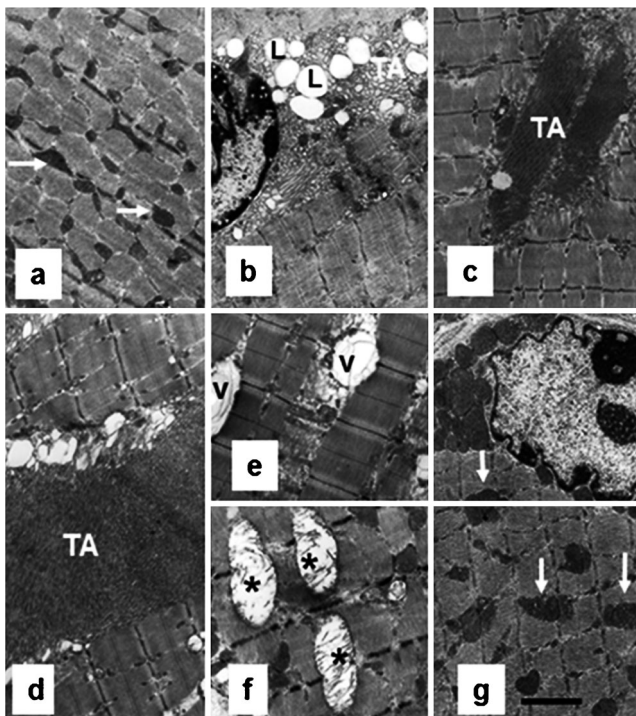


Fig. 2 ØY + T:O joining improves myofibrillar ultrastructure in old mice (a–g). Representative TEM image of a portion of a gastrocnemius muscle from a young mouse exhibiting normal myofibrillar cytoarchitecture and sarcomere organization (a) with an abundance of mitochondria (arrow). In striking contrast, muscle from an old mouse exhibits a perturbed muscle ultrastructure (b), including intramyofibrillar lipid (IML) accumulation (L) and the formation of large areas of tubular aggregation (TA). Muscle from an aged parabiont in the Y:O pairing shows no IML accumulation and relatively smaller areas of TA formation (c). In contrast, muscles from aged partners in the ØY:O pairing (d–f) show larger areas of TA formation and vacuolated (v) and swollen mitochondria with broken cristae (asterisk). Notably, the ØY + T:O pairing restores normal cytoarchitecture and sarcomere organization with abundant hypertrophied mitochondria in aged mice (g). Bar 2.5 µm

reductions in the V_v % of vacuolated mitochondria (by 39.5 %) and IML (47 %) in aged parabionts were also seen in

Table 1 Morphometric data with respect to the volumetric composition (V_v %^a) of normal-looking mitochondria (NMIT), vacuolated mitochondria (VMIT), intramyofibrillar lipid (IML) accumulation, and tubular aggregation (TA) in skeletal muscles of young (Y), old (O), and old mice in various parabiotic pairings with untreated (Y:O), castrated (ØY:O), and castrated+1-cm testosterone-treated young males (ØY + T:O). Values are given as means ± SEM. In each column, means with unlike superscripts are significantly ($P < 0.05$) different

Treatment	NMIT	VMIT	IML	TA
Y	5.55±1.17 ^A	0.25±0.05 ^A	0.17±0.05 ^A	0 ^A
O	1.73±0.33 ^B	2.23±0.33 ^B	2.32±0.01 ^B	9.30±2.14 ^B
Y:O	4.40±0.55 ^A	1.35±0.29 ^C	1.23±0.21 ^C	5.93±0.55 ^B
ØY:O	1.92±0.16 ^B	2.81±0.91 ^B	3.02±0.85 ^B	18.45±4.63 ^C
ØY + T:O	10.86±2.24 ^C	1.08±0.07 ^C	0.69±0.09 ^D	3.63±0.44 ^D

^a V_v % is the volume density expressed as a percentage of the myofibrillar volume

comparison with aged controls. Intriguingly, the ØY + T:O pairing was even more effective than the Y:O pairing in reversing such age-related changes in muscle ultrastructure. The V_v % of normal-looking mitochondria was increased to 627 % and 195 % over the values measured in young and old controls, respectively. Significant ($P < 0.05$) reductions were additionally noted in the V_v % of vacuolated mitochondria (51.6 %), IML (70.3 %), and TA (61 %) in these parabionts compared with those of old controls. In contrast, muscle in the ØY:O pairing failed to show a reversal of these age-associated changes.

Muscle hypertrophy of old mice in ØY + T:O pairing is associated with stimulation of Notch signaling

As shown in Fig. 3a, b, a significant ($P < 0.01$) decrease occurred in Notch-1 levels in gastrocnemius muscles from old mice when compared with young animals. Following Y:O or ØY + T:O pairings, the muscle from old parabionts exhibited increased Notch-1 expression in comparison with old controls. No upregulation of Notch-1 expression was seen in old mice in the ØY:O pairing. Notch-1 expression was associated with cell proliferation as demonstrated by the increased expression of PCNA in muscle lysates ($P < 0.05$, Fig. 3a, c). Notch-1 expression was significantly and positively correlated with changes in serum testosterone levels ($r = 0.93$; $P < 0.02$) throughout the groups.

Discussion

The central hypothesis of this study is that testosterone is obligatory for the restoration of the systemic environment that supports muscle growth and improves muscle pathology in aging. We have tested this hypothesis by using a heterochronic parabiosis model between young and old mice with additional manipulations. The results of the present study confirm and extend earlier findings by demonstrating that the ØY + T:O pairing attenuates the age-related decrease in muscle mass, induces muscle fiber hypertrophy, improves skeletal muscle ultrastructure, and fully restores Notch-1 expression in aged mice. Notably, these improvements in skeletal muscles do not occur in aged mice in the ØY:O pairing. Importantly, we further show that such a reversal of the aging muscle phenotype can be achieved with normal levels of testosterone (1.7 ± 0.3 ng/ml) in aged heterochronic parabionts as opposed to supra-physiological levels (8.0 ± 3.1 ng/ml) of testosterone required for muscle hypertrophy in old control (non-parabiosed) mice, as noted in our previous study (Kovacheva et al. 2010). Thus, we speculate that the observed muscle hypertrophy in old partners with normal levels of testosterone, as opposed to supra-physiological levels (Kovacheva et al. 2010), in the ØY + T:O pairing arises from

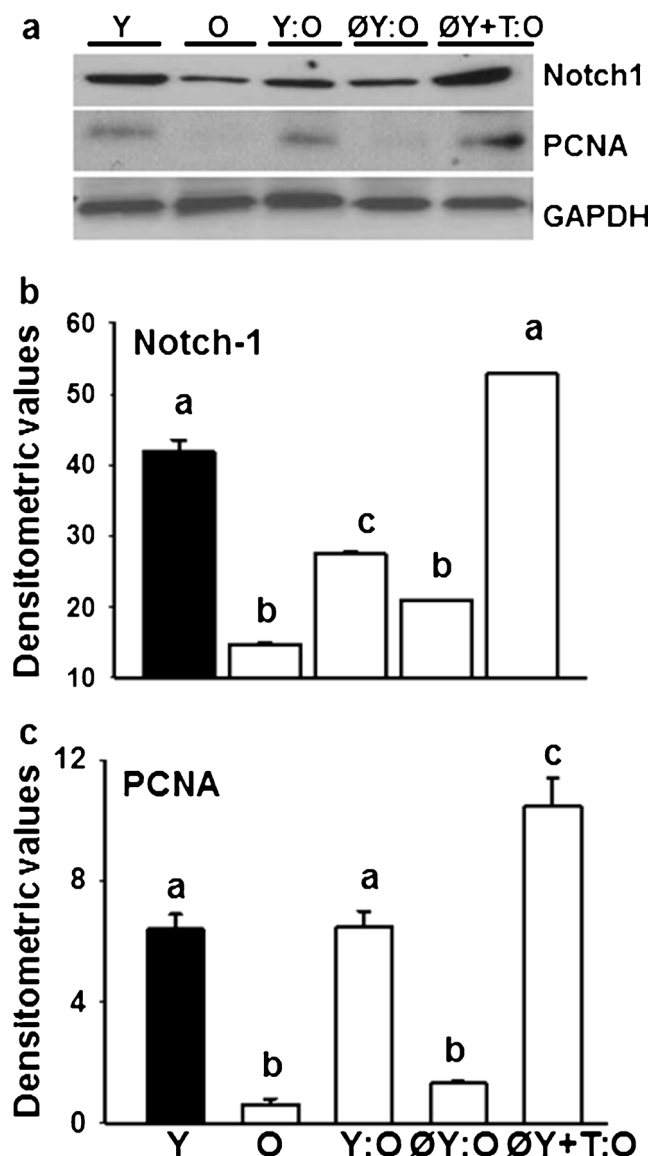


Fig. 3 Muscle hypertrophy in old mice in ØY + T:O pairing is associated with the stimulation of Notch signaling. **a** Western blots of muscle lysates show that the ØY + T:O, but not the ØY:O, pairing fully restores Notch-1 expression to levels seen in young (Y) controls. Activation of Notch signaling in these heterochronic old parabionts is associated with increased expression of proliferating cell nuclear antigen (PCNA) in muscle lysates (GAPDH D-glyceraldehyde-3-phosphate dehydrogenase as a loading control). **b**, **c** Quantification of band intensities. Values are means \pm SEM. Means with unlike superscripts are significantly ($P < 0.05$) different

the activation of an old muscle stem cell niche. Given the adverse effects associated with testosterone administration in the elderly (Cunningham and Toma 2011; Spitzer et al. 2013), our study has important clinical implications.

The diverse ultrastructural changes seen in aged muscles, including mitochondrial swelling with broken cristae, mitochondrial vacuolization, increased IML accumulation, and the presence of TA, are consistent with those described in the earlier literature in humans and in rodents (Kaminska et al.

1998; Agbulut et al. 2000; Corsetti et al. 2008; Crane et al. 2010). Notably, TA formation can also be seen in several human muscle disorders, including myopathies with exercise-induced cramps and muscle pain, myoasthenic disorders, and in some familial myopathies (Niakan et al. 1985; Rosenbergh et al. 1985; Mahjneh et al. 2007; Jain et al. 2008). We do not know the way in which TA formation and myopathies are connected. The pathophysiology of TA formation during aging also remains unknown but might be a manifestation of perturbed muscle structure and function in aging. The amount of oxidative stress increases as an organism ages and is postulated to be a major causal factor for the loss of muscle mass and function in aging (Braga et al. 2008; Kovacheva et al. 2010; Crane et al. 2010; Sinha-Hikim et al. 2013). Increased oxidative stress has also been implicated in mitochondrial damage and IML accumulation in aged muscles (Martin et al. 2007; Bonnard et al. 2008). Furthermore, Bonard and colleagues (2008) have demonstrated that the increased generation of oxidative stress, triggered by a high-fat and high-sucrose diet or streptozotocin treatment, leads to a reduction in mitochondrial density and marked alterations in mitochondrial structure. These effects are blocked by the attenuation of oxidative stress either through the normalization of glycemia or by antioxidant treatment. Pertinent here is that testosterone is able to reduce oxidative stress in aged muscles (Kovacheva et al. 2010). Collectively, these data suggest that testosterone restores muscle architecture in aged parabionts, possibly through the suppression of oxidative stress.

Notch signaling is essential for the activation, proliferation, and myogenic progression of satellite cells (Conboy et al. 2003, 2005; Shadrach and Wagers 2011). Our previous studies on both elderly men (Sinha-Hikim et al. 2006) and aged mice (Kovacheva et al. 2010) indicate an involvement of Notch signaling in testosterone-mediated muscle growth during aging. Consistent with a role of Notch signaling in muscle growth, we have found increased Notch-1 expression in aged muscle in the Y:O pairing. Interestingly, no upregulation of Notch-1 expression has been detected in muscle from old parabionts in ØY:O pairings. In contrast, the ØY + Y:O pairing results in a marked upregulation of Notch-1 expression in aged muscle to levels even higher than those seen in young controls. Muscle Notch-1 expression is positively correlated to the changes in circulating testosterone levels ($r = 0.93$; $P < 0.02$). Notably, previous work examining the effects of heterochronic parabiosis between young and old mice has shown the activation of Notch signaling and the proliferation and rejuvenation of aged satellite cells (Conboy et al. 2005). Together, these data suggest that, in the present model, testosterone restores the aged systemic milieu to its youthful state, through the stimulation of Notch signaling, and induces hypertrophy in aging muscle.

Although our study has important implications for the rapidly expanding field of the modulation of the local

environment that promotes muscle growth in aging, it has some limitations. One limitation is that we have not used this model of *in vivo* or *in vitro* heterochronic parabiosis to characterize the stimulatory effects of testosterone on aged muscle progenitor cells. An additional short-fall of our study is that the functionality of testosterone-induced muscle hypertrophy has not been determined. Clearly, both these points merit further investigation.

In summary, our results indicate that testosterone is one of the serum factor(s) necessary for the muscle growth seen in aged mice in an experimental heterochronic parabiosis model. A deeper understanding of the mechanism by which testosterone improves the local environment that supports muscle regeneration and growth in aging might provide new entry points for therapeutic strategies in clinical settings.

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