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Enobosarm (GTx-024) modulates adult skeletal muscle mass independently of the androgen receptor in the satellite cell lineage

Vanessa Dubois[1], Ioannis Simitsidellis[2], Michaël R. Laurent[1,3], Ferran Jardi[4], Philippa T. K. Saunders[2], Dirk Vanderschueren[4], and Frank Claessens[1]

[1]Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium; [2]Medical Research Council Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom; [3]Gerontology and Geriatrics, KU Leuven, Leuven, Belgium; [4]Clinical and Experimental Endocrinology, Department of Clinical and Experimental Medicine, KU Leuven, Leuven, Belgium

Androgens increase skeletal muscle mass, but their clinical use is hampered by lack of tissue selectivity and subsequent side-effects. Selective androgen receptor modulators (SARMs) elicit muscle-anabolic effects while only sparingly affecting reproductive tissues. The SARM GTx-024 (enobosarm) is being investigated for cancer cachexia, sarcopenia, and muscle wasting diseases. Here, we investigate the role of muscle androgen receptor (AR) in the anabolic effect of GTx-024. In mice lacking AR in the satellite cell lineage (satARKO), the weight of the androgen-sensitive levator ani muscle was lower, but decreased further upon orchidectomy. GTx-024 was as effective as dihydrotestosterone (DHT) in restoring levator ani weights to sham levels. Expression of the muscle-specific androgen-responsive genes S-adenosylmethionine decarboxylase and myostatin was decreased by orchidectomy and restored by GTx-024 and DHT in control mice, while expression was low and unaffected by androgen status in satARKO. In contrast, insulin-like growth factor IEa expression was not different between satARKO and control muscle, decreased upon castration, and was restored by DHT and GTx-024 in both genotypes. These data indicate that GTx-024 does not selectively modulate AR in the satellite cell lineage and that cells outside this lineage remain androgen-responsive in satARKO muscle. Indeed, residual AR positive cells were present in satARKO muscle, coexpressing the fibroblast-lineage marker vimentin. AR positive, muscle-resident fibroblasts could therefore be involved in the indirect effects of androgens on muscle. In conclusion, both DHT and GTx-024 target AR pathways in the satellite cell lineage, but cells outside this lineage also contribute to the anabolic effects of androgens.

Androgens play important roles in diverse biological processes such as development and maintenance of the male phenotype. In addition, they have effects on several nonreproductive tissues including bone, smooth muscle, and skeletal muscle (1–3). Their biological effects are exerted by activating the androgen receptor (AR). This ligand-inducible transcription factor binds to specific DNA sequences called androgen response elements (AREs) to regulate transcription of target genes (4).

Despite their well-documented effect of increasing muscle mass and strength (5, 6), the clinical use of androgens is limited due to a lack of tissue selectivity and potential subsequent side effects. Together with their poor oral bioavailability and pharmacokinetic profile, this has stimulated the development of selective AR modulators (SARMs). A well-established body of evidence supports their in vivo tissue selectivity in animal models, as assessed by their ability to increase levator ani muscle weight and bone mineral density (BMD) while only sparingly affecting the weight of androgenic tissues (7).

GTx-024 (enobosarm) is a nonsteroidal SARM which increases muscle mass with only limited effects on seminal

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vesicles in preclinical studies (8). Trials in healthy elderly men (9) and in cancer patients with muscle wasting (10) have reported increased lean body mass and improved physical function in the GTx-024 treated groups compared to placebo, making this compound a strong candidate for further clinical development.

Skeletal muscle is a complex tissue and the impact of androgens can be mediated via one or all of the AR positive cell types. Indeed, androgens can have direct effects on cells of the satellite cell lineage including satellite cells, myoblasts, and myocytes, which all express the AR (1, 11, 12). In addition, indirect effects may contribute to the eventual muscle hypertrophy, with endothelial cells and fibroblasts within the muscle being candidate target cells since AR expression has been reported in these cell types (13, 14).

Whether the anabolic effect of GTx-024 on muscle is due to selective action on muscle cell AR is presently unknown. To further clarify this issue, we performed a castration and drug replacement study in mice selectively lacking the AR in satellite cells and hence in myoblasts and myocytes (satARKO, satellite cell-specific AR knockout). The nonaromatizable androgen dihydrotestosterone (DHT) was used as a positive control.

Materials and Methods

Animals

Mice with a satellite cell-specific knockout of the AR (satARKO) (AR^{fl}/Y;MyoD-$iCre^{+/-}$) and control mice (AR^{fl}/Y; MyoD-$iCre^{-/-}$) (C57BL/6J genetic background) were generated as described elsewhere (15). The animals were housed in standard cages at 20°C with a 12h-dark/light cycle according to our institutional guidelines. They had ad libitum access to tap water and standard chow. Offspring were weaned at 3 weeks of age and genotyped by PCR-based analysis of DNA samples obtained via tail biopsy. The experimental protocol was conducted with approval of the KU Leuven ethical committee (P078–2010).

Experimental design

At 12 weeks of age, male mice were randomly divided into 4 groups which each contained 8 mice per genotype. Animals were weighed and body composition was assessed by whole-body dual-energy X-ray absorptiometry (DXA). In group 1, mice were sham-operated (sham) under sodium pentobarbital anesthesia. In groups 2, 3, and 4, mice were orchidectomized (orx). Mice from groups 3 and 4 were treated with 7 mg/kg/d dihydrotestosterone (DHT) (#10300 from Sigma-Aldrich, St. Louis, MO, USA) or 3 mg/kg/d GTx-024 (#43974 from Sigma-Aldrich) for two weeks. The drugs were dissolved in dimethylsulfoxide-polyethylene glycol 300 (1:9, vol/vol) and administered via daily subcutaneous injections in the cervical region. The sham and orx animals were treated with vehicle during the treatment period. At the end of the treatment, animals were weighed and body composition was assessed by whole-body DXA. Seminal vesicles, levator ani muscle as well as several limb muscles (gastrocnemius, extensor digitorum longus, soleus) were collected and weighed after euthanasia by cardiac puncture.

Dual-energy X-ray absorptiometry

Whole body lean and fat mass were analyzed in vivo using the PIXImus mouse densitometer (Lunar Corp., Madison, WI, USA) with ultrahigh resolution (0.18 × 0.18 pixels, 1.6 line pairs/mm) and software version 1.45.

Serum IGF-I measurement

After acid-ethanol extraction, serum IGF-I concentrations were measured by an in-house RIA in the presence of an excess of IGF-II (25 ng/tube) (16).

Quantitative real-time PCR

Total RNA was extracted from murine muscle using TRIzol reagent (Invitrogen) according to the manufacturer's protocols. After digestion with DNase I (Fermentas, St Leon-Rot, Germany), cDNA was synthesized from 300 ng (levator ani muscle) or 1 μg (gastrocnemius muscle) RNA using the RevertAid M-MuLV Reverse Transcriptase kit (Fermentas) and random hexamer primers (Fermentas). The PCR reaction mixtures (10 μl) contained 1x Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.15 μM of each primer, and 50 nM ROX Reference Dye (Invitrogen). The 7500 FastReal-Time PCR system (Applied Biosystems, Foster City, CA, USA) was used with the 'Fast RT-PCR' two-step protocol (2 minutes at 50°C, 20 seconds at 95°C and 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C). The primer sequences are listed in **a href="http://press.endocrine.org/doi/suppl/10.1210/en.2015–1479/suppl_file/en-15–1479.pdf"Supplemental Table 1. All primers were designed to hybridize to separate exons, and generation of single correct amplicons was checked by DNA**

Table 1. Antibody Table. Details of the primary antibodies used for fluorescent immunohistochemistry.

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Androgen receptor (AR)	Synthetic peptide derived from near N-terminus of human AR	Rabbit Anti-Human Androgen Receptor (AR) Monoclonal Antibody (Clone SP107)	Spring Bioscience; M4070	Rabbit monoclonal	1:300
Myogenin	Recombinant protein containing rat myogenin amino acid 30 − 224	Anti-Myogenin antibody [F5D]	Abcam; ab1835	Mouse monoclonal	1:50
CD31	Synthetic peptide corresponding to C terminus of mouse CD31	Anti-CD31 antibody	Abcam; ab28364	Rabbit polyclonal	1:500
Vimentin	Synthetic peptide corresponding to residues surrounding Arg45 of human vimentin	Vimentin (D21H3) XP Rabbit mAb	Cell Signaling Technology; 5741	Rabbit monoclonal	1:600
Laminin	Protein purified from the basement membrane of Englebreth Holm-Swarm (EHS) sarcoma (Mouse)	Anti-Laminin antibody	Abcam; ab11575	Rabbit polyclonal	1:300
Pax7	Purified internal fragment of human recombinant PAX7 expressed in E.Coli	PAX7 Antibody	Thermo Scientific; PA1–117	Rabbit polyclonal	1:500

sequencing and in melting curve assays. Gene expression values are expressed relative to the levels of 18S rRNA.

Luciferase reporter assay

Mouse C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 4,5 g/l glucose, 4 mM L-glutamine, and penicillin (100 IU/ml)-streptomycin (100 μg/ml) (Sigma-Aldrich), and supplemented with 10% fetal calf serum (Invitrogen). For transfection experiments, cells were seeded in 96-well plates at a density of 10^4 cells per well in DMEM supplemented with 5% charcoal-stripped serum (Sigma-Aldrich) and transiently transfected using X-tremeGENE transfection reagent (Roche, Basel, Switzerland) with 10 ng of expression plasmid for full-size human AR (17), 100 ng of luciferase reporter plasmid containing four copies of the ARE of interest, and 10 ng of β-galactosidase expression plasmid (Stratagene, La Jolla, CA, USA), which served as an internal control for transfection efficiency. The luciferase reporter plasmids were generated as described before (18), and the ARE sequences are listed elsewhere (15). Cells were lysed after stimulation for 24 hours with 100 nM DHT or GTx-024 in the presence or absence of 100 μM MDV3100 (#SRP016825M from Sequoia Research Products, Pangbourne, UK), and luciferase and β-galactosidase activities were measured and calculated as previously described (19).

Double antibody fluorescent immunohistochemistry

Double antibody fluorescent immunohistochemistry was performed as described previously (20). Briefly, levator ani and gastrocnemius muscles were fixed in 10% neutral buffered formalin (Sigma-Aldrich) overnight at 4°C, rinsed with PBS and then stored in 70% ethanol at 4°C. After embedding into paraffin wax, 5 μm sections were cut onto micro slides, deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval in a decloaking chamber containing 0.01 M citrate buffer. The tissue sections were then incubated with 3% hydrogen peroxide in methanol for 30 minutes at room temperature to block endogenous peroxidase activity, rinsed with water, and blocked for 30 minutes in goat serum (Biosera, Kansas City, MO, USA) diluted 1:4 in Tris-buffered saline (TBS; 50 mM Tris pH 7.4, 0.85% saline) containing 5% bovine serum albumin (BSA). Primary antibodies were diluted in goat serum/TBS/BSA and incubated at 4°C overnight. Details of the primary antibodies are provided in Supplemental Table 2. Secondary antibody (goat antirabbit peroxidase [Abcam, ab7171] or goat antimouse peroxidase [Abcam, ab6823]) was diluted 1:500 in goat serum/TBS/BSA and applied to sections for 45 minutes at room temperature. Finally, slides were incubated with the Tyramide Signal Amplification™ kit (Perkin Elmer, Waltham, MA, USA; Fluorescein for AR and Cyanine 3 for the other markers) for 10 minutes, then with DAPI (Sigma-Aldrich) diluted 1:500 in TBS to enable nuclear counterstaining. Washes between the several incubations were performed three times for 3 minutes each using TBS. Immunostaining of negative controls, which did not show any antiserum immunolabeling, included sequential elimination of either the primary or secondary antibody from the staining procedure. Sections were examined using a Zeiss LSM 510 Meta-confocal microscope equipped with a digital camera and images were analyzed by ImageJ software. The percentage of AR positive cells corresponding to fibroblasts (identified as vimentin positive) in the LA was calculated out of 286 ± 73 AR positive cells per animal. All procedures were carried out using coded slides to avoid bias.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (Version 6.00 for Windows, San Diego, CA, USA). Fisher's exact test was used for categorical variables, while for ordinal variables Student's *t* test and one-way ANOVA were used to analyze differences between two or more groups, respectively. Two-way ANOVA was used in experiments with more than two independent variables. If overall ANOVA revealed significant difference, Bonferroni's post hoc test was used to analyze differences between groups. All statistical tests were performed two-tailed. Data are presented as means ± SEM, and $P < .05$ was considered statistically significant.

Results

GTx-024 is a tissue-selective compound with anabolic effect on murine androgen-sensitive muscle

To validate the doses used and to confirm the tissue-selectivity of GTx-024 in our experimental setting, a pilot experiment was performed in which the weight of levator ani (LA) and seminal vesicles (SV) of control mice were compared among the different treatment groups. Orchidectomy (orx) at 12 weeks of age led to markedly reduced LA and SV weights after two weeks, and both DHT and GTx-024 increased the weight of these organs when drug treatment was initiated immediately after orx (**Figure 1**). However, whereas DHT and GTx-024 restored LA weight to sham level (**Figure 1A**), they had a differential effect on SV weight. Indeed, DHT supplementation fully normalized SV weight, while only partial restoration was observed in the GTx-024-treated group (**Figure 1B**). Gastrocnemius (GASTR) muscle mass was not affected by orx with or without drug replacement (**Supplemental Figure 1A**), which correlates with the lower levels of AR protein (**Supplemental Figure 1 B and C**). Thus, we conclude that GTx-024 acts as a SARM with an anabolic effect on LA muscle equivalent to physiological androgen concentrations in this experimental setting.

Muscle AR is not essential for modulation of muscle mass by GTx-024

To investigate whether the anabolic effect of GTx-024 is mediated via AR in muscle cells, we performed the same castration experiment with drug replacement in the satARKO model. In these mice, the AR was selectively ablated in the muscle progenitor cells called satellite cells, leading to a more than two-fold reduction in LA weight (15) (sham conditions in **Figure 2A**).

It is known that lean body mass or appendicular muscle mass in mice is considerably less sensitive than LA muscle (11), especially in short-term experiments. Indeed, body weight and body composition were not different between the groups, neither at baseline nor after the treatment period (**Supplemental Figure 2 A and B**). In addition, limb muscle mass was not different between satARKO and control mice and unaffected by orx without or with DHT or GTx-024, as evidenced by the similar weight of gastrocnemius (GASTR), extensor digitorum longus (EDL), and soleus (SOL) muscles in the different treatment groups (**Supplemental Figure 2C**). Therefore, we focused in further experiments on perineal muscle, which is widely used as a read-out for androgens in rodents including in SARM development (21).

Orx decreased LA mass in control mice, an effect that was also observed in satARKO mice (**Figure 2A**). Interestingly, GTx-024 reversed the orx effect on LA muscle of satARKO mice, to the same extent as did DHT (**Figure 2A**). These observations were confirmed in another perineal muscle, the bulbocavernosus (BC) (**Figure 2B**). As a control for the in vivo effectiveness of the treatments, the SV weights for each condition are depicted in **Figure 2C**.

The rescue of LA mass to sham level observed in castrated DHT- as well as GTx-024-treated satARKO mice may reflect an indirect nonmuscle AR contribution. Alternatively, residual muscle AR expression could be responsible for this anabolic response. To elucidate this question, we measured the expression of S-adenosylmethionine decarboxylase 1 (Amd1) and myostatin (Mstn), two genes shown to be strongly androgen-regulated in skeletal muscle (15, 22). Indeed, a more than 10-fold reduction in Amd1 and Mstn mRNA was observed in LA muscle of satARKO compared to control mice (sham conditions in **Figure 3A** and **Figure 3B**). Orx decreased Amd1 and Mstn transcript levels in control mice but no further decrease was observed in satARKO mice (**Figure 3A** and **Figure 3B**), demonstrating that muscle AR is sufficient for androgen regulation of these muscle-specific genes. In addition, these findings confirm effective disruption of muscle AR in satARKO, at least at the functional level. Orx-mediated decrease in Amd1 and Mstn mRNA was reversed by DHT and also by GTx-024 in control mice (**Figure 3A** and **Figure 3B**), indicating that GTx-024 action on muscle is, at least in part, direct via muscle AR activation. Importantly however, neither DHT nor GTx-024 were able to induce Amd1 or Mstn expression in satARKO LA (**Figure 3A** and **Figure 3B**) although they had a clear effect on muscle mass, supporting the hypothesis of an indirect nonmuscle AR contribution to muscle mass by androgens and GTx-024. Similar Amd1 expression patterns were observed in GASTR muscle (**Supplemental Figure 3A**). Mstn transcript levels were however not altered by orx with or without drug replacement in GASTR muscle, albeit lower in satARKO compared to control samples (two-way ANOVA, $P = .02$ for genotype) (**Supplemental Figure 3B**).

We previously identified two conserved AREs in the promotor and in exon 2 of the Mstn gene, referred to as ARE1 and ARE2, both of them binding the DNA-binding domain of the AR and conferring androgen-responsiveness to a heterologous promotor (15). ARE1 is part of an AR binding site found in the chromatin of primary human myoblasts by ChIP-on-Chip analysis (23). In a transient transfection assay in the C2C12 muscle cell line, ARE1 and ARE2 based reporter genes displayed responsiveness to DHT and GTx-024, which was strongly reduced in the presence of the AR antagonist MDV3100 (**Supplemental Figure 4**). Thus, in line with the above-mentioned in vivo findings, these in vitro data indicate that Mstn gene transcription is a good readout of muscle-specific androgen responsiveness, and that muscle AR is involved in the anabolic effect of GTx-024.

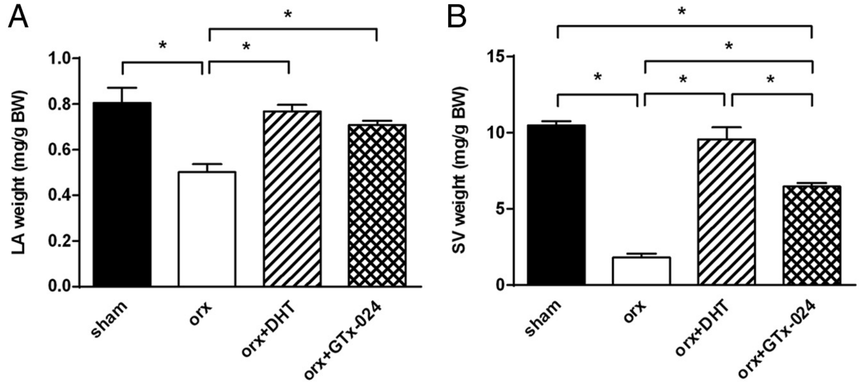

Figure 1. GTx-024 is a tissue-selective compound with anabolic effect on muscle. (A and B) Levator ani (LA) muscle weight (A) and seminal vesicle (SV) weight (B) of 14-week-old control mice that were sham-operated (sham), orchidectomized (orx) or orchidectomized and treated with dihydrotestosterone (orx+DHT) or GTx-024 (orx+GTx-024) at 12 weeks of age (n = 3). Organ weights were corrected for body weight (BW). Error bars indicate SEM; $^*P < .05$.

Insulin-like growth factor IEa (IGF-IEa), an IGF-I isoform locally expressed within muscle, is an important positive regulator of muscle mass (24), and its expression is regulated by androgens (25, 26). Indeed, orx decreased IGF-IEa transcript levels in LA of control mice, an effect that was reversed by DHT and also by GTx-024 (**Figure 3C**). Importantly however, the similar IGF-IEa transcript levels in control and satARKO sham-operated animals as well as the decrease in IGF-IEa mRNA in castrated satARKO mice (**Figure 3C**) indicate that androgen regulation of IGF-IEa is indirect via nonmuscle AR. Orx-mediated decrease in IGF-IEa mRNA was reversed by DHT and also by GTx-024 in both genotypes (**Figure 3C**), opening up the possibility that IGF-I signaling may be involved in the indirect nonmuscle AR contribution to muscle mass by androgens and GTx-024. Serum IGF-I levels were similar in satARKO and control mice and unaffected by orx without or with DHT or GTx-024 (**Supplemental Figure 5**).

Altogether, we conclude that both muscle cell and non-

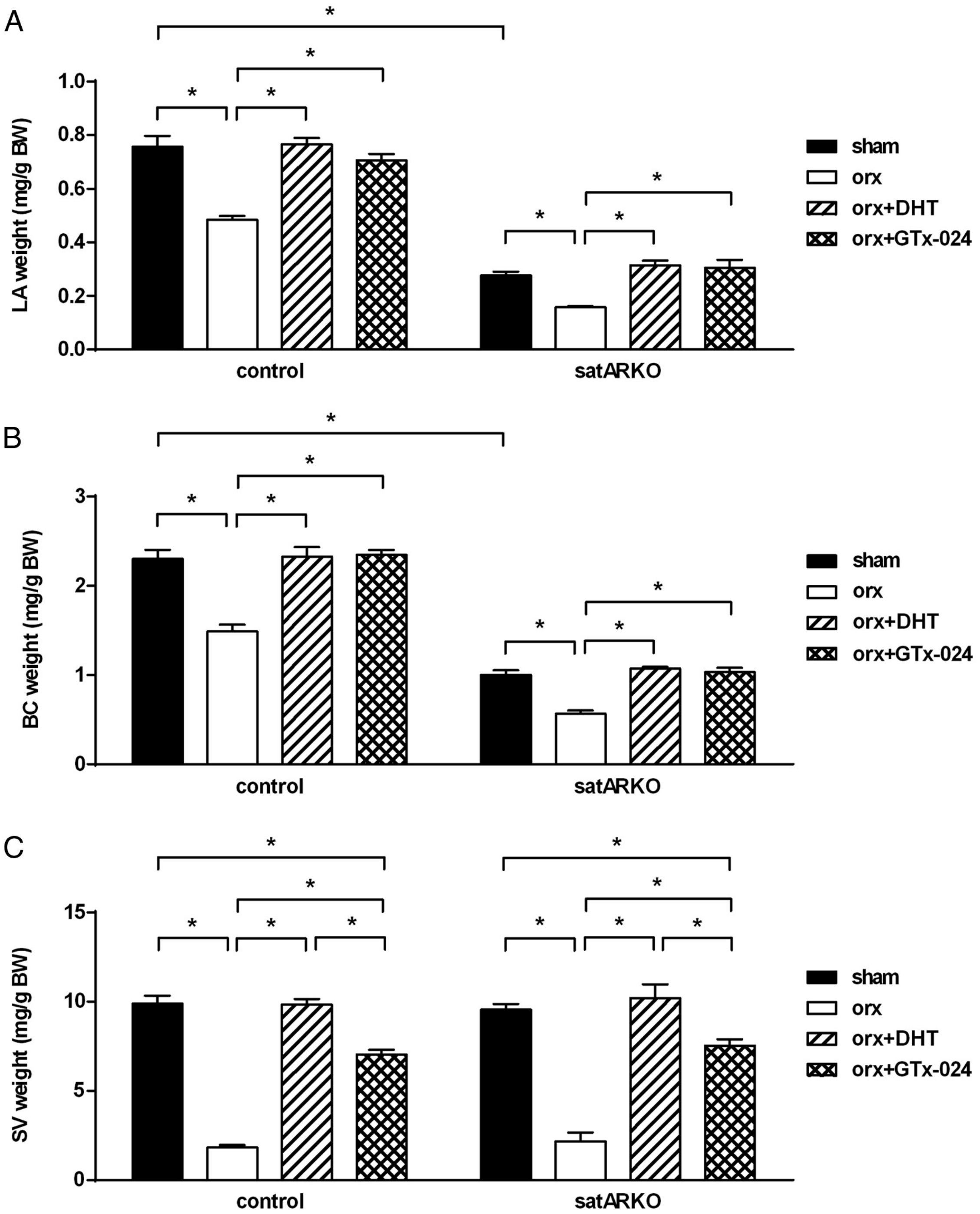

Figure 2. GTx-024 effects on muscle mass in the satARKO model. Levator ani (LA) weight (A), bulbocavernosus (BC) weight (B) and seminal vesicle (SV) weight (C) of 14-week-old control and satARKO mice that were sham-operated (sham), orchidectomized (orx) or orchidectomized and treated with dihydrotestosterone (orx+DHT) or GTx-024 (orx+GTx-024) at 12 weeks of age (n = 7–8). Organ weights were corrected for body weight (BW). Error bars indicate SEM; $^*P < .05$.

muscle cell AR play a role in DHT and GTx-024 action on muscle. The tissue-selectivity of GTx-024 is however not explained by selective targeting of muscle cell AR, as evidenced by retained efficacy in the satARKO model.

Fibroblasts may be involved in indirect androgen action on muscle

We next examined which cell types within the muscle other than the muscle cells themselves may mediate the indirect anabolic effect of androgens. Although AR protein is expressed predominantly in satellite cells and myonuclei, endothelial cells and fibroblasts within the muscle have also both been reported to express the AR (13, 14). Therefore, LA muscle sections from control and satARKO mice were double stained for the AR on the one hand and for myogenin, CD31 or vimentin (vim) on the other hand (markers for muscle fibers, endothelial cells and fibroblasts, respectively). In control mice, we observed AR+

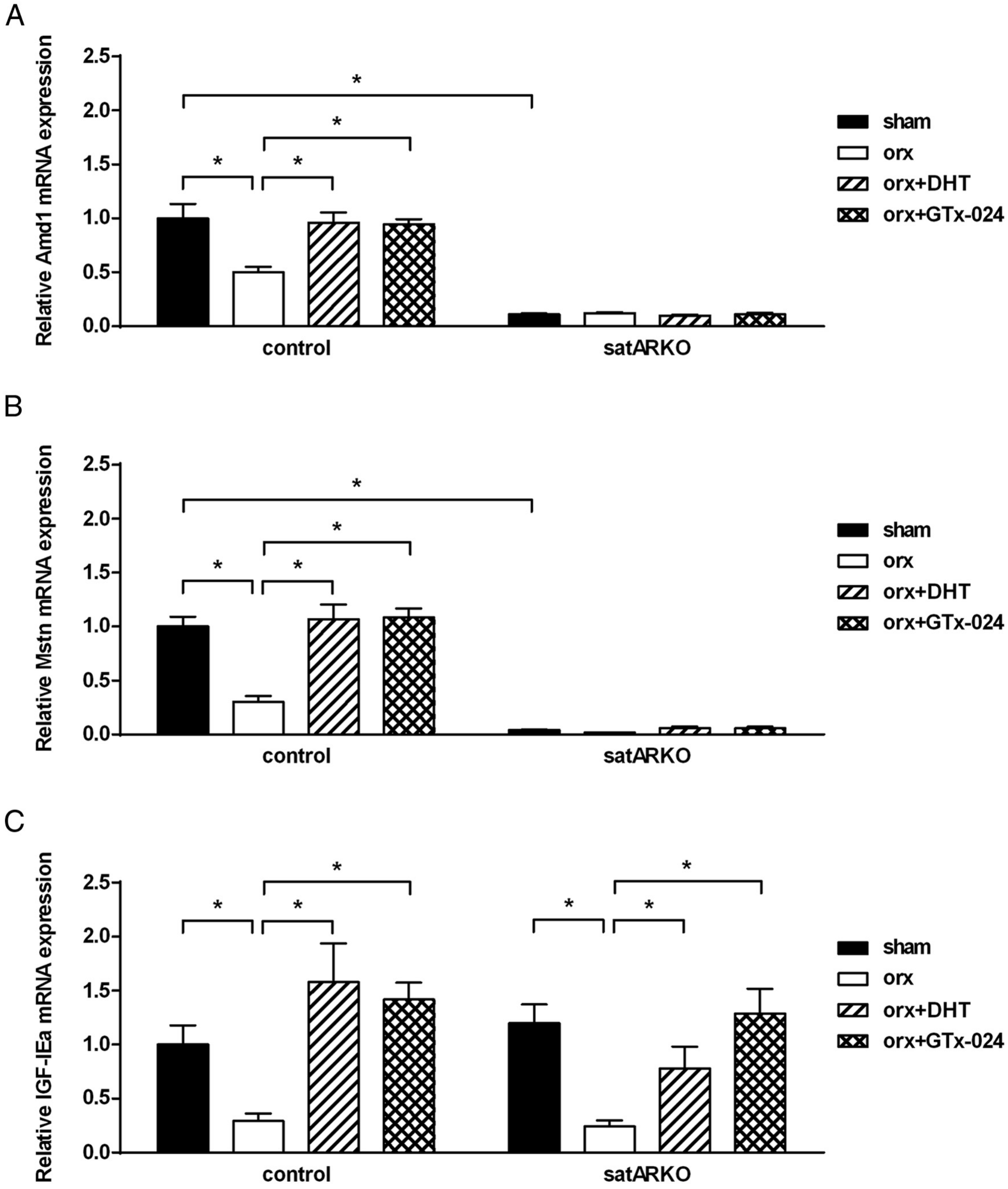

Figure 3. Amd1, Mstn, and IGF-IEa mRNA expression in levator ani muscle. Quantitative real-time PCR analysis of S-adenosylmethionine decarboxylase (Amd1) (A), myostatin (Mstn) (B), and insulin-like growth factor IEa (IGF-IEa) (C) mRNA in levator ani muscle of 14-week-old control and satARKO mice that were sham-operated (sham), orchidectomized (orx) or orchidectomized and treated with dihydrotestosterone (orx+DHT) or GTx-024 (orx+GTx-024) at 12 weeks of age (n = 7–8). Error bars indicate SEM; $*P < .05$.

nuclei coexpressing not only the muscle marker myogenin (**Figure 4A**) but also the fibroblast marker vim (**Figure 4B**). The stainings revealed no coexpression of AR and CD31 (**Figure 4C**). In satARKO LA muscle, the overall number of AR+ cells was considerably reduced (quantification in **Figure 6A**), in line with the strongly reduced mRNA levels reported previously in (15). Still, we observed many remaining AR+ nuclei, which appeared to be located almost entirely outside of muscle fibers (white arrows in **Figure 4A**). The nonsatellite cell nature of these remaining AR+ nuclei was confirmed by their localization outside of the basal lamina (**Figure 5A**) as well as the absence of Pax7 coexpression (**Figure 5B**). Most of the AR+ nuclei in satARKO LA were identified as fibroblasts, as determined by positive staining to vim (**Figure 4B**).

A more detailed examination of the latter double staining revealed that, while control LA muscle consists mostly of AR+ vim- cells (white arrows in upper panel of **Figure 6B**) with some cells being AR+ vim+ (red arrows in upper panel of **Figure 6B**), the vast majority of AR+ cells in satARKO LA muscle also express vim (red arrows in lower panel of **Figure 6B**). Quantification of AR+ and vim+ cells is shown in **Figure 6C**. These findings were confirmed in GASTR muscle (**Supplemental Figure 6**). Altogether, the intranuclear presence of AR suggests that fibroblasts could be involved in the indirect anabolic effect of androgens on skeletal muscle.

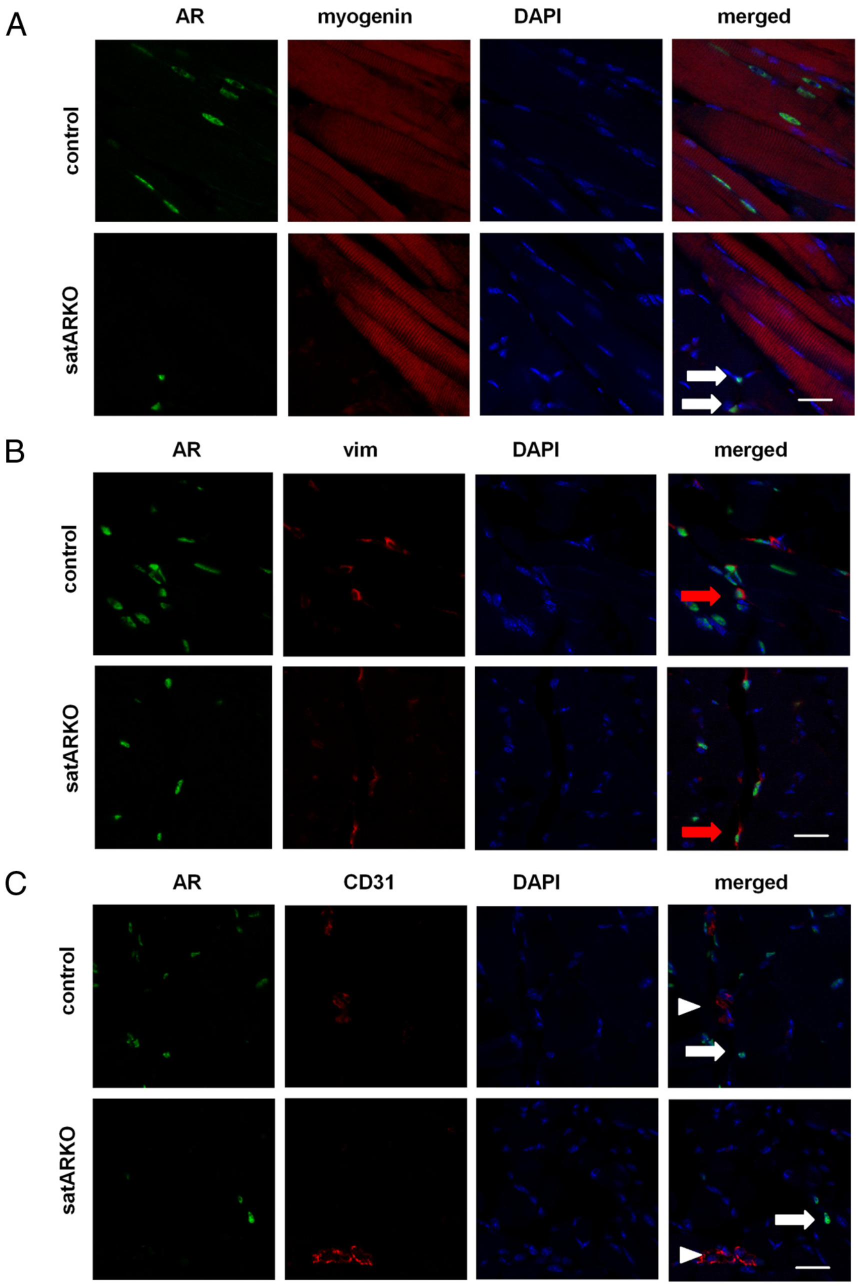

Figure 4. Microscopy of cell types identified by double staining for AR and cell type-specific markers. A, Levator ani (LA) muscle sections from 8-week-old control and satARKO mice were stained for AR (green), myogenin (red), and DAPI (blue). Myogenin is a marker for muscle cells. The remaining AR+ nuclei in satARKO LA muscle appear to be located outside of the muscle fibers (white arrows). B, LA muscle sections from 8-week-old control and satARKO mice were stained for AR (green), vimentin (red), and DAPI (blue). Vimentin (vim) is a marker for fibroblasts. Positive staining for vim was detected in AR+ cells (red arrows). C, LA muscle sections from 8-week-old control and satARKO mice were stained for AR (green), CD31 (red), and DAPI (blue). CD31 is a marker for endothelial cells. CD31 staining was detected around blood vessels (white arrowheads) and did not colocalize with AR+ cells (white arrows). Scale bar 20 μm.

Discussion

Muscle wasting is a hallmark of aging (27) and also occurs in various chronic disorders such as cancer (27, 28). Androgens could potentially be exploited in these patient groups, as they increase both muscle mass and strength (5, 6). Their anabolic action is thought to be mediated by a dual mechanism, ie, direct activation of muscle AR as well as indirect action through nonmuscle AR pathways (1). However, as androgen treatment is associated with potential cardiovascular and prostate cancer risks, SARMs were developed as an alternative strategy to counteract muscle atrophy (7). GTx-024 or enobosarm (also known as Ostarine and S-22) is clinically well-characterized, and has consistently demonstrated increases in lean body mass across various populations (29). The molecular mechanisms behind its tissue-selectivity and anabolic action on muscle itself however remain elusive. The aim of the present study was therefore to investigate whether the anabolic effect of GTx-024 is mediated via AR in muscle cells. To this end, we performed a castration and drug replacement experiment in mice selectively lacking the AR in satellite cells and hence in myoblasts and myocytes (satARKO) (15), and compared the effects of GTx-024 with those of DHT.

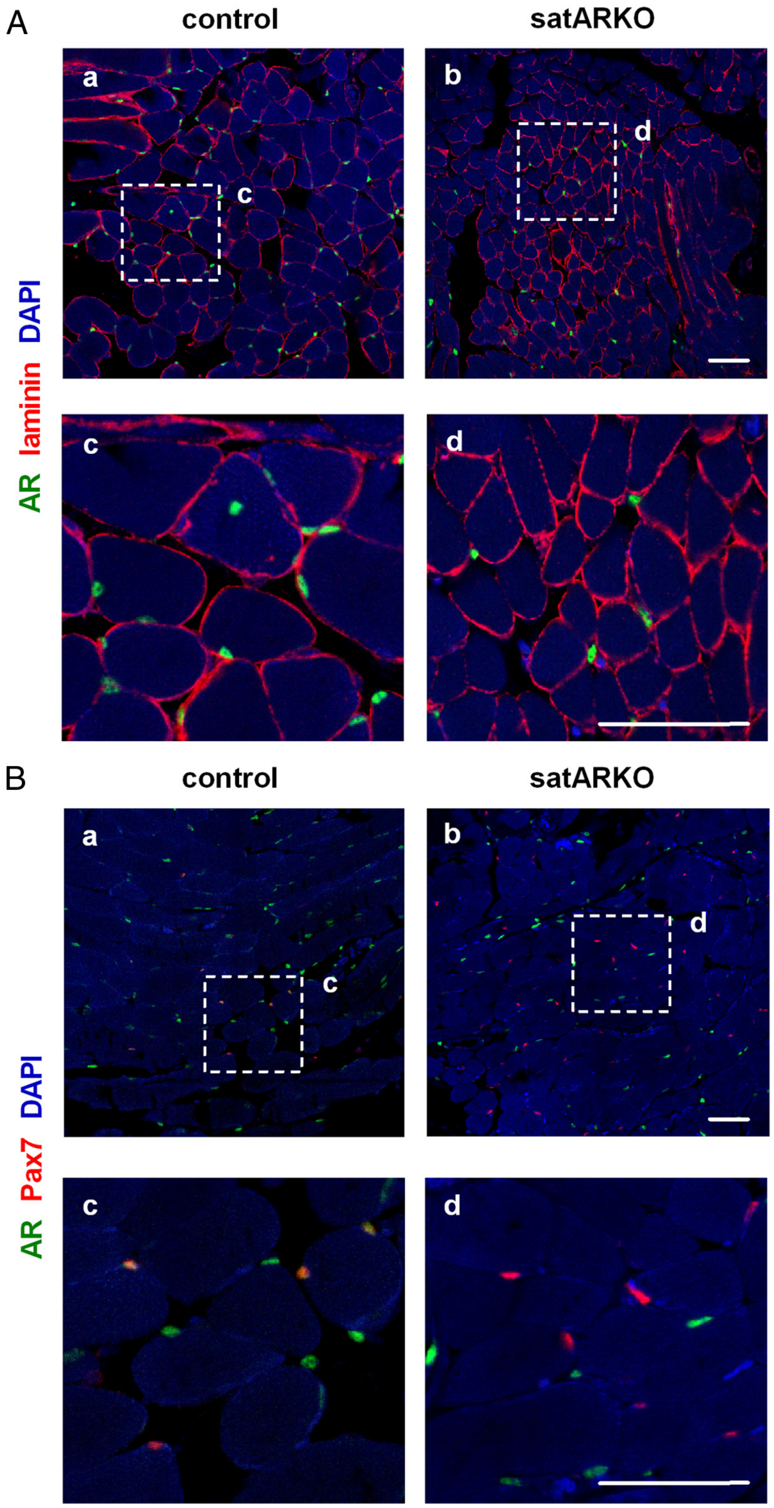

Figure 5. The remaining AR+ nuclei in satARKO muscle are not satellite cells. A, Levator ani (LA) muscle sections from 8-week-old control and satARKO mice were stained for AR (green), laminin (red), and DAPI (blue). Laminin is a structural component of the basal lamina. B, LA muscle sections from 8-week-old control and satARKO mice were stained for AR (green), Pax7 (red), and DAPI (blue). Pax7 is a marker for satellite cells. The nonsatellite cell nature of the remaining AR+ nuclei in satARKO muscle is confirmed by their localization outside of the basal lamina as well as the absence of Pax7 coexpression. Scale bar 50 μm.

Skeletal muscles differ markedly in their responsiveness to androgens. For example, the perineal skeletal muscles LA and BC are highly androgen responsive and depend on androgens for their normal maintenance and function, whereas the limb skeletal muscle EDL is relatively unresponsive to androgens and does not depend on androgens to maintain fiber size (30). In accordance, selective AR ablation in muscle reduces BC/LA but not limb muscle mass (11, 15). Immunohistochemical staining of muscle sections revealed that the BC/LA complex contains much more AR protein than do less responsive muscles like GASTR or EDL (13, 31), a finding that was confirmed in this study. Thus, differences in AR protein content of skeletal muscles seem to underlie differences in androgen responsiveness. This difference in androgen sensitivity is illustrated by the fact that in this study two weeks of treatment with DHT or GTx-024 were sufficient to increase LA mass in orx animals but not to alter lean body mass, in contrast with the increase in lean mass observed upon eight weeks of treatment with DHT or the structurally related GTx-007 compound (32). Due to its high androgen responsiveness, the LA muscle is widely accepted as read-out for androgen anabolic action in preclinical and pharmacological studies including in SARM development (21). For this reason, we decided to focus on LA muscle to study the mechanisms of GTx-024 action.

A direct role for muscle AR in mediating androgen anabolic action was demonstrated by various mouse models in which the AR was specifically ablated in progenitor (15) or mature (11, 12) muscle cells. These three models all showed a muscle phenotype, albeit generally mild. To determine the implication of muscle AR in mediating GTx-024 action, we assessed the expression of two muscle genes, Amd1 and Mstn, in response to GTx-024 treatment. Amd1 is a key enzyme in the synthesis pathway of polyamines, which are increased in rodent models of muscle hypertrophy (33). Mstn is a strong negative regulator of muscle growth, since disruption of the Mstn gene induces a dramatic increase in muscle mass (34). These genes are androgen-regulated in skeletal muscle, as Amd1 (22) and Mstn (35) mRNA levels were decreased in orx mice and restored by testosterone administration. Recently, we identified muscle AR as a mediator of this androgen regulation. Indeed, Mstn as well as Amd1 transcript levels were reduced in satARKO muscle (15). GTx-024 upregulated mRNA levels of both genes in LA muscle of castrated control mice, indicating a role for muscle AR in GTx-024 action.

None of the muscle-specific ARKO models fully reproduces the muscle phenotype of the global ARKO (22, 36), suggesting that muscle cells are not the sole target for androgen action in muscle. In accordance, cell-specific overexpression of wild type AR in skeletal muscle of testicular feminized (Tfm) rats is not sufficient to rescue BC/LA mass (37). Additional evidence for indirect androgen action on muscle is provided by the observation that orx further decreases BC/LA mass in satARKO mice, an effect that is fully reversed by DHT supplementation (15). Thus, the satARKO model could be further exploited to demonstrate a tissue-selective mechanism via muscle AR. However, while regulation of the muscle-specific androgen target genes Amd1 and Mstn was completely ablated in satARKO, GTx-024 still reversed the orx effect on BC/LA mass as efficiently as DHT in vivo. Hence, our data suggest that GTx-024 action is both direct via muscle AR and indirect via nonmuscle AR pathways, just as for other androgens.

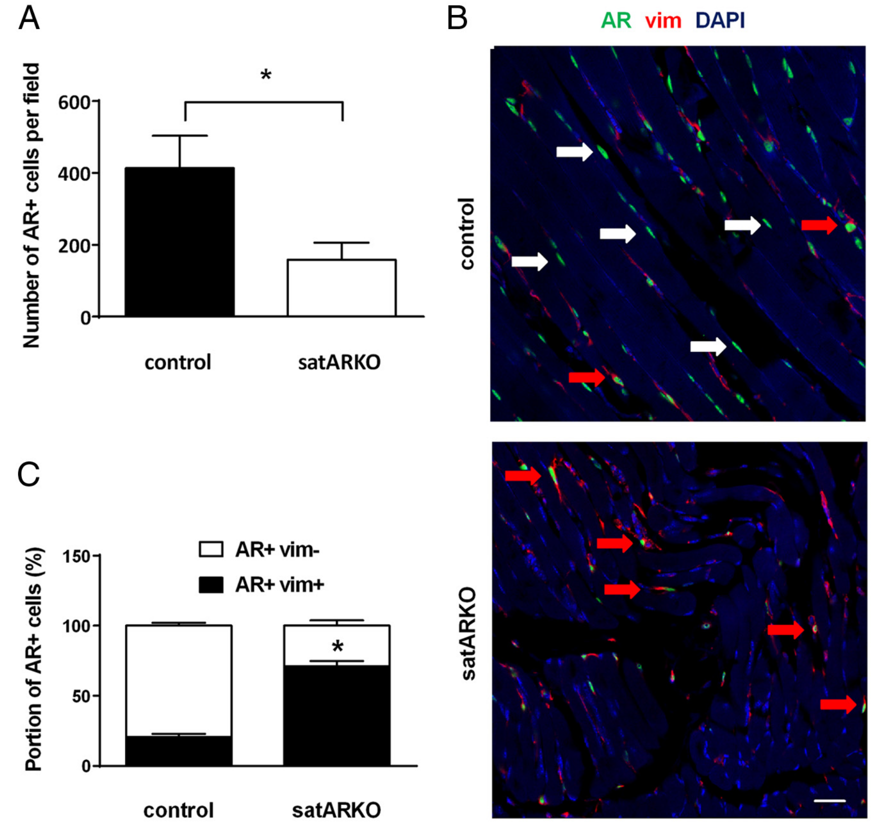

Figure 6. Fibroblasts may be involved in indirect androgen action on muscle. A, Number of AR+ cells per field in levator ani (LA) muscle sections from 8-week-old control and satARKO mice (n = 3). B, LA muscle sections from 8-week-old control and satARKO mice were stained for AR (green), vimentin (red), and DAPI (blue). While control LA muscle consists mostly of AR+ vim- cells (white arrows in upper panel) with some cells being AR+ vim+ (red arrows in upper panel), the vast majority of AR+ cells in satARKO LA muscle also expresses vim (red arrows in lower panel). Scale bar 20 μm. C, Percentage of AR+ cells that stained negatively (white) or positively (black) for vim was calculated from LA muscle sections from 8-week-old control and satARKO mice (n = 3). Error bars indicate SEM; *$P < .05$.

A first potential pathway of indirect androgen action on muscle is by affecting the nonmuscular fraction of the tissue. Indeed, AR expression within the muscle is not restricted to satellite cells and myonuclei, but has also been described in endothelial cells and fibroblasts (13, 14). Therefore, in this study, LA muscle sections were double stained for the AR on the one hand and for CD31 or vim on the other hand (markers for endothelial cells and fibroblasts, respectively). In our conditions, CD31+ cells were scarce throughout the LA and did not show AR expression. However, resident fibroblasts within the LA express nuclear AR, thus suggesting that these cells could be involved in the androgen action on muscle. This hypothesis is further supported by the fact that in satARKO LA muscle, which shows androgen responsiveness in the absence of muscle AR, the vast majority of remaining AR+ cells also expresses vim. Also in GASTR muscle, vimentin is expressed in some AR+ cells from control mice as well as in most AR+ cells from satARKO animals. Thus, although our study focused on LA muscle, our findings can be extended to limb skeletal muscles.

Several studies support the involvement of muscle fibroblasts in the development, maintenance and regeneration of muscle structure and function. Mice devoid of Tcf4, which is abundantly expressed in muscle fibroblasts but not in myogenic cells, display an abnormal muscular morphology, with several muscles being aberrantly split along with an altered muscle fiber type (38). Similarly, ablation of Tcf4 during muscle regeneration leads to premature satellite cell differentiation and smaller myofibers (39). The exact mechanisms underlying the paracrine control of myoblast proliferation and differentiation by fibroblasts are unclear. Early studies have shown that in cultures composed of both myogenic and fibroblast-like cells, myoblasts exhibit a prolonged proliferative phase resulting in delayed but increased production of fused myotubes. Subsequent experiments with conditioned media indicated that the fibroblast-like cells produce soluble myogenic growth factors, but failed to determine their identity (40). Recently, FGF-2 was proposed as a possible mediator, since neutralizing antibodies to FGF-2 were able to block the fibroblast effects on myotubes. However, inhibition due to FGF-2 was only noticed in coculture experiments allowing direct cell-cell contact between both cell types and not in conditioned media experiments (41). Thus, the exact nature and identity of the fibroblast-derived paracrine factors stimulating myogenesis remain presently unknown.

Although androgen effects on fibroblasts have been intensively studied in prostate cancer (42), androgen action on muscle fibroblasts remains to be investigated. Recently, a mouse model was generated in which the AR was ablated in mesenchymal cells (43). In these mutant mice, the BC/LA muscle complex failed to develop. Moreover, as the number of proliferating undifferentiated myoblasts was reduced, the authors suggested that the mesenchymal AR may regulate the proliferation of muscle myoblasts in a paracrine way (43). Since muscle fibroblasts are from mesenchymal origin, this study supports the involvement of fibroblasts in indirect androgen action on muscle. Additional evidence is provided by in vitro experiments, in which androgen treatment of the C3H 10T1/2 pluripotent mesenchymal cell line upregulated myogenic differentiation markers (44). To confirm however that the indirect pathway, potentially via muscle-resident fibroblasts, affects adult muscle homeostasis, studies using an inducible Cre-LoxP model targeting these fibroblasts (with minimal changes in other organs) would be required.

Transcript levels of IGF-IEa were not different between satARKO and control muscle but were decreased upon castration in both genotypes, indicating indirect androgen regulation of IGF-IEa via nonmuscle AR. DHT as well as GTx-024 rescued the orx-mediated decrease in IGF-IEa mRNA, suggesting that local IGF-I signaling may be involved in the indirect nonmuscle AR contribution to muscle mass by androgens and GTx-024. There were no differences in serum IGF-I levels. There is increasing evidence that, in contrast to circulating IGF-I (45), locally produced IGF-I is an important mediator of androgen action in muscle. Indeed, androgen treatment increases and orchidectomy decreases IGF-I mRNA in muscle (12, 25). However, as both fibroblasts (46) and muscle cells (47) produce IGF-I, further studies are needed to determine whether, in addition to a direct effect on IGF-I production by muscle cells (26), androgens stimulate IGF-I production by fibroblasts with subsequent proliferative effects on neighboring muscle cells, or alternatively, enhance the secretion of myogenic factors by fibroblasts which will lead to increased IGF-I production by the muscle cells themselves.

Although in this study we were interested in the local effects of androgens on muscle, it must be taken into account that androgens might also promote muscle function by acting on other organs or systems (1, 48). A limited number of studies have assessed this possibility, focusing mainly on the effects of androgens on muscle innervation and, in particular, on the highly androgen-sensitive spinal motor neurons supplying the LA (49, 50). Although these studies suggest that the neuronal AR is not crucial for maintaining LA mass, this has not been confirmed yet with a neuron-specific AR knockout model.

In summary, the mechanism of action of GTx-024 on skeletal muscle is partly direct via muscle AR activation. In addition, part of the anabolic effect seems to be indirect via nonmuscle AR pathways. Muscle fibroblasts may play a role in these indirect pathways, although additional studies are needed to clarify the exact molecular mechanisms of this indirect activity. Moreover, further investigation is required to confirm whether other SARMs exist or can be designed which have a more muscle-specific action.

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Address all correspondence and requests for reprints to: Frank Claessens, Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, KU Leuven, Campus Gasthuisberg O&N1 PO Box 901, Herestraat 49, 3000 Leuven, Belgium. Phone: +32 16 330 253. Fax: +32 16 345 995. E-mail: frank.claessens@med.kuleuven.be.

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