Androgen-mediated improvement of body composition and muscle function involves a novel early transcriptional program including IGF1, mechano growth factor, and induction of β -catenin

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Abstract

Androgens promote anabolism in the musculoskeletal system while generally repressing adiposity, leading to lean body composition. Circulating androgens decline with age, contributing to frailty, osteoporosis, and obesity; however, the mechanisms by which androgens modulate body composition are largely unknown. Here, we demonstrate that aged castrated rats develop increased fat mass, reduced muscle mass and strength, and lower bone mass. Treatment with testosterone or 5 α -dihydrotestosterone (DHT) reverses the effects on muscle and adipose tissues while only aromatizable testosterone increased bone mass. During the first week, DHT transiently increased soleus muscle nuclear density and induced expression of IGF1 and its splice variant mechano growth factor (MGF) without early regulation of the myogenic factors MyoD, myogenin, monocyte nuclear factor, or myostatin. A genome-wide microarray screen was also performed to identify potential pro-myogenic genes that respond to androgen receptor activation *in vivo* within 24 h. Of 24 000 genes examined, 70 candidate genes were identified whose functions suggest initiation of remodeling and regeneration, including the type II muscle genes for myosin heavy chain type II and parvalbumin and the chemokine monocyte chemoattractant protein-1. Interestingly, *Axin* and *Axin2*, negative regulators of β -catenin, were repressed, indicating modulation of the β -catenin pathway. DHT increased total levels of β -catenin protein, which accumulated in nuclei *in vivo*. Likewise, treatment of C2C12 myoblasts with both IGF1Ea and MGF C-terminal peptide increased nuclear β -catenin *in vitro*. Thus, we propose that androgenic anabolism involves early downregulation of *Axin* and induction of IGF1, leading to nuclear accumulation of β -catenin, a pro-myogenic, anti-adipogenic stem cell regulatory factor.

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Introduction

Androgens are important endocrine regulators of male sexual development and maintenance of muscle, bone, adipose tissue, and body composition (Vermeulen 1998, Vermeulen *et al.* 1999). Testosterone, the major circulating androgen, can act directly or be converted to the more potent androgen 5 α -dihydrotestosterone (DHT) by 5 α -reductase, or to estrogens by aromatase (Russell & Wilson 1994, Simpson *et al.* 1994). Both testosterone and DHT activate the androgen receptor (AR), a nuclear receptor that functions as a transcription factor in hormone-sensitive cells (Chang *et al.* 1995, Heinlein & Chang 2002). After reaching peak levels in early adulthood, androgen levels decline with age in both sexes (Tenover 1994, Lamberts *et al.* 1997).

This loss of endogenous androgens parallels several symptoms of aging, including decreased muscle mass and function (Snyder *et al.* 1999), increased visceral fat (Katznelson *et al.* 1998) and bone loss (Katznelson *et al.* 1996). Treatment with testosterone improves muscle mass and strength, bone density, and reduces visceral fat in a variety of subjects (Bardin 1996, Katznelson *et al.* 1996, Bhasin *et al.* 1997, 2000, Swerdloff & Wang 2003). Thus, restoring androgens to youthful levels could potentially be used to manage sarcopenia, osteoporosis, visceral obesity, and frailty.

The mechanisms by which androgens promote anabolism in adult animals are largely unknown. AR is detectable in bone and muscle cells, but levels are low compared with reproductive tissues such as the prostate and levator ani muscle (Antonio *et al.* 1999,

Monks *et al.* 2004). Nevertheless, castration in rats reduces contractile force in muscles of the hindlimb (Brown *et al.* 2001) and produces ultrastructural signs of degeneration and reduced protein synthesis (Ustunel *et al.* 2003). Treatment with anti-androgens limits gains in muscle strength during weight training (Ruzic *et al.* 2003). Conversely, repletion with testosterone increases lean mass and muscle strength, improves nitrogen balance, and induces myofiber hypertrophy (Sinha-Hikim *et al.* 2002). Thus, testosterone regulates both the mass and function of skeletal muscle in adults.

At the cellular level, the effects of testosterone on body composition might involve recruitment or activation of resident myogenic precursor cells to existing myofibers (Chen *et al.* 2005b). Satellite cells cultured from porcine muscle express AR, and AR agonists delay their differentiation (Doumit *et al.* 1996). Furthermore, in muscle biopsies from men that exhibited gains in myofiber volume and strength following 20 weeks of testosterone treatment, satellite cell number was increased (Sinha-Hikim *et al.* 2003). Other studies report changes in the local expression of insulin-like growth factor 1 (IGF1) in muscle samples from patients receiving anabolic androgens (Sheffield-Moore 2000, Ferrando *et al.* 2002). As IGF1 and its related splice variants stimulate satellite cell proliferation and promote muscle hypertrophy (Musaro *et al.* 2001, Hill & Goldspink 2003, Goldspink & Yang 2004), these data suggest that androgens regulate muscle mass by this mechanism. Some *in vitro* data support the concept that androgens act directly on satellite or other precursor cells. Murine C2C12 cells, which resemble myogenic precursors, do not express AR, but when AR expression is produced by transfection, AR-selective ligands increase myogenin expression and accelerate myoblast differentiation and fusion (Lee 2002, Vlahopoulos *et al.* 2005). Mouse C3H10T1/2 fibroblast cells express endogenous AR, and DHT inhibits their differentiation into adipocytes and promotes the expression of MyoD and myosin heavy chain type II (MHC2; Compston 2001, Singh *et al.* 2003). In addition to satellite cells, AR is expressed in mature myofibers (Saartok *et al.* 1984, Sar *et al.* 1990) in several types of motoneurons (Lumbroso *et al.* 1996, Piccioni *et al.* 2001), and in intramuscular fibroblasts (Monks *et al.* 2004), and thus could influence growth and function through activation in these cells. Finally, androgens regulate systemic levels of IGF1, GH, and thyroid hormone, and may oppose the actions of glucocorticoids, any of which could contribute to the effects of androgen on muscle (Link *et al.* 1986, Hickson *et al.* 1990, Banu *et al.* 2002, Ferrando *et al.* 2002).

It has been proposed that testosterone could promote the differentiation of mesenchymal multipotent cells into the myogenic lineage while inhibiting adipogenic differentiation by modulating nuclear translocation of β -catenin (Bhasin *et al.* 2006).

β -Catenin, through its action in cell adhesion and Wnt signal transduction, plays a critical role in embryonic development and regulation of adult stem cell populations in various tissues (Clevers 2006). *In vitro*, β -catenin is both necessary and sufficient for myogenesis and inhibits adipogenesis (Ross *et al.* 2000, Petropoulos & Skerjanc 2002). Moreover, β -catenin is essential for adult skeletal muscle growth and regeneration *in vivo* (Polesskaya *et al.* 2003, Reya & Clevers 2005, Armstrong *et al.* 2006), and myonuclear β -catenin is up-regulated during overload-induced muscle hypertrophy in adults (Armstrong & Esser 2005). Muscle regrowth following atrophy is associated with down-regulation of glycogen synthase kinase-3 β (GSK3 β), a negative regulator of β -catenin (van der Velden *et al.* 2007, Schakman *et al.* 2008). Finally, β -catenin promotes self-renewal of satellite stem cells (Perez-Ruiz *et al.* 2008). Together, these findings suggest that Wnt/ β -catenin signaling plays an active role in the maintenance of body composition in adults. However, the role for β -catenin in androgen-mediated muscle growth has not been studied.

An important step towards understanding androgenic signaling in regulating body composition will be to define the processes governed by AR and the cell type(s) in which AR exerts its anabolic effect. Since many studies use testosterone, the relative contributions of the AR and estrogen receptors are ambiguous, and some report that estrogen is an essential component of androgenic anabolism (Bilezikian *et al.* 1998, Vandenput *et al.* 2002). Furthermore, the genes targeted by AR in muscle, fat, and bone are unknown. To address these issues, we validated the castrated rat model and characterized the effects of DHT in the soleus muscle and identified genes that respond to DHT within the first week of treatment and are potential downstream effectors of anabolic action.

Materials and methods

Animal studies and analysis of body composition

All animal studies described in this report were approved by the Institutional Animal Care and Use Committee. Sprague-Dawley rats (Taconic, Hudson, NY, USA) were purchased following orchidectomy (ORX) or sham orchidectomy (SHAM) at 10 weeks of age and maintained for 11 weeks post-surgery with *ad libitum* access to food and water. Animals aged 21 weeks were then analyzed by dual photon emission X-ray absorptiometry (DEXA) to quantify lean, fat, and bone mass using a Hologic 4500A instrument at baseline and at indicated times during treatment by s.c. injection with 3 mg/kg per day DHT, 10 mg/kg per day testosterone, or vehicle (0.4 ml propylene glycol; $n=9$

per group). In a second time-course experiment, 11-week-post-ORX rats were treated with DHT or vehicle as above for 4, 7, 14, and 21 days ($n=10$ per group) with a vehicle group matched with each time point, and then the soleus muscles were collected for RNA, DNA, protein extraction, or formalin fixed for histological examination. Finally, a shorter experiment was conducted with older ORX rats aged 6 months. They were treated with vehicle or DHT for 1, 4, and 7 days ($n=4$ per group) to focus on earlier gene expression changes and confirm previous quantitative real-time PCR (qRT-PCR) results.

Contractile measurements

Animals were sedated with 3:1 ketamine:xylazine, shaved at the left hind limb, and placed on a 39 °C heating pad. The sciatic nerve was placed over a platinum bipolar electrode connected to an Astro-Med S48 stimulator. Then the soleus was surgically isolated at its insertion point, and the tendon was severed and sutured to a T10 force transducer housed in a micromanipulator and connected to a P220 amplifier using 2·0 silk. The Achilles tendon was severed to minimize the influence of contraction by the gastrocnemius. The sciatic nerve and soleus were bathed in 37 °C mineral oil and Rat Ringer's respectively. A force–tension curve was obtained by stimulating the sciatic nerve at supramaximal voltage (1·2 V) for 0·5 ms while stretching the muscle across 1 mm increments. Once the stretch distance that produced maximal twitch strength was identified, the sciatic nerve was stimulated with 1·2 V, 100 Hz, 400 ms, and square pulse waves to obtain peak tetanic tension. The data were collected and analyzed using Astro-Med software (Warwick, RI, USA). The soleus was dissected and weighed after recording. Peak tetonic tension was then normalized per unit mass as previously described (Brown *et al.* 2001, Brown & Taylor 2005).

Histomorphometry

Soleus muscles were fixed in formalin and embedded in paraffin, and multiple serial cross-sections were produced. Hemotoxylin- and eosin-stained muscle sections were examined and photographed using a Nikon Eclipse E1000M microscope/digital camera system. The number of nuclei was counted in an area covering 10–20 fibers three times per muscle section ($n=10$ sections/group). The number of fibers in that area was counted, and the nuclei per fiber and μm^2 of area were calculated using Bioquant software (San Diego, CA, USA). Differences in fiber size or nuclear number were tested by statistical *t*-test. Nuclei labeled by hemotoxylin or by anti- β -catenin antibodies

by immunohistochemistry were counted per muscle cross-sectional area of soleus muscles. Sections were visualized and photographed using a Nikon Eclipse E1000M microscope/digital camera system.

Immunohistochemistry

Immunostaining for β -catenin was performed on formalin-fixed, paraffin-embedded tissue sections of rat soleus muscles ($n=10$ rats/sections per group). Rat duodenum sections were used as a positive control. Sections were dewaxed and rehydrated. Mouse monoclonal anti- β -catenin antibodies (Sigma, clone 6F9, 1:500) were detected using biotinylated anti-mouse IgG secondary antibody and avidin-conjugated HRP system (Vectastain ABC, Invitrogen), and stained using nickel diaminobenzidine (DAB). For counting the proportion of β -catenin-stained nuclei, sections were immunostained by DAB (without nickel and therefore brown) and counterstained with hemotoxylin (blue). The sample sections were counterstained with eosin and visualized using a Nikon Eclipse E1000M microscope/digital camera system. The ratio of DAB stained to hemotoxylin-stained nuclei was reported as percent β -catenin-positive nuclei.

C2C12 myoblast cell culture

Mouse C2C12 myoblasts (American Type Culture Collection, ATCC, Rockford, MD, USA) were maintained in DMEM with 1 g/l D-glucose, sodium pyruvate (110 mg/l), fetal bovine serum 10%, L-glutamine (2 mM), and penicillin–streptomycin 1% (all Gibco, Invitrogen) at 37 °C in a humidified atmosphere of 5% CO₂. Cells were passed at 60–70% confluence and tested in OptiMem (Invitrogen) media at 100% confluence.

Immunocytochemistry

Confluent C2C12 myoblasts were treated with recombinant mouse IGF1 (Sigma) at 10 and 30 ng/ml, or the C-terminal peptide 1–24 of mechano growth factor (ctMGF, Phoenix Pharmaceuticals, Burlingame, CA, USA) at 30 and 60 ng/ml for 25 min, fixed with 4% paraformaldehyde in PBS, permeabilized with 0·5% Triton X-100 in PBS, and blocked with 5% normal goat serum. The fixed cells were immunostained for myonuclear β -catenin using primary mouse monoclonal anti- β -catenin antibodies (clone 6F9, Sigma) 1:50, goat anti-mouse Alexa Fluor 488 secondary antibodies (Invitrogen) 1:100, counterstained with the fluorochrome 4',6-di-amidino-phenyl-indole (DAPI) nuclear stain, and photographed using a Nikon Eclipse E1000M

microscope camera system. Photos were processed for false color for DAPI nuclear staining and merged using Adobe Photoshop 7.0.

Western immunoblot

Whole soleus muscle lysates were prepared in lysis buffer (2% SDS, 62.5 mM Tris, pH 6.8, 10% glycerol, 1% 2-mercaptoethanol, 1× complete protease inhibitors (Roche), and phosphatase inhibitor cocktails (Sigma)) and assayed for total protein using the BCA method (Pierce Biotechnology, Rockford, IL, USA). About 40 µg of protein were subjected to SDS-PAGE using 12.5% polyacrylamide gels (Pierce Biotechnology), transferred to nitrocellulose, and detected using mouse monoclonal IgG1 anti-β-catenin (clone 6F9, Sigma), polyclonal rabbit anti-phospho-β-catenin Ser33/37 Thr41 IgG1 1:400 (#9561 Cell Signaling, Danvers, MA, USA), rabbit monoclonal anti-GSK3β IgG1 1:400 (#9315 Cell Signaling), rabbit polyclonal anti-phospho-GSK3β (Ser9) IgG1 1:400 (#9336 Cell Signaling), mouse monoclonal anti-axin (H98) IgG1 1:400, rabbit polyclonal anti-axin2/conductin IgG1 1:200, polyclonal mouse anti-β-tubulin (D10) IgG1 1:200, mouse anti-actin 1:400, and mouse anti-lamin IgG1 1:200 primary antibodies (all Santa Cruz Biotechnology, Palo Alto, CA, USA), followed by HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology) 1:17 000 and processed for enhanced chemiluminescence (Pierce Biotechnology). Bands were visualized using Biomax MR Kodak film. Nuclear protein from C2C12 myoblasts was prepared using the nuclear protein isolation kit (NE-PER, Pierce Biotechnology) according to the manufacturer's instructions. Control and ctMGF or IGF1-treated C2C12 cells were homogenized in 750 µl cytoplasmic extraction reagent and centrifuged at 16 000 *g* for 5 min. The supernatant, consisting of cytosolic protein, was collected and the remaining pellet was treated with 150 µl nuclear extraction reagent. The samples were then centrifuged at 16 000 *g* for 10 min, and the supernatant was collected for nuclear proteins. Protein concentrations were determined using BCA method with a NanoDrop ND-1000 spectrophotometer. Band optical density measurements were generated by a Bio-Rad GS-800 calibrated densitometer (Bio-Rad Labs, Hercules, CA, USA), and the data were analyzed by AlphaEase software.

TOPFLASH β-catenin plasmid reporter transfection and assay

To measure β-catenin nuclear translocation, the TCF/LEF β-catenin TOPFLASH firefly and control *Renilla* luciferase reporter plasmids (Milipore, Bedford, MA, USA) were cotransfected into suspended C2C12

myoblasts using 1 µg DNA with Lipofectamine 2000 (Invitrogen), and plated into 96-well plates at 10 000 cells per well. After 24 h, the growth media were replaced with serum-free media, and the confluent myoblasts were treated with water vehicle, IGF1, or ctMGF as above. After 16 h, both firefly and *Renilla* luciferase activity were measured using a commercially available luciferase assay system kit (Dual-Glo, Promega) and quantified using an EnVision luminescence detector (Perkin Elmer, Shelton, CT, USA). All values were first normalized to control *Renilla* luciferase activity, and IGF1 and ctMGF values normalized to vehicle-treated wells.

Quantitative RT-PCR

Total RNA was prepared from soleus muscle using TRIzol (Gibco) following the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed using the Perkin Elmer Taqman 7700 (Perkin-Elmer) with gene-specific primers and fluorescence-labeled probes (5'-reporter dye, 6-carboxyfluorescein (FAM); 3'-quencher dye, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA)), which were designed using Primer Express (version 1.5) software and synthesized by Applied Biosystems (Foster City, CA, USA). The primer and probe sequences are listed in Table 1. *Mgf* and *Igf1ea* were detected by SYBR Green (Stratagene, Cedar Creek, TX, USA) as described elsewhere (Hill & Goldspink 2003). Three aliquots of RNA from each sample underwent three independent reverse transcription reactions, resulting in nine measurements. From these measurements, a mean and s.d. of measurement were derived, and both vehicle- and DHT-treated samples were normalized to glucuronidase expression. DHT values were compared with time-matched vehicle values and analyzed by *t*-test. Values with *P* values < 0.05 were considered significantly different. Data were confirmed in multiple independent experiments.

Microarrays

Total RNA was collected from soleus (Trizol, Gibco) from rats treated with vehicle or DHT (3 mg/kg per day, *n*=6 per group) from two separate experiments and treated with DNase I (as directed by the manufacturer, Genhunter Nashville, TN, USA), and purified using Qiagen RNeasy (Qiagen) columns. Complete details of the microarray protocol are available (van't Veer *et al.* 2002). Briefly, RNA samples were labeled in an *in vitro* transcription reaction with the fluorescent dyes Cy3 and Cy5. RNA from vehicle-treated samples labeled with Cy3 was then mixed with RNA from DHT samples labeled with Cy5 and competitively hybridized to 25 000 feature rat oligonucleotide arrays (Agilent Technologies, Palo Alto, CA, USA).

Table 1 Gene primers and probe sets

Gene	Accession number	Primer/probe sequence
<i>Axin2</i>	NM_024355	Forward 5'-GGAGCACCGGTCCAACAC-3' Reverse 5'-TGGTTTGTGGGTCTCTTCATA-3' Probe 5'-CCCTGGCCCTCTTACCCTCTGGC-3'
<i>Ctnnb1</i> (β -catenin)	NM_053357	Forward 5'-GCCACAGCTCCCCTGACA-3' Reverse 5'-ATATGTTGCCACGCCTTCATT-3' Probe 5'-AGTTGCTCCACTCCAG-3'
<i>Ccl2</i> (<i>Mcp1</i>)	NM_031530	Forward 5'-CCAATGAGTCGGCTGGAGAA-3' Reverse 5'-GAGCTTGGTGACAAATACTACAGCTT-3' Probe 5'-AATCACCAGCAGCAGGTGTCCCAA-3'
<i>Ccl7</i> (<i>Mcp3</i>)	NM_001007612	Forward 5'-GCCGCGCTTCTGTGTGT-3' Reverse 5'-TGGATGAATTGGTCCCCTCTG-3' Probe 5'-CTGCTCACAGCTGCTGCTTTCACCG-3'
<i>Egr1</i>	NM_012551	Forward 5'-CCATGA ACGCCCGTATGC-3' Reverse 5'-CATGCAGATTCGACACTGGAA-3' Probe 5'-TCGCCGCTTTTCTCGCTCGGAT-3'
<i>Gusb</i>	NM_017015	Forward 5'-GGAGTCGGGCCCAACCT-3' Reverse 5'-GCTCTGCTTCTTGGGTGATGT-3' Probe 5'-VIC-ATGCCGGTCCCTTCCAGCTTCAA-3'
<i>Igf1Ea</i>	NM_001082478	Forward 5'-GCTTGCTCACCTTTACCAGC-3' Reverse 5'-AATGTACTTCCTTCTGGGTCT-3' Detected by SYBR Green method
<i>Mgf</i>	NM_001082478	Forward 5'-GCTTGCTCACCTTTACCAGC-3' Reverse 5'-AAATGTACTTCCTTCTCTCTC-3' Detected by SYBR Green method
<i>Mnf</i>	NM_010911	Forward 5'-TACCGCTTCGTCCAGAATGTG-3' Reverse 5'-GCCTTCGCGGCGAACT-3' Probe 5'-CCTCTGACCTTCAGCTGGCCGC-3'
<i>Myh4</i>	NM_019325	Forward 5'-GGAGCGGGCCGACATC-3' Reverse 5'-TTCGCTTATGACTTTGGTGTA-3' Probe 5'-CCGAGTCCCAGGTCAACAAGCTGC-3'
<i>MyoD</i>	NM_176079	Forward 5'-GCCCGGTCTGCACTCATG-3' Reverse 5'-GAGTGTCAATTAAGCTTCATTTTGG-3' Probe 5'-ATGGTGCCCTGGGTCCCTCATG-3'
<i>Mstn</i>	NM_019151	Forward 5'-TTGGATGAGAATGGGCATGAT-3' Reverse 5'-AAAAAGGGATTACGCCCATCTT-3' Probe 5'-TTGCTGTAACTTCCCAGGACCAGGA-3'
<i>Nr4a3</i> (<i>Nor-1</i>)	NM_031628	Forward 5'-TGAAGGAAGTTGTGCGTACAGATAG-3' Reverse 5'-TCATCATACAGATCGGAGGAGATG-3' Probe 5'-GTCTGCCTTCCAAACCAAGAGCC-3'
<i>Pvalb</i>	NM_022499	Forward 5'-GACACCACTCTTCTGGAAAATGC-3' Reverse 5'-GCCTGGGTCCCTCCCTACAG-3' Probe 5'-AAACAATAAAGGCTGTACCCATCGGACACC-3'

Samples were also labeled in reverse and hybridized to a second microarray. Extensive quality control and normalization measures assure the overall validity of the experiment and have been previously described (van't Veer *et al.* 2002). The two fluorescent measurements (Cy5 (red) and Cy3 (green)) provide two intensities for comparison. Cy5 and Cy3 ratio intensities were converted to fold change. *P* values for differences between hybridization signals were calculated using an error ratio model (Weng *et al.* 2006). Genes were selected by applying a filter using absolute fold change >1.5 and *P*<0.05 for both ratio experiments, with genes regulated the same direction both times, which yielded 70 genes.

Results

Effects of castration on body composition

To compare testosterone, which can be converted to estrogen, with DHT, which cannot be converted to estrogen, we examined both hormones' effects on body composition and muscle strength in castrated male rats (orchidectomized, ORX). Age- and weight-matched male rats aged 10 weeks underwent ORX or sham operation and were left untreated for 11 weeks. DEXA revealed that ORX significantly increased fat mass, and decreased lean body mass (LBM) and bone mineral content (BMC), a measure of the extent of mineralized skeleton (Fig. 1A). These data confirm previous

Figure 1 Effect of androgen depletion on lean body mass, fat mass, and bone mineral content (BMC). Graphed values are mean body composition values of the 21-week-old rats, 11 weeks after SHAM surgery or orchidectomy (ORX) as measured by DEXA ($\pm$ S.E.M.). (A) Mean pretreatment values for SHAM ($n=9$) versus all ORX animals ($n=27$). *Indicates $P<0.05$, t -test for SHAM versus all ORX rats. All ORX animals were randomized to three groups prior to treatment. Androgen induced changes in body composition. (B) Mean group DEXA measurements of change in lean mass, fat mass, and bone mineral content graphed as a function of time (error bar represents S.E.M.). Data are different from baseline measurements taken immediately before treatment. Δ , ORX + testosterone (10 mg/kg per day); $\square$, ORX + 12 DHT (3 mg/kg per day); $\blacksquare$, ORX + VEH; $\bullet$, SHAM + VEH. *Indicates different from ORX 13 ($P<0.05$, one-way ANOVA, Fisher's PLSD only shown at final time point). $N=9$ per group. (C) Soleus weight and strength after androgen treatment. All values are mean $\pm$ S.E.M., and asterisks indicate different from ORX ($P<0.05$, one-way ANOVA, Fisher's PLSD). Peak tetanic tension (Po) of the right soleus, soleus wet weight, and Po divided by soleus weight. Note Y-axis scale does not begin at the value 0 for visual clarity in (A) for BMC and (C).

observations showing that androgen depletion in rats (Vanderschueren *et al.* 2000) and in humans (Wang *et al.* 2000) negatively affects body composition, and establishes these animals as suitable models for investigating androgenic modulation of body composition.

Effects of androgen treatment on body composition

We then determined whether treatment with androgens influences body composition in ORX rats. ORX rats were randomized into groups ($n=9$) based on LBM and were then treated daily for 8 weeks with 3 mg/kg per day DHT or 10 mg/kg per day testosterone. The doses were selected based on pilot pharmacokinetic studies in ORX rats showing them as the minimal doses required to maintain prostate weights to that measured in SHAM rats while producing the maximal increase in periosteal bone formation rate. The average $C_{\max}$ and AUC for this dose were 1.8 ng/ml and 32 000 pg*h/ml respectively, which is near the physiologic range for

testosterone in rats (Oliva *et al.* 2006) and is similar to that used previously (Hanada *et al.* 2003, Gao *et al.* 2005). DEXA scans were performed at 4, 6, and 8 weeks to determine the effects on body composition. After 6 weeks of treatment, sham-operated rats, testosterone, and DHT showed similar increases in LBM (Fig. 1B). By 8 weeks, both testosterone and DHT fully restored LBM accretion rate to that observed in intact animals. Likewise, both testosterone and DHT were equivalent in their ability to inhibit increases in fat mass, beginning 4 weeks after treatment (Fig. 1B). In contrast, only testosterone treatment increased BMC compared with control animals, and only after the full 8 weeks of treatment (Fig. 1B). Seminal vesicle and prostate weights were measured and show that both testosterone and DHT were fully effective in restoring these organs (data not shown). These data confirm that the doses of testosterone and DHT given were roughly equivalent, in that they equally supported the growth and maintenance of androgen-dependent organs.

Effects of androgens on contractile force of the soleus

To characterize the effects of androgens on muscle function, the contractile properties of the soleus muscle were measured at the end of the 8-week treatment. The soleus was chosen because it is a relatively homogenous type I fiber muscle in the hind limb widely used in regeneration studies. ORX rats exhibited a significant loss of muscle strength compared with sham rats as measured by peak tetanic tension (Fig. 1C). Treatment of ORX rats with either testosterone or DHT restored peak tetanic strength to sham levels. Soleus mass measurements from ORX rats were significantly lower than in sham rats, indicating that androgen deficiency for a total of 19 weeks produces significant atrophy. Androgen treatment did not produce statistically meaningful increases in soleus mass after 8 weeks of treatment (8.3% increase, $P=0.34$, testosterone; 8.8% increase, $P=0.33$, DHT; Fig. 1C). Both testosterone and DHT increased muscle quality as a function of strength to mass ratio (Fig. 1C). There were no significant differences in soleus muscle relaxation time half-life after withdrawal of electrical stimulus or the time to peak tetanic tension after initiation of electrical stimulation. Thus, both testosterone and DHT restore contractile strength to the soleus of ORX rats.

Histological examination of androgen-treated soleus

Several reports suggest that androgens modulate the number of myogenic precursors and affect fiber number and/or diameter (Bhasin *et al.* 1997, 2003, Sinha-Hikim *et al.* 2002). To examine the sequence of events that precede androgenic myoanabolism, ORX rats were treated daily with vehicle or 3 mg/kg per day DHT for 4, 7, 14, or 21 days. At each time point, soleus was collected and cross-sections were prepared. As expected from the timing of the experiment (before changes in lean mass are evident), no consistent effect was observed during this time frame on fiber area. When the total number of nuclei per area was examined, DHT transiently increased nuclear density at day 7 (Fig. 2). These visual data were confirmed by measuring DNA content, which was also transiently increased by DHT (data not shown). These data suggest that DHT increases myonuclear number in the soleus without affecting fiber diameter during the initial phases of anabolism.

Gene expression changes in the soleus with androgen treatment

We next examined the possibility that the early phases of androgenic anabolism involve in the regulation of myogenic regulatory factors (MRFs) such as MyoD, myogenin, the satellite cell marker monocyte nuclear

Figure 2 Histology of soleus muscle 7 days after DHT treatment. ORX rats were treated for 4, 7, 14, or 21 days with vehicle or DHT (3 mg/kg per day) $n=10$ per group, and the soleus was collected and processed for histological examination. Representative cross-sectional fields of day 7 samples are shown above. Nuclear number per square micron (mean $\pm$ s.e.m.) at each time point (* different from vehicle $P<0.05$, t -test) is shown below.

factor (MNF), the negative regulator of myogenesis myostatin (McPherron *et al.* 1997) or IGF1, or its splice variant MGF, which are critically involved in soleus muscle regeneration (DeVol *et al.* 1990, Adams *et al.* 1999, Semsarian *et al.* 1999, Hameed *et al.* 2003). The RNA abundance for each was measured in soleus total RNA during the first 3 weeks of androgen treatment by qRT-PCR. There were no significant changes in the RNA expression of the classic MRF genes (Fig. 3). In contrast, we observed increased expression of both IGF1Ea (classic circulating) and its splice variant MGF (Fig. 4). Thus, DHT does not regulate, within the first 21 days of treatment, the expression of these myogenic factors associated with muscle except for IGF1 and MGF (Cornelison & Wold 1997, Liu *et al.* 2003, Polesskaya *et al.* 2003, Seale *et al.* 2003).

Microarray screen for androgen-responsive genes in the soleus

To characterize the immediate early response to DHT, a nonbiased microarray approach was employed. ORX rats aged 21 weeks were treated with a single injection of 3 mg/kg per day DHT or with vehicle ($n=6$ each). After 24 h, the soleus was collected and total RNA was prepared as above. A second experiment was then performed using the same design for confirmation. Genes were selected for further analysis if their RNA was altered by DHT treatment (absolute 1.5-fold change and $P<0.05$) in both experiments.

Figure 3 Expression of classic myogenic regulators during DHT treatment time-course experiments. RNA was collected from the soleus of animals treated for the indicated time points with vehicle or DHT (3 mg/kg per day), and RNA levels of *MyoD*, myostatin, *Mnf*, and myogenin were determined by quantitative qRT-PCR. All values were first normalized within samples to glucuronidase, a housekeeping gene not affected by treatment or time in this experiment (not shown), and then to vehicle. For each time point, the DHT value normalized to the vehicle value, which was set to 1. Error bars represent S.E.M.

Selected results were confirmed by qRT-PCR in independent experiments (see below). The genes, accession numbers, mean fold change results, *P* values, and functional annotations are listed in Tables 2 and 3.

Identification of early androgen-responsive genes in muscle

Seventy genes met the above criteria. These genes were categorized by their putative functions as those affecting tissue remodeling, inflammation/immune modulation, glucose metabolism, neurogenesis, and transcriptional and signaling cascades. The regulated genes include fast twitch MHC subtype-4/fiber type-2b (*Myh4*) and parvalbumin (*Pvalb*); both components of type-2 fast twitch muscle that is selectively lost in hypogonadal men and replaced during androgen treatment (Table 2; Anderson *et al.* 1988, Sinha-Hikim *et al.* 2002, Racay *et al.* 2006). Multiple genes implicated in tissue remodeling (Holmbeck *et al.* 1999, Ichimura *et al.* 2004, Koh *et al.* 2005) were identified and tabulated separately (Table 4), including plasminogen activator inhibitor-1 (*Serpine1*), matrix metalloproteinase-14 (*Mmp14*), connective tissue growth factor (*Ctgf*), kidney injury molecule-1 (*Kim1*), mast cell protease-4 (*Mcpt4*), and heat shock protein 70 (*Hspa1a*). Several of these genes are proposed to play important roles in muscle homeostasis, for example the IGF1-binding protein, CTGF, is involved in muscle, bone, and liver cell regeneration, and inhibits the transforming growth factor (TGF)/bone morphogenetic protein (BMP) pathway that limits muscle

growth (Ohnishi *et al.* 1998, Pummila *et al.* 2007, Hayata *et al.* 2008, Smerdel-Ramoya *et al.* 2008). In terms of inflammation, chemokine C-C ligand-7, *Ccl7* and chemokine C-C ligand-2, *Ccl2* were up-regulated by microarray and confirmed by qRT-PCR (Fig. 5). These RNAs code for cytokine molecules involved in local inflammation signaling and monocyte recruitment via chemotaxis (Van Damme *et al.* 1993, Wuyts *et al.* 1994).

Another subset of genes we identified suggests a role for specific transcription and signaling cascades. This set of genes includes BMP receptor type-II (*Bmpr2*), which was up-regulated in only one of the 24-h microarray screens; however, it was up-regulated by DHT in other experiments and, due to its important function, it was manually selected for confirmation. BMP receptors transduce signals from the BMP and TGF- β superfamily, which includes several inhibitors of myogenesis such as BMP2, TGF- β 2, and myostatin (Shi & Massague 2003). Other genes with less clear function in muscle were regulated, including the nuclear orphan receptor (*Nr4a3*, Nor-1) and the transcription factor early growth response-1 (*Egr1*, EGR1), both of which were repressed at 24 h (Table 3). Axin2 (*Axin2*), a constituent of the Wnt-regulatory complex involved in the degradation of β -catenin, was down-regulated (Table 3). Though typically regulated largely at the post-translational level and not detected by the microarray screen, this observation prompted us to

Figure 4 Expression of *Igf1ea* (classic circulating) and *Igf1ec* (mechano growth factor, *Mgf*). Aged ORX rats ($n=4$ per group) were treated with vehicle or DHT (3 mg/kg per day) for 1, 4, and 7 days in one experiment (left panel) and soleus RNA collected individually, and the rats were again treated with vehicle or DHT for 4, 7, and 21 days in the second experiment (right panel) and soleus RNA collected and pooled in three replicates of 3–4 (from $n=10$ rats per group) and processed for total RNA. RNA for *Igf1ea* and IGF1 splice variant *Mgf* was assayed by qRT-PCR for gene expression relative to vehicle. All values were first normalized within samples to glucuronidase gene expression. For each time point, the DHT value was normalized to the vehicle value, which was set to 1. Error bars represent S.E.M. * $P < 0.005$, *t*-test.

Table 2 5 α -Dihydrotestosterone (3 mg/kg) up-regulated genes, soleus, aged castrated rats after 24 h

Sequence name	Sequence description	Accession number	Class/pathway(s)	Fold change	P value
<i>Myh4</i>	Myosin heavy chain type-2b, fast twitch	L24897	ATPase/muscle contraction, fast twitch	9.49	<0.001
<i>Pvalb</i>	Parvalbumin	NM_022499	Calcium-binding protein/fast twitch, neurogenesis	4.63	<0.001
<i>Actn3</i>	Actinin, α -3 skeletal muscle specific	NM_133244	Muscle structure	2.16	<0.001
<i>Ctgf</i>	Connective tissue growth factor	NM_022266	Receptor ligand/ECM remodeling/inflammation	2.14	0.016
<i>Ggps1</i>	Geranylgeranyl diphosphate synthase 1	NM_001007626	Isoprenyl synthase/protein lipidation	2.08	<0.001
<i>Ccl7</i>	Chemokine (C–C motif) ligand 7 (<i>Mcp3</i>)	NM_001007612	Receptor ligand/ECM remodeling, inflammation	2.01	<0.001
<i>Mcpt4</i>	Mast cell protease 4	NM_019321	Protease/ECM remodeling, inflammation	1.98	0.017
<i>Ccl2</i>	Chemokine (C–C motif) ligand 2 (<i>Mcp1</i>)	AF058786	Receptor ligand/ECM remodeling, inflammation	1.95	<0.001
<i>Slc6a18</i>	Solute carrier family 6, member 18	NM_017163	Neurotransmitter transporter/hypotonic stress response	1.87	<0.001
<i>Pard3</i>	Par-3 partitioning defective 3	NM_031235	PKC-binding protein/axonogenesis	1.86	0.005
<i>Cyp2c</i>	Cytochrome P-450 2c	J02657	Male-specific CYP450 oxidase/testosterone metabolism	1.84	0.031
<i>Grm2</i>	Metabotropic glutamate receptor 2	M92075	Amino acid receptor/pain signaling	1.84	<0.001
<i>Agtr1</i>	Angiotensin II receptor, type-1	NM_031009	Surface receptor/angiogenesis	1.78	0.019
<i>Slco1b2</i>	Solute carrier organic anion transporter member 1b2	AF147740	Surface receptor/steroid hormone and anion transport	1.78	0.043
<i>Ptger2</i>	Prostaglandin E receptor EP2 subtype	U48858	Receptor ligand/chondrogenesis, inflammation	1.75	<0.001
<i>Slc16a13</i>	Solute carrier family 16 member 13	NM_001005530	Surface receptor/pyruvate transport	1.72	<0.001
<i>Serpine1</i>	Serine proteinase inhibitor, clade E, member 1	NM_012620	Serine protease inhibitor/inflammation	1.70	0.003
<i>Kim-1</i>	Kidney injury molecule-1	AF035963	Cell adhesion molecule/kidney regeneration	1.69	0.018
<i>Cntn3</i>	Contactin 3, fibronectin type-III	NM_019329	Cell adhesion molecule/ECM remodeling/inflammation	1.67	0.016
<i>Cnr2</i>	Cannabinoid receptor 2	NM_020543	Surface receptor/ECM remodeling/inflammation	1.66	0.001
<i>Hspa1a</i>	Heat shock protein 70	L16764	Chaperone and nuclear receptor cofactor/signaling	1.61	<0.001
<i>Gzmb</i>	Granzyme B, serine protease	M34097	Serine protease/inflammation, circadian rhythm	1.58	0.007
<i>Mybpc2</i>	Myosin-binding protein-c, fast twitch	XM_214945	Myosin-binding protein/muscle structure, fast twitch	1.58	0.011
<i>Col17a1</i>	Procollagen, type XVII, α -1	XM_219976	Extracellular matrix molecule/structural	1.58	0.002
<i>Tpm1</i>	Tropomyosin 1 (α)	NM_019131	Calcium-binding ATPase/muscle contraction	1.57	<0.001
<i>Tnfaip6</i>	TNF- α -induced protein	XM_001065494	Unknown class/ECM remodeling/inflammation	1.57	<0.001
<i>Pck1</i>	Phosphoenol pyruvate carboxykinase-1	NM_198780	Kinase/gluconeogenesis, glucose homeostasis	1.56	0.048
<i>Mbnl2</i>	Muscleblind-like 2	XM_214253	Unknown class/muscle development	1.56	0.020
<i>Syn1</i>	Synapsin I	NM_019133	Synapse cytoskeleton anchor/neurotransmitter release	1.55	0.025
<i>Spinlwl1</i>	Eppin precursor, serine protease inhibitor	XM_001071681	Serine protease inhibitor/ECM remodeling/inflammation	1.52	0.011
<i>Arid1b</i>	AT-rich interactive domain 1b	NM_172157	Nuclear transcription factor/ischemic stress	1.52	0.001
<i>Igsf7</i>	Immunoglobulin superfamily, member 7 CD300d	XM_213514	Surface receptor/ECM remodeling, inflammation	1.51	<0.001
<i>Slc2a3</i>	Solute carrier family 2 member 3 (GLUT3)	NM_017102	Glucose transporter/glucose homeostasis	1.51	0.005
<i>Lyz</i>	Lysozyme	NM_012771	Polysaccharide hydrolase/anti-inflammatory	1.51	0.003

Table 3 5 α -Dihydrotestosterone (3 mg/kg) down-regulated genes, soleus, aged castrated rats after 24 h

	Sequence description	Accession number	Class/pathway(s)	Fold change	P value
Sequence name					
<i>Prkce</i>	Protein kinase C epsilon	NM_017171	Protein kinase/inflammation, insulin secretion	−1.50	0.003
<i>Axin2</i>	Axin 2 (conductin), axil	NM_024355	Signal transduction/Wnt inhibitor, β -catenin degradation	−1.50	<0.001
<i>Gnao</i>	Guanine nucleotide-binding protein, α activating activity polypeptide o	NM_017327	G-protein/muscuranic cholinergic signal	−1.51	0.001
<i>Itga6</i>	Integrin α -6	AJ312934	Cell adhesion receptor/cell contact and recognition	−1.51	0.025
<i>Pfkfb3</i>	6-Phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3	NM_057135	Metabolic enzyme/glycolysis	−1.52	<0.001
<i>Gstt2</i>	Glutathione S-transferase theta 2	D10026	GST/xenobiotic metabolism	−1.52	<0.001
<i>Hnt</i>	Neurotrimin	NM_017354	Cell adhesion molecule/ axonogenesis	−1.52	0.025
<i>Sertad2</i>	Serta domain containing 2	NM_001024903	E2F transcription factor/ unknown function	−1.53	0.002
<i>Grm4</i>	Glutamate receptor, metabotropic 4	NM_022666	Surface receptor/GABAergic inhibitor	−1.56	<0.001
<i>Irx5</i>	Iroquois homeobox protein 5	NM_001030044	Transcription factor/ mesoderm development	−1.56	<0.001
<i>Ches1</i>	Checkpoint suppressor 1	XM_234377	Transcription factor/ mesoderm development	−1.57	0.049
<i>Kng1</i>	Kininogen 1	NM_012696	Protease inhibitor/ inflammation/senescence	−1.59	0.024
<i>Col10a1</i>	Collagen α -1 type X	AJ131848	Extracellular matrix protein/ structural	−1.61	0.030
<i>Phactr1</i>	Phosphatase and actin regulator 1	NM_214457	Phosphatase/actin cytoskeletal organization	−1.61	0.003
<i>Aox1</i>	Aldehyde oxidase 1	NM_019363	Oxidase/retinoic acid synthesis (ALS pathophysiology)	−1.61	0.005
<i>Arhgef5</i>	Rho guanine nucleotide exchange factor 5	140898	G-protein/oxidation, neurogenesis	−1.62	0.003
<i>Lpd</i>	Liposidin, acyl-CoA synthase	AF208125	Fatty acid synthase/ fatty acid metabolism	−1.62	0.031
<i>Fbp1</i>	Fructose-1,6-biphosphatase 1	NM_012558	Phosphatase enzyme/ gluconeogenesis	−1.63	0.039
<i>Kalrn</i>	Kalirin-12A RhoGEF kinase	84009	Kinase/cell adhesion, axonogenesis	−1.64	<0.001
<i>Jun</i>	Jun oncogene	X17215	Transcription factor/ oncogene	−1.64	0.005
<i>Tnfsf13b</i>	Tumor necrosis factor superfamily, member 13b	AI059288	Unknown class/ECM remodeling, inflammation	−1.65	0.008
<i>Cyp26b1</i>	Cytochrome P-450, family 26, subfam b, polypeptide 1	NM_181087	Oxidase/retinoic acid inactivation	−1.70	<0.001
<i>Nr4a2</i>	Nurr1	U01146	Nuclear orphan receptor/ neurogenesis	−1.71	0.013
<i>Egr2</i>	Early growth response-2, zinc finger protein krox-20	AB032419	Nuclear transcription factor/ synaptic transmission	−1.73	0.001
<i>Nppc</i>	c-type natriuretic peptide	D90219	Receptor ligand/vasoactive neuropeptide	−1.74	0.008
<i>Mmp14</i>	Matrix metalloproteinase 14	NM_031056	Protease/ECM remodeling, inflammation	−1.76	<0.001
<i>Arntl</i>	Aryl hydrocarbon receptor nuclear translocator-like	AF015953	Unknown class/circadian rhythm/protein catabolism	−1.86	<0.001
<i>Cldn4</i>	Claudin 4	304407	Cell adhesion molecule/tight junctions	−1.86	<0.001

Table 3 Continued

	Sequence description	Accession number	Class/pathway(s)	Fold change	P value
<i>Sema6a</i>	Semaphorin 6a	XM_341612	Transmembrane protein/neurogenesis/axon guidance	-1.91	0.001
<i>Tacstd1</i>	Tumor-associated calcium signal transducer 1	AJ001044	Transmembrane protein/cell adhesion, calcium signaling	-1.95	0.001
<i>Trp63</i>	Transformation-related protein 63	NM_019221	p53-like nuclear transcription factor/differentiation	-1.95	0.016
<i>Nr4a3</i>	Nuclear receptor subfam 4, grp A, member 3 (NOR-1)	NM_017352	Nuclear orphan receptor/proliferation mesoderm	-1.98	<0.001
<i>Egr1</i>	Early growth response 1	NM_012551	Nuclear transcription factor/proliferation/differentiation	-2.23	<0.001
<i>Fabp1</i>	Fatty acid-binding protein 1	M35991	Fatty acid-binding protein/fatty acid metabolism	-2.42	0.005
<i>Slc15a1</i>	Solute carrier family 15 member 1	D50664	Surface receptor/peptide transporter	-2.45	0.002
<i>Cldn3</i>	Claudin3	NM_031700	Cell adhesion molecule/tight junctions	-2.58	0.036

measure β -catenin RNA, which showed modest up-regulation at 1, 4, and 7 days by qRT-PCR (Fig. 6). Therefore, during the first 24 h, DHT regulates the expression of a specific set of genes that might be downstream mediators of AR in muscle tissue.

Based on the microarray results, the expression of *Myh4*, *Parvl*, *Ccl2*, and *Ccl7* were confirmed by qRT-PCR (Fig. 5). Other potentially myogenic transcription factors were confirmed by qRT-PCR to be regulated by DHT including *Nr4a3*, *Egr1*, and *Bmpr2*. These genes were studied in separate time-course experiments ranging from 1 to 21 days after DHT stimulation in soleus muscle (Fig. 7). The *Bmpr2* gene is induced twofold after 1–4 days and falls to basal levels through 21 days (Fig. 7). *Nr4a3* exhibits a biphasic expression pattern, with levels suppressed after 1–4 days and

induced twofold afterwards (Fig. 7). *Egr1* expression was repressed throughout both experiments (Fig. 7). Thus, the microarray experiment detected real and reproducible changes in gene expression, as all seven genes were in fact androgen responsive.

Androgenic repression of *Axin* and *Axin2* in the soleus

The microarray observation of decreased *Axin2* expression was confirmed by qRT-PCR (Fig. 8A). *Axin2* acts as a negative feedback regulator of the Wnt-signaling pathway in the colon (Lustig *et al.* 2002). *Axin1* and *Axin2* have 45% homology and are functionally redundant in mice (Chia & Costantini 2005). Thus, we examined protein levels of AXIN and

Table 4 Microarray identified early genes involved in muscle or tissue regeneration

Sequence name	Common name	Up/down regulation	RT-QPCR confirmed	Citation
<i>Myh4</i>	Myosin heavy chain type-2b	Up	Yes	Sinha-Hikim <i>et al.</i> (2002)
<i>Pvalb</i>	Parvalbumin	Up	Yes	Anderson <i>et al.</i> (1988) and Racay <i>et al.</i> (2006)
<i>Ctgf</i>	Connective tissue growth factor	Up	ND	Ohnishi <i>et al.</i> (1998) and Hayata <i>et al.</i> (2008)
<i>Ccl2</i>	Monocyte chemoattractant protein-1	Up	Yes	Shireman <i>et al.</i> (2007)
<i>Ccl7</i>	Monocyte chemoattractant protein-3	Up	Yes	Schenk <i>et al.</i> (2007)
<i>Serpine1</i>	Plasminogen activator inhibitor-1	Up	ND	Koh <i>et al.</i> (2005)
<i>Kim-1</i>	Kidney injury molecule-1	Up	ND	Ichimura <i>et al.</i> (2004)
<i>Hspa1a</i>	Heat shock protein 70	Up	ND	Miyabara <i>et al.</i> (2006)
<i>Mcpt4</i>	Mast cell protease-4	Up	ND	Zweifel <i>et al.</i> (2005)
<i>Mmp14</i>	Matrix metalloprotease-14	Down	ND	Holmbeck <i>et al.</i> (1999)

Figure 5 Confirmation of microarray expression of genes involved in muscle regeneration. Aged ORX rats ($n=4$ per group) were treated for the times indicated with vehicle or DHT (3 mg/kg per day). The soleus was collected from individual rats ($n=4$ per group) and processed for total RNA. RNA for myosin heavy chain type-2b (*Myh4*), parvalbumin (*Pvalb*), monocyte chemoattractant protein-1 and -3 (*Ccl2* and *Ccl7*) were assayed by qRT-PCR for gene expression relative to vehicle. All values were first normalized within samples to glucuronidase gene expression. For each time point, the DHT value was normalized to the vehicle value, which was set to 1. * $P<0.005$ *t*-test. Error bars represent S.E.M.

AXIN2 in soleus extracts and found that DHT repressed expression (Fig. 8A and B). As both axin types inhibit β -catenin, these findings further suggested to us that β -catenin is functionally induced.

Finally, it has been reported that growing muscle features inactivation via serine-9 phosphorylation of the β -catenin inhibitor GSK3 β (Chin *et al.* 2005). If true with DHT treatment, such an observation would also suggest activation of β -catenin signaling. Thus, we measured serine-9 phosphorylation of GSK3 β after DHT treatment and found increased serine-9 phosphorylated (and thus inactivated) GSK3 β after 7 days (Fig. 8C).

Androgenic induction of β -catenin in the soleus

The above data suggested that β -catenin protein, which is regulated post-translationally by axin expression, IGF1 and GSK3 β phosphorylation, is induced by DHT in muscle. Western blot data confirmed the accumulation of β -catenin protein, with levels ~ 20 -fold greater than those in controls during the first 21 days of response to DHT (Fig. 6B). We also observed decreased levels of serine 33/37/41 phosphorylated β -catenin, which triggers β -catenin degradation and is consistent with downregulation of axin-dependent GSK3 β activity and upregulation of β -catenin levels. To determine the subcellular localization of β -catenin, sections of soleus

muscle from rats treated with DHT or with vehicle for 7 days were examined by immunohistochemistry. Antibodies against β -catenin labeled nuclei with a significantly higher proportion of β -catenin-positive nuclei evident in the DHT-treated specimens (Fig. 9). Though we did not definitively identify the cell types expressing β -catenin, we were careful to exclude nuclei that were not intimately associated with myofibers such as cells in or around fatty deposits or blood vessels. The specificity of the staining was confirmed by omitting the primary antibody, which resulted in loss of all signals and parallel staining of intestinal sections, which

Figure 6 DHT upregulates expression of Wnt pathway regulator β -catenin through transcription and repression of phosphorylation. Rats were treated with vehicle or DHT (3 mg/kg per day) for the times indicated. The soleus was collected individually ($n=4$ per group, top panels) or pooled ($n=6$ per group, bottom panels), and processed for total RNA and protein. (A) Gene expression levels of β -catenin were determined by qRT-PCR. All values were normalized within samples to glucuronidase. For each time point, the DHT value was normalized to the vehicle value, which was set to 1. * $P<0.05$, *t*-test. Error bars represent S.E.M. (B) Immunoblot of total β -catenin and phospho- β -catenin (top panel). Bands represent individual rats at day 7 of treatment. The optical density (OD) values of DHT bands were normalized to vehicle values. * $P<0.05$, *t*-test.

Figure 7 Expression of regulated genes in the soleus of ORX rats treated with vehicle or DHT (3 mg/kg per day) in two time-course experiments for the times indicated. The soleus was collected individually for the short term ($n=4$ per group, left panels) and in three pooled replicates of 2 (from $n=6$ rats per group, right panels) and processed for total RNA. Gene expression levels of nuclear orphan receptor-1 (*Nr4a3*), bmp type II receptor (*Bmpr2*), and early growth response-1 (*Egr1*) were determined by qRT-PCR. All values were normalized within samples to glucuronidase. For each time point, the DHT value was normalized to the vehicle value, which was set to 1. * $P<0.005$, t -test. Error bars represent s.e.m.

showed typical β -catenin patterns (data not shown). These data demonstrate that β -catenin protein is rapidly induced by DHT in the soleus and accumulates in nuclei during the early stages of anabolism.

IGF1 and ctMGF induce β -catenin nuclear translocation in C2C12 myoblasts

Our findings of androgenic induction of *Igf1* and *Mgf* prompted us to explore the potential direct link of these IGF1 variants to β -catenin in cultured C2C12 myoblasts since IGF1 induces β -catenin nuclear translocation in other cells (Satyamoorthy *et al.* 2001, Chen *et al.* 2005a). Both IGF1 and ctMGF rapidly increased total nuclear β -catenin protein expression in C2C12 myoblasts (Fig. 10A and C) and induced β -catenin-mediated TCF/LEF signaling using the TOPFLASH β -catenin reporter assay (Fig. 10B). Finally, nuclear localization of β -catenin in C2C12 cells after IGF1 and ctMGF treatment was confirmed by immunocytochemistry (Fig. 10C). Thus, in myoblast-like cells, IGF1 and MGF are sufficient to upregulate β -catenin protein and induce nuclear localization and transcriptional activity.

Discussion

Androgens are important regulators of body composition during postnatal development and aging in both genders. In clinical studies, testosterone maintains, restores and/or increases LBM and BMC, and decreases fat mass. However, testosterone can be converted to estradiol (E_2); thus, clinical studies involving testosterone remain equivocal for understanding the molecular

Figure 8 DHT downregulates expression of Wnt pathway negative regulators, *Axin2* and *Axin*. (A) Rats were treated with vehicle or DHT (3 mg/kg per day) for the times indicated. The soleus was collected individually ($n=4$ per group top panels) and processed for total RNA and protein. Gene expression levels of *Axin2* were determined by qRT-PCR. All values were normalized within samples to glucuronidase. For each time point, the DHT value was normalized to the vehicle value, which was set to 1. * $P<0.005$, t -test. Error bars represent s.e.m. (B) Immunoblot of total AXIN and AXIN2. Bands represent individual rats at day 7 of treatment. The optical density (OD) values of DHT bands were normalized to vehicle values. (C) Immunoblot for total GSK3 β and serine-9-phosphorylated GSK3 β . DHT upregulates expression of serine-9-phosphorylated GSK3 β . Rats were treated with vehicle or DHT (3 mg/kg per day) for 7 days. The soleus was collected individually ($n=4$) and processed for protein extraction. Bands represent individual rats at day 7 of treatment. The optical density (OD) values of DHT bands were normalized to vehicle values.

Figure 9 Expression of β -catenin in muscle nuclei with DHT treatment. ORX rats were treated for 4, 7, 14, or 21 days with vehicle or DHT (3 mg/kg per day), and then soleus was collected and processed for histological examination. (A) Representative cross-sectional fields of day 7 samples are shown above. Muscle nuclei labeled with β -catenin-specific antibody as visualized by immunohistochemistry using nickel DAB. (B) The number of β -catenin-positive nuclei was counted per mm² of cross-sectional area. $N=10$ rats/sections per group. $*P<0.05$, t -test. (C) The total number of nuclei was counted (without nickel) following hematoxylin counterstaining for each section and the proportion calculated and graphed. $*P<0.05$, t -test.

basis of androgenic myoanabolism. We explored this problem by establishing a suitable animal model for clarifying the role of AR on body composition and identifying genomic and molecular responses in muscle tissue downstream of androgen stimulation.

ORX-induced androgen deficiency results in decreased LBM, greater fat mass, and decreased BMC (Fig. 1A). Examination of the soleus confirmed that previous data showing muscle weight and strength are decreased by androgen loss (Boissonneault 2001, Brown *et al.* 2001). Both testosterone and DHT restored muscle contractile strength and induced LBM accumulation (Fig. 1B and C). Moreover, both testosterone and DHT suppressed the accumulation of fat mass similar to that of sham-operated rats. However, only testosterone increased whole-body BMC above that of vehicle-treated ORX rats, suggesting an important role for aromatization to E_2 for maintenance of bone mass. This observation is in agreement with other studies suggesting that DHT is less effective than testosterone in preventing cancellous bone loss (Vanderschueren *et al.* 1992) and that aromatization to estrogens is important for skeletal homeostasis (Vanderschueren *et al.* 1997). Note that whole-body BMC measurements are not likely to detect small but important changes in the extent of mineralization in the periosteum, as it constitutes a small fraction of total bone and is stimulated by androgens in rats (Hanada *et al.* 2003).

The role of AR in muscle growth at the cellular and molecular level was then examined in the soleus, a common model for regeneration studies. Interestingly, soleus contractile strength was restored in the absence of large gains in soleus mass after 8 weeks of treatment (Fig. 1C), suggesting a positive effect on muscle efficiency. Seventeen-week studies in OVX rats show

Figure 10 IGF1 and its splice variant mechano growth factor C-terminal peptide (ctMGF) promote nuclear translocation of myogenic Wnt regulator β -catenin in C2C12 myoblasts. Cells were treated with vehicle, IGF1 (10 and 30 ng/ml), and ctMGF (30 and 60 ng/ml). (A) Immunoblot of total nuclear β -catenin after 25 min of treatment. Lamin is load control. (B) The β -catenin TOPFLASH reporter plasmid containing TCF/LEF promoter linked to the firefly luciferase gene was cotransfected into C2C12 myoblasts with a constitutively active *Renilla* luciferase plasmid, and the cells were plated in 96-well plates and stimulated with IGF1 and ctMGF at the above concentrations for 16 h. β -Catenin-responsive firefly luciferase reporter activity was first normalized to *Renilla* luciferase activity, and the IGF1 and ctMGF values were normalized to the vehicle value, which was set to 1. $*P<0.01$, one-way ANOVA, Fisher's PLSD. (C) Near confluent C2C12 myoblasts in chamber slides were washed and placed in serum-free media with vehicle, IGF1 (30 ng/ml), or ctMGF (60 ng/ml) for 25 min just prior to fixation. The cells were prepared for immunocytochemistry, stained with anti- β -catenin antibodies, and counterstained with nuclear DAPI stain.

that DHT induces gains in soleus weight (data not shown); thus, the experiments presented here were probably not long enough to detect significant gains in muscle weight. To characterize the sequence of events preceding, and therefore likely involved in initiating, muscle anabolism, we focused on the first 21 days following DHT treatment. We find that DHT might promote the proliferation or recruitment of cells early during the regeneration process, as an increase in nuclear density was detected only in 7 days post-treatment (Fig. 2). We then examined whether key myogenic factors are regulated, but over this time we found no differences in the expression of MyoD or myogenin, the satellite cell marker MNF (Garry *et al.* 2000), or myostatin (Fig. 3). This observation is in agreement with recent findings in ARKO mice where classic MRF genes were not regulated, and the overall gene profile suggested that androgens delay differentiation allowing clonal expansion of myoblasts (MacLean *et al.* 2008). These data do not exclude the possibility that these genes are involved in androgenic anabolism, as they could be regulated in a small proportion of cells, by a post-transcriptional mechanism, or at later time points. It will be important to determine whether and when the classical MRF transcriptional cascade and satellite cell activation occur.

The microarray analysis revealed that a relatively small number of genes respond to DHT in the soleus after 24 h. Using our statistical criteria, 70 genes responded to DHT (Tables 2 and 3). In a parallel experiment in which prostate was studied, approximately ten times as many genes were DHT-responsive at 24 h, even though more stringent statistical criteria were applied (Nantermet *et al.* 2004). Thus, the soleus is relatively unresponsive, possibly reflecting the lower levels of AR in muscle compared with prostate (Krieg 1976, Michel & Baulieu 1980).

Examination of the function of these genes provides insight into the initial response of the soleus to DHT. MHC type-2b and parvalbumin were induced, both of them are type II muscle-specific proteins, lending validity to the model since androgen depletion results in the loss of type II muscle and repletion increases (Sinha-Hikim *et al.* 2002). When taken together, these gene expression data suggest an initiation of events similar to muscle regeneration, which initially involves cytokine-mediated inflammation, extracellular matrix, vascular remodeling, and, at later stages, recruitment of myogenic progenitor cells and satellite cells followed by differentiation into new muscle myofibers (Seale & Rudnicki 2000, Goetsch *et al.* 2003, Charge & Rudnicki 2004). In terms of cytokine-mediated inflammation, two cytokine-encoding RNAs, *Ccl2* and *Ccl7*, were induced. Their proteins are potent chemotactic agents and thus could recruit cells to the regenerating muscle, as has been proposed for CCL2 (Warren *et al.* 2004) and CCL7 after myocardial

infarction (Schenk *et al.* 2007). Interestingly, CCL2 knockout mice exhibit impaired regeneration and abnormal fat accumulation after muscle injury (Shireman *et al.* 2007, Contreras-Shannon *et al.* 2007). Moreover, side population and CD45+ cells induced by Wnt/ β -catenin signaling are important for regeneration of the soleus in response to toxin-induced muscle damage (Asakura *et al.* 2002, Poleskaya *et al.* 2003) and represent specific subsets of immune cells distinct from satellite cells. We also demonstrate the androgenic induction of *Mgf*, an IGF1 splice variant, up-regulated with muscle damage and exercise (Hameed *et al.* 2003, Rigamonti *et al.* 2009). There is also evidence for matrix remodeling, as *Mmp14* rapidly responded to DHT. Since muscle matrix undergoes significant changes during embryogenesis (Visse & Nagase 2003) and regeneration (Charge & Rudnicki 2004), this gene could function in that process. It is interesting to note the induction of genes that are also induced during exercise, which includes IGF1, MGF, β -catenin, HSPA1A, SERPINE1, CCL2, and CCL7 (Tables 2 and 4), since the rats were single-housed and not allowed vigorous exercise.

The gene induction for a receptor for BMP-signaling molecules, *Bmpr2*, is interesting, given the role of this family in repressing myogenesis (e.g. myostatin (Thomas *et al.* 2000) and TGF- β (Massague *et al.* 1986)). However, the type II BMP receptor requires a type I receptor for classical Smad-mediated signal transduction (Foletta *et al.* 2003); thus, this observation does not necessarily indicate enhanced myoinhibitory BMP/TGF- β signaling. In fact, the *Drosophila* homolog of *Bmpr2*, *wishful thinking*, is required for proper formation of the neuromuscular junction (Marques *et al.* 2002), potentially suggesting a neuromodulatory function of this gene.

The microarray study also identified genes whose function in muscle tissue has not been studied. These include the transcription factors EGR1, which functions in inflammation, proliferation, differentiation, and apoptosis, and NR4A3, an orphan nuclear receptor that acts as a transcriptional activator in a ligand-independent manner (Thiel & Cibelli 2002, Wansa *et al.* 2003). qRT-PCR reveals that both *Egr1* and *Nr4a3* RNAs are initially repressed, and while *Egr1* exhibited sustained repression, *Nr4a3* was induced during the time nuclear content was elevated (see Figs 2 and 7). Two *Nr4a3* gene-disrupted mouse strains have been reported, one of which reports embryonic lethality due to accumulation of mesodermal cells in the primitive streak (DeYoung *et al.* 2003). NR4A3 also regulates in oxidative metabolism in muscle (Pearen *et al.* 2008), suggesting that androgens could modulate metabolism through control of NR4A3 expression.

The most notable finding from the microarray was inhibition of the Wnt-signaling molecule Axin2, an inhibitor of β -catenin. This phenomenon has been reported in the testosterone-treated ORX mouse

prostate (Wang *et al.* 2008). Axin mRNA and protein were repressed through 7 days (Fig. 8) corresponding to increased GSK3 β serine-9 phosphorylation, and decreased phosphorylation and nuclear accumulation of β -catenin protein (Figs 9 and 10). β -Catenin is both essential and sufficient for P19 cell myogenesis and inhibits adipogenesis *in vitro* (Ross *et al.* 2000, Petropoulos & Skerjanc 2002). Furthermore, β -catenin signaling is required for regeneration of the soleus (Poleskaya *et al.* 2003). Upregulation of both total muscle and myonuclear β -catenin occurs during exercise, load-induced muscle hypertrophy, and myocardial recovery after heart failure *in vivo* (Sakamoto *et al.* 2004, Armstrong & Esser 2005, Braz *et al.* 2009), and the Wnt/ β -catenin pathway promotes insulin/IGF1-mediated reserve cell activation and myotube hypertrophy *in vitro* (Rochat *et al.* 2004). The anti-atrophy effects of IGF1 in glucocorticoid-treated rats are via the AKT/GSK3 β / β -catenin pathway (Schakman *et al.* 2008). Moreover, β -catenin expression is necessary for physiological growth of muscle in adult animals (Armstrong *et al.* 2006). Our data apparently oppose the current view that *Axin2* expression is a direct indicator of β -catenin signaling. However, the observation of DHT-mediated IGF induction, AXIN protein downregulation, and serine-9 phosphorylation of GSK3 β (Fig. 8C) indicates that multiple mechanisms are in effect, which could result in a special case where *Axin2* downregulation may not signify reduced β -catenin signaling. Interestingly, β -catenin and AR physically and functionally interact in prostate and neuronal cells (Pawlowski *et al.* 2002, Yang *et al.* 2002), suggesting crosstalk between androgen and Wnt signaling. To explore how androgens affect β -catenin signaling, we stimulated mouse myoblasts with IGF1 or ctMGF, and observed that both promote rapid upregulation and nuclear translocation of β -catenin (Fig. 10). Thus, we suggest that by inducing IGF1 and its muscle-specific splice variants, AR promotes the growth of muscle by activating the β -catenin pathway. Downregulation of β -catenin attenuates myocardial remodeling by promoting precursor cell differentiation, while upregulation induces precursor cell proliferation during muscle regeneration (Otto *et al.* 2008, Perez-Ruiz *et al.* 2008, Zelarayan *et al.* 2008). Thus, these data are consistent with the current understanding of β -catenin in muscle remodeling. Though the current data do not prove this hypothesis, the role of β -catenin in promoting myogenesis and muscle regeneration and repressing adipogenesis provides a unifying concept for androgen-mediated changes in body composition.

Declaration of interest

There are no conflicts of interest that would compromise the impartiality of this research.

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