Impaired skeletal muscle development and function in male, but not female, genomic *androgen receptor* knockout mice

Helen E. MacLean,* W. S. Maria Chiu,* Amanda J. Notini,* Anna-Maree Axell,* Rachel A. Davey,* Julie F. McManus,* Cathy Ma,* David R. Plant,† Gordon S. Lynch,† and Jeffrey D. Zajac*

*Department of Medicine, University of Melbourne, Austin Health, Heidelberg, Victoria, Australia; and †Department of Physiology, University of Melbourne, Melbourne, Victoria, Australia

ABSTRACT **To identify mechanisms of anabolic androgen action in muscle, we generated male and female genomic androgen receptor (AR) knockout (ARKO) mice, and characterized muscle mass, contractile function, and gene expression. Muscle mass is decreased in ARKO males, but normal in ARKO females. The levator ani muscle, which fails to develop in normal females, is also absent in ARKO males. Force production is decreased from fast-twitch ARKO male muscle, and slow-twitch muscle has increased fatigue resistance. Microarray analysis shows up-regulation of genes encoding slow-twitch muscle contractile proteins. Real-time PCR confirms that expression of genes encoding polyamine biosynthetic enzymes, ornithine decarboxylase (*Odc1*), and S-adenosylmethionine decarboxylase (*Amd1*), is reduced in ARKO muscle, suggesting androgens act through regulation of polyamine biosynthesis. Altered expression of regulators of myoblast progression from proliferation to terminal differentiation suggests androgens also promote muscle growth by maintaining myoblasts in the proliferate state and delaying differentiation (increased *Cdkn1c* and *Igf2*, decreased *Itg1bp3*). A similar pattern of gene expression is observed in orchidectomized male mice, during androgen withdrawal-dependent muscle atrophy. In conclusion, androgens are not required for peak muscle mass in females. In males, androgens act through the AR to regulate multiple gene pathways that control muscle mass, strength, and fatigue resistance.—MacLean, H. E., Maria Chiu, W. S., Notini, A. J., Axell, A.-M., Davey, R. A., McManus, J. F., Ma, C., Plant, D. R., Lynch, G. S., Zajac, J. D. Impaired skeletal muscle development and function in male, but not female, genomic *androgen receptor* knockout mice. *FASEB J.* 22, 2676–2689 (2008)**

Key Words: hypertrophy · anabolic · knockout · strength · polyamine

DESPITE THE WIDESPREAD use of anabolic androgens to enhance athletic performance, the mechanisms of androgen action in skeletal muscle remain poorly understood. Androgens are required to maintain normal muscle mass and strength in men, since suppression of testosterone levels reduces these parameters (1). Conversely, exogenously administered androgens have anabolic effects on muscle (2). Randomized controlled trials demonstrated a dose-dependent response of lean body mass and leg strength to testosterone in eugonadal young and elderly men (3, 4). Anecdotal reports from anabolic steroid use in female athletes are highly suggestive that androgens also increase muscle strength in women (5). However, it is yet to be determined whether the low testosterone levels in females play a physiological role in developing normal peak muscle mass, and the mechanisms of androgen action in male muscle are still unknown.

Androgens act predominantly through the androgen receptor (AR), a member of the ligand-dependent nuclear transcription factor family. Both testosterone and dihydrotestosterone bind and activate the AR to regulate target gene expression (6). Although both naturally occurring AR mutant mice (*Tfm*) and AR knockout (ARKO) mouse models have been generated (7), no systematic analysis of AR-null skeletal muscle phenotype has been performed, and thus the AR-mediated actions of androgens in muscle remain undefined. In addition, testosterone in males can also be aromatized to estradiol, to act *via* the estrogen receptor (ER) (8). Male muscle contains aromatase enzyme activity (9), suggesting the potential for testosterone action *via* estradiol in muscle. Male mice lacking ERβ also show altered muscle function (10); however, the relative importance of AR- *vs.* ER-mediated pathways in muscle is unknown.

The *AR* gene is expressed widely, including in myoblasts, myofibers and satellite cells of males and females (11). The *AR* is also expressed in motor neurons, which are also a direct target for androgen action (12) and may contribute to regulation of muscle mass. Andro-

[1] Correspondence: Austin Hospital, Heidelberg, VIC 3084, Australia. E-mail: hmaclean@unimelb.edu.au

doi: 10.1096/fj.08-105726

0892-6638/08/0022-2676 © FASEB

gens may also modulate levels of circulating insulin-like growth factor 1 (IGF1) (13), which is also a potent anabolic agent in muscle. Further, androgen-dependent behavioral modifications, such as modulation of activity levels, could potentially impact on muscle mass.

The major unanswered questions regarding the anabolic actions of androgens in skeletal muscle are the following. *1*) Are these actions mediated predominantly *via* the AR? *2*) What is their role in development of normal muscle mass in females *vs.* males? *3*) What are the functional consequences of androgen action in skeletal muscle? *4*) What are the target genes that mediate these actions in muscle? To answer these questions, we have used the cre/*lox*P system to create male and female global ARKO mice.

MATERIALS AND METHODS

Mice

AR^{lox} mice were generated as described prevously (14), with exon 3 flanked by *lox*P sites. AR^{lox} heterozygous mice were back-crossed onto a C57BL/6 background for >6 generations prior to experimental analysis. CMV-cre transgenic mice were obtained with permission from Dr. Ursula Lichtenberg (Institute for Genetics, University of Cologne, Cologne, Germany) and were maintained on a C57BL/6 background. Control littermates were used for both males [wild type (WT)] and females (WT or CMV-cre heterozygotes), as described. Mice were housed in a conventional facility, and standard chow and water were provided *ad libitum*. Studies were performed with the approval of the Austin Health Animal Ethics Committee, Melbourne Health Animal Ethics Committee, and the University of Melbourne Animal Ethics Committee.

Tissue collection

Wet weight of tissues was determined to an accuracy of 0.1 mg, from the mean mass bilaterally. Body and muscle mass were measured in 9 and 12 wk ARKO and WT male littermates ($n \geq 24$/group at 9 wk, $n \geq 12$/group at 12 wk), and in 9 wk ARKO females, and WT and CMV-cre heterozygote female littermates ($n \geq 12$/group). Data from WT and CMV-cre females were pooled, as there was no statistical difference between all parameters in these groups (data not shown).

IGF1 assay

Serum IGF1 was assayed in 9- and 12-wk WT and ARKO males ($n=12$/group), using the IGF1 RIA (Bioclone; Marrickville, NSW, Australia) according to manufacturer's instructions, except the assay was scaled down 2-fold to 50 μl serum/mouse.

cDNA synthesis, reverse transcriptase-polymerase chain reaction (RT-PCR), and PCR

Total RNA isolation and cDNA synthesis were performed as described previously (15). To detect expression of the normal and exon 3-deleted *AR* genes, RT-PCR was performed on 500 ng cDNA using primers flanking exon 3 (14).

To detect the AR^{lox} allele in the spiking experiment, genomic DNA from gastrocnemius muscle of an ARKO female and an AR^{lox} heterozygote female was combined (100 ng/reaction), and PCR primers within exon 3 of the *AR* gene and the *neo* cassette were used (14). The amount of control AR^{lox} female DNA varied from 100% to 0.1%. For the 100% ARKO female samples, DNA from two independent ARKO females was used in separate reactions. The *GAPDH* gene was amplified to control for amount of DNA.

Quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) was performed in duplicate using 500 ng cDNA in a 25-μl reaction, using TaqMan gene expression assays and the 7500 real-time PCR system (Applied Biosystems, Scoresby, VIC, Australia). Relative expression was determined using the $\Delta\Delta C_T$ method, as previously described (15). To quantitate *AR* gene expression, up to 7 samples/group were analyzed using the mouse *AR* gene expression assay (assay ID: Mm00442688_m1), which amplifies between exons 2 and 3 with a probe against the exon2/exon 3 boundary. To compare gene expression in WT and ARKO gastrocnemius muscle, Q-PCR was performed on 12 samples/group, and 9 samples/group were used for the orchidectomy *vs.* orchidectomy plus testosterone groups, using gastrocnemius muscle collected in our previous study (16). Preoptimized or custom TaqMan assays were used as listed in Supplemental Data.

Microarray analysis

Microarray analysis was performed by the Australian Genome Research Facility using the Affymetrix system (Millennium Sciences, Surrey Hills, VIC, Australia). Gastrocnemius muscle RNA from WT and ARKO males ($n=2$/group) was analyzed, using Affymetrix mouse genome 430 2.0 array. This array contains >45,000 probe sets representing >34,000 mouse genes, with 11 pairs of oligonucleotide probes for each sequence. Androgen-responsive genes were identified using normalized signal intensity to identify genes with ≥1.6-fold-change in duplicate samples. For genes with more than one probe set in the array, data from all probes were combined in the statistical analyses. GO mining was performed using the online Affymetrix NetAffx analysis center (https://www.affymetrix.com/analysis/index.affx).

In vitro physiology

In vitro physiological analyses were performed on muscles from 9 wk WT males, WT females, and ARKO males ($n=6$/group), using two hind limb muscles: the fast-twitch extensor digitorum longus and the slow-twitch soleus. Contractile properties, including maximum tetanic force, specific force, fatigue, time-to-peak tension and half-relaxation time were assessed as described previously (16).

Statistical analysis

For comparison of means for more than 2 groups, data were analyzed by 1-way ANOVA with Tukey's *post hoc* analysis; for 2 groups, unpaired Student's *t* test was used. Fatigue was analyzed by general linear model univariate analysis with Tamhane's *post hoc* test, as Levene's test of equality of error indicated unequal variance across genotypes. GO distributions were analyzed by χ^2 analysis. All analyses were performed using SPSS 11, except the GO χ^2 analysis, which was calculated using the Affymetrix online GO tool.

RESULTS

Generation of male and female ARKOs

We have a floxed AR mouse line (AR^{lox}), in which exon 3 of the AR gene (on the X chromosome) is flanked by *lox*P sites (**Fig. 1*A***). Exon 3 encodes the second zinc finger of the DNA binding domain, and deletion maintains the mRNA reading frame, leading to AR protein that lacks DNA binding and has ablation of genomic actions (14). To generate global ARKO males, the ARΔ line was created (Fig. 1*B*). Because hemizygous ARKO males are androgen insensitive and infertile, the cre/*lox*P system was required to generate global ARKO females (Fig. 1*B*).

To confirm the cre-mediated deletion of the AR^{lox} allele in ARKO females, we used primers within exon 3 to amplify DNA from a number of tissues including tail, kidney, heart, and skeletal muscle, and no band was detectable (data not shown). To further quantitate the degree of AR^{lox} deletion in the ARKO females, we spiked genomic DNA from ARKO female muscle with varying concentrations of control AR^{lox} heterozygous female muscle DNA, and carried out PCR using primers specific for the AR^{lox} allele, amplifying both exon 3 (data not shown) and the *neo* cDNA sequence (Fig. 1*C*). In each case, the AR^{lox} allele could be amplified from ARKO female DNA containing down to 0.1% control AR^{lox} DNA, but not from the 100% ARKO female DNA, suggesting a very high efficiency of AR^{lox} deletion.

AR mRNA expression in muscle from 9-wk-old WT and ARKO males was measured by Q-PCR, using a probe homologous to exon 3. The level of *AR* gene expression in different muscles varied in different WT muscles, with the highest expression in the known androgen-responsive levator ani muscle (Fig. 1*D*). As expected, no WT *AR* mRNA was detectable in muscle from ARKO males using this exon 3-specific probe (Fig. 1*D*). However, reverse transcriptase PCR using primers flanking exon 3 amplified the AR^{Δ} mRNA in ARKO male muscle samples (Fig. 1*E*), confirming that the mutant gene is still expressed.

We previously showed that ARKO males have decreased serum testosterone levels (14). In the present study, we measured serum IGF1 levels in 9- and 12-wk-old WT and ARKO males and showed no difference in IGF1 levels between the two groups (**Table 1**).

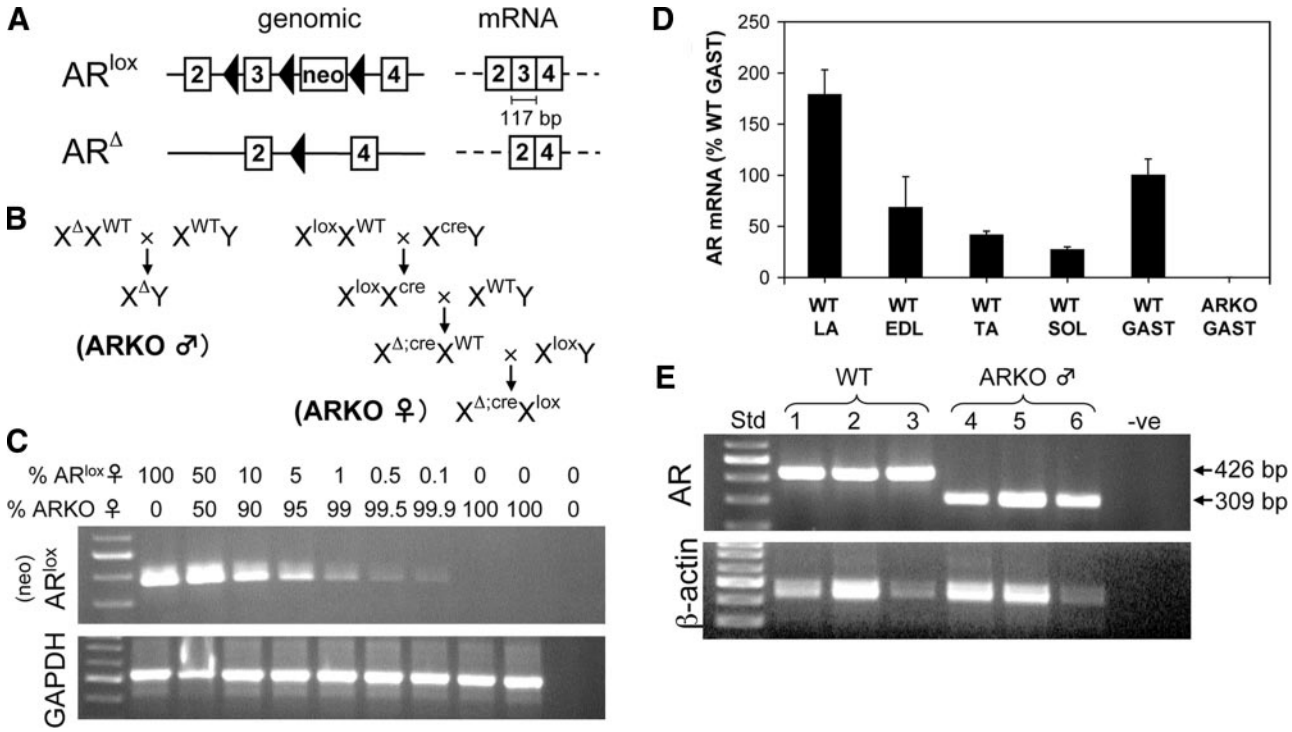

Figure 1. Generation and characterization of global ARKO mice. *A*) Diagram of targeted *AR* gene locus, including AR^{lox} allele with *lox*P sites (◀) flanking exon 3 and *neo* cassette in intron 3, and AR^{Δ} allele with deletion of exon 3 (117 bp). *B*) Steps required to breed ARKO male and ARKO female mice. X^{WT}, WT *AR* gene allele on the X chromosome; X^{lox}, AR^{lox} allele; X^{Δ}, AR^{Δ} allele; X^{cre}, X-linked CMV-cre transgene. *C*) PCR spiking experiment using primers specific for the AR^{lox} allele (*neo* cDNA sequence), demonstrating *AR* deletion in genomic DNA of ARKO female gastrocnemius muscle. AR^{lox} ♀, AR^{lox} heterozygote female; ARKO ♀, $AR^{\Delta,lox}$/CMV-cre heterozygote female. GAPDH shows amplification of a control genomic locus. *D*) Quantitative real-time PCR using exon 3-specific probe, showing expression of normal *AR* mRNA in WT male muscles: levator ani (LA), extensor digitorum longus (EDL), tibialis anterior (TA), soleus (SOL), gastrocnemius (GAST), and ARKO male gastrocnemius (ARKO GAST) (n=3/group, mean±SE). *E*) RT-PCR from WT and ARKO male gastrocnemius muscle (n=3/group) using primers flanking *AR* exon 3, demonstrating AR^{Δ} mRNA (309 bp band) in ARKO males.

TABLE 1. *Serum IGF1 levels in WT and ARKO males*

Age (wk)	WT (ng/ml)	ARKO (ng/ml)	*P*
9	253.8 ± 23.9 (12)	222.1 ± 16.4 (12)	0.286
12	213.8 ± 13.1 (13)	221.9 ± 19.9 (12)	0.740

Values are means ± SE; number/group in parentheses. *P* values calculated using Student's *t* test, ARKO *vs.* age-matched WT.

ARKO males have reduced muscle mass but ARKO females are normal

Body mass and muscle mass were examined in ARKO males at 9 and 12 wk of age, and in ARKO females at 9 wk. At both ages, body mass in ARKO males was intermediate between that of WT males and females (**Fig. 2*A***, **Table 2** and data not shown), with a 12–13% reduction in body mass compared with WT males ($P<0.001$). In contrast, there was no difference in body mass between ARKO females and control females (WT and CMV-cre heterozygotes) (Fig. 2*A*).

We measured the mass of a number of hind limb muscles, including the fast-twitch tibialis anterior (TA) and extensor digitorum longus (EDL), the slow-twitch soleus (SOL), and the mixed-fiber gastrocnemius (GAST) muscles. At 9 wk of age, the mass of all hind limb muscles was significantly reduced in ARKO males compared with WT males (Fig. 2*B–E*), with up to a 20% reduction in mass of individual muscles. This change in absolute mass reflects both direct (muscle-specific) and indirect effects of AR deletion, but even when adjusted for total body mass, which is itself androgen-dependent (14, 16), TA mass was still significantly reduced in 9 wk ARKO males (Fig. 2*F*). At 12 wk, a similar significant decrease in absolute muscle mass was observed in all muscles of the ARKO *vs.* WT males (Table 2). The percentage decrease in muscle mass in ARKO males was smaller when adjusted for total body mass (Table 2); however, when adjusted for heart mass, which is not androgen-dependent and controls for differences in total body size, muscle mass was still significantly reduced in ARKO males, by 7–16% (Table 2). The highly androgen-dependent levator ani muscle, a perineal muscle that is present in males but fails to develop in female rodents (17), did not develop in ARKO males (data not shown).

ARKO females were examined at 9 wk of age. In contrast to the ARKO males, there was no difference in muscle mass between ARKO females and control WT and CMV-cre heterozygote female littermates (Fig. 2*B–E*).

ARKO male muscles have reduced strength and increased resistance to fatigue

To determine the AR-mediated actions of androgens on muscle function, we analyzed muscle contractile properties *in vitro* from fast-twitch EDL and slow-twitch SOL muscles in ARKO males and WT males and females. As no effect of AR deletion was observed on ARKO female muscle mass, ARKO females were not included in the functional studies. Maximum tetanic force, a measure of muscle contractile strength, was significantly decreased in the EDL from ARKO males *vs.* WT males ($P=0.001$), down to the level of WT females (**Fig. 3*A***); however, there was no difference in the SOL muscle between ARKO and WT males (Fig. 3*A*). To determine whether the changes in maximum tetanic force were due to changes in intrinsic contractile function, specific force was calculated (16). There was no difference in EDL-specific force between the ARKO males, WT males, and WT females (Fig. 3*B*), suggesting the decrease in strength in the ARKO males is caused by the decreased muscle mass.

To measure the ability of muscles to resist fatigue, they were stimulated to contract with maximum force every 4 s for 4 min, with tetanic force produced during this period determined. For the EDL muscle, there was no difference in the fatigue resistance of ARKO males, WT males, and WT females (Fig. 3*C*). In contrast, for the SOL muscle, WT females were more fatigue resistant than WT males, evidenced by a higher force maintained over the contraction period ($P<0.001$)

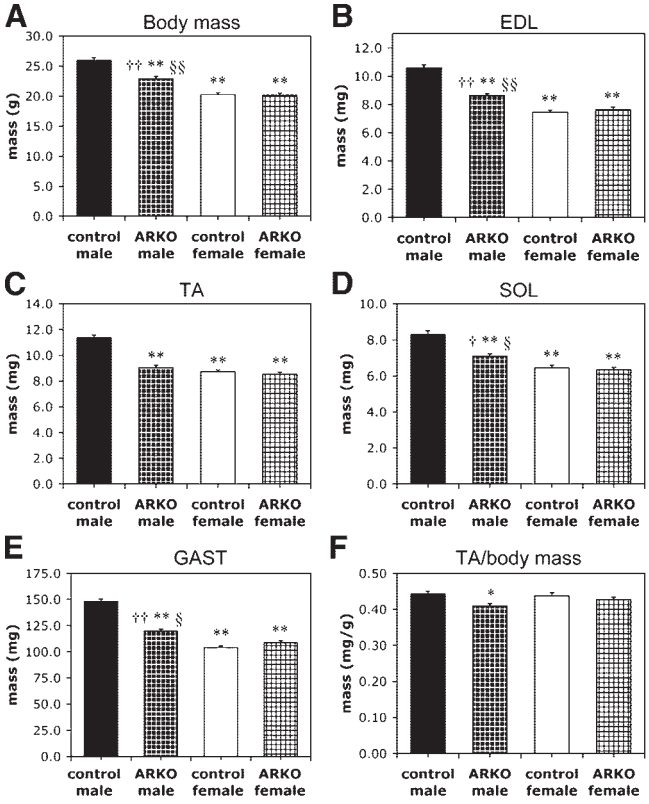

Figure 2. ARKO muscle mass. Nine-week control (WT) male ($n=21$), ARKO male ($n=26$), control (WT and CMV-cre heterozygote) female ($n=21$) and ARKO female ($n=11$) mice. *A*) Body mass. *B*) EDL mass. *C*) TA mass. *D*) SOL mass. *E*) GAST mass. *F*) TA/body mass. Data presented as mean ± SE. $^{*}P < 0.05$, $^{**}P \leq 0.001$ *vs.* control male; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$ *vs.* control female; $^{\S}P < 0.05$, $^{\S\S}P < 0.001$ *vs.* ARKO female (1-way ANOVA, Tukey's *post hoc* test).

TABLE 2. *Muscle mass in 12-wk-old WT and ARKO males*

Measure	Wild-type	ARKO	Decrease in ARKO (%)	*P*
Body (g)	29.1 ± 0.3 (17)	25.4 ± 0.6 (19)	12.6	0.000
Heart (mg)	149.2 ± 3.5 (17)	134.9 ± 3.7 (19)	9.6	0.009
Heart/body (mg/g)	5.12 ± 0.10 (17)	5.34 ± 0.11 (19)	NC	NS
EDL (mg)	10.6 ± 0.3 (12)	8.1 ± 0.2 (13)	23.1	0.000
EDL/body (mg/g)	0.364 ± 0.011 (12)	0.322 ± 0.006 (13)	11.4	0.002
EDL/heart (mg/mg)	0.071 ± 0.003 (12)	0.069 ± 0.002 (13)	13.2	0.008
TA (mg)	12.3 ± 0.2 (12)	9.3 ± 0.2 (13)	25.0	0.000
TA/body (mg/g)	0.425 ± 0.004 (12)	0.368 ± 0.009 (13)	13.3	0.000
TA/heart (mg/mg)	0.082 ± 0.002 (12)	0.069 ± 0.002 (13)	15.8	0.000
SOL (mg)	12.3 ± 0.2 (12)	9.3 ± 0.2 (13)	25.0	0.000
SOL/body (mg/g)	0.425 ± 0.004 (12)	0.368 ± 0.009 (13)	13.3	0.000
SOL/heart (mg/mg)	0.082 ± 0.002 (12)	0.069 ± 0.002 (13)	15.8	0.000
GAST (mg)	163.3 ± 2.3 (14)	127.8 ± 1.7 (23)	21.7	0.000
GAST/body (mg/g)	5.60 ± 0.06 (14)	5.27 ± 0.08 (23)	5.9	0.008
GAST/heart (mg/mg)	1.07 ± 0.02 (14)	0.99 ± 0.02 (20)	7.6	0.010

Mass shown as absolute mass and adjusted for total body mass and heart mass. Values are means ± SE; number/group in parentheses. *P* values calculated using Student's *t* test, ARKO *vs.* WT. EDL, extensor digitorum longus; TA, tibialis anterior; SOL, soleus; GAST, gastrocnemius; NC, no change; NS, nonsignificant.

(Fig. 3*C*). Furthermore, ARKO males were equivalent to WT females and more fatigue resistant than WT males ($P<0.05$) (Fig. 3*C*).

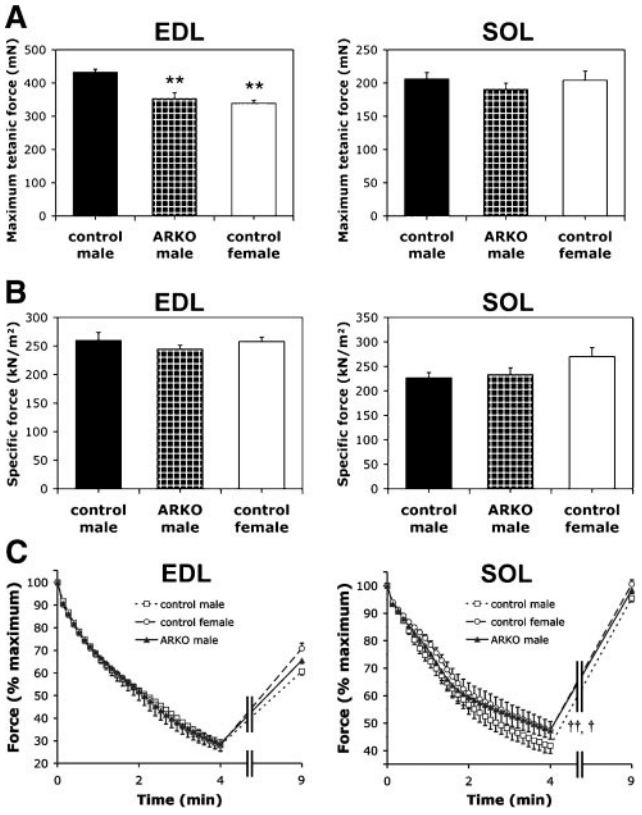

Figure 3. ARKO muscle function. *In vitro* analyses in fast-twitch EDL and slow-twitch SOL muscles from 9 wk WT males, ARKO males, and WT females. *A*) Maximum tetanic force. *B*) Specific force. *C*) Fatigue. Data presented as mean ± SE; $n = 6$/group. $^{*}P < 0.05$, $^{**}P \leq 0.001$ *vs.* WT male (1-way ANOVA, Tukey's *post hoc* test). $^{\dagger}P < 0.05$, ARKO male *vs.* WT male; $^{\dagger\dagger}P < 0.001$, control female *vs.* WT male (general linear model univariate analysis, Tamhane's *post hoc* test).

Altered gene expression in ARKO male muscle

To identify target genes mediating the structural and functional differences in the ARKO male muscle, microarray analysis was performed on WT and ARKO male gastrocnemius muscle. A suite of 93 genes was identified that showed ≥1.6-fold differences in expression, comprising 46 genes down-regulated in the ARKO and 47 genes up-regulated (**Table 3** and data not shown). Genes in a number of functional categories were identified, including contractile/structural, transcriptional regulation, immune/inflammatory response, growth factors/signal transduction, energy metabolism, and cell cycle and transport. Gene ontology (GO) mining performed on the androgen-responsive genes identified significantly overrepresented functional processes, with the most highly overrepresented being polyamine biosynthesis (χ^2 analysis, $P<0.001$). S-adenosylmethionine decarboxylase 1 (*Amd1*), ornithine decarboxylase 1 (*Odc1*) and spermine oxidase (*Smox*) were all down-regulated in the ARKO. A cluster of genes with a smaller magnitude up-regulation in the ARKO male muscle was characteristic of a fast-to-slow-twitch phenotype change (**Fig. 4**). Other genes previously demonstrated to play a role in regulation of muscle atrophy or hypertrophy were unchanged (**Table 4**).

Microarray results were confirmed by Q-PCR on a subset of genes, chosen based on their overrepresentation from GO mining, their putative role in regulation of proliferation/differentiation or signaling pathways, or their potential role in skeletal muscle. Q-PCR demonstrated that expression of *Amd1* was decreased 10-fold in ARKO muscle and *Odc1* was decreased 3-fold (**Fig. 5*A***). Expression of other regulatory genes, *Wnt4*, frizzled 4 (*Fzd4*), cyclin dependent kinase inhibitor 1c (*Cdkn1c*) ($p57^{Kip2}$) and protein phosphatase 3, catalytic subunit α (*Ppp3ca*) (calcineurin A) was significantly increased in ARKO muscle, and integrin β1 binding protein 3 (*Itgb1bp3*) was significantly decreased (Fig.

TABLE 3. *Changes in gene expression from 9-wk-old ARKO vs. WT male gastrocnemius muscle from microarray analysis*

Gene	Accession no.	Name	Mean WT	Mean ARKO	Fold change in ARKO	*P*	Process or function
			Structural/muscle				
Actr3b	BB125424	Actin related protein 3b	1.95	0.29	6.6 decrease	0.021	Actin filament formation
Itgb1bp3	AK009352	Integrin beta 1 binding protein 3	1.90	0.51	3.7 decrease	0.078	Negative regulation of myoblast differentiation
D130058I21Rik	BB458758	RIKEN cDNA D130058I21	1.40	0.67	2.1 decrease	<0.001	Cytoskeleton
Casq2	NM_009814	Calsequestrin 2 (cardiac calsequestrin)	0.70	1.30	1.9 increase	<0.001	Muscle contraction
Sorbs2	BB251748	Sorbin and SH3 domain containing 2 (Arg binding protein 2)	1.32	0.73	1.8 decrease	0.009	Actin cytoskeleton
Kif3c	BM898710	Kinesin family member 3C	1.30	0.75	1.7 decrease	<0.001	Microtubule-based movement
Mybph	NM_016749	Myosin binding protein H	0.50	1.45	2.9 increase	0.008	Muscle contraction
Myl4	NM_010858	Myosin, light polypeptide 4, alkali; atrial, embryonic	0.64	1.34	2.1 increase	0.002	Muscle contraction
Tnnt1	NM_011618	Troponin T1, skeletal, slow	0.74	1.38	1.9 increase	0.043	Muscle contraction
Csrp3	NM_013808	Cysteine and glycine-rich protein 3 (muscle LIM protein)	0.73	1.19	1.6 increase	0.035	Myogenesis
Myl2	NM_010861	Myosin, light polypeptide 2, regulatory, cardiac, slow	0.80	1.22	1.5 increase	0.032	Muscle contraction
			Polyamine biosynthesis				
Amd1	NM_009665	S-adenosylmethionine decarboxylase 1	1.69	0.35	4.8 decrease	<0.001	Spermine/spermidine biosynthesis
Smox	BC004831	Spermine oxidase	1.58	0.55	2.9 decrease	<0.001	Spermine catabolism
Odc1	C81193	Ornithine decarboxylase, structural	1.44	0.59	2.5 decrease	<0.001	Polyamine biosynthesis
			Transcriptional regulation				
Irx3	NM_008393	Iroquois related homeobox 3 (*Drosophila*)	1.42	0.58	2.4 decrease	<0.001	Transcriptional regulation
Ankrd6	BM225135	Ankyrin repeat domain 6	1.31	0.81	1.6 decrease	0.002	Transcriptional regulation
Cited2	NM_010828	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.53	1.55	2.9 increase	<0.001	Transcriptional regulation
Dbp	BC018323	D site albumin promoter binding protein	0.62	1.19	1.9 increase	0.007	Transcriptional regulation; circadian rhythm
Peg3	AB003040	Paternally expressed 3	0.68	1.28	1.9 increase	<0.001	Transcriptional regulation; imprinting
Mkl1	BM196656	Megakaryoblastic leukemia)/ myocardin-like 1 (myocardin related transcription factor A)	0.75	1.22	1.6 increase	0.010	Transcriptional regulation
Nsbp1	NM_016710	Nucleosome binding protein 1	0.77	1.20	1.6 increase	0.007	Transcriptional regulation
Pdlim1	NM_016861	PDZ and LIM domain 1 (elfin)	0.80	1.24	1.6 increase	0.009	Transcriptional regulation

TABLE 3. (*continued*)

Gene	Accession no.	Name	Mean WT	Mean ARKO	Fold change in ARKO	*P*	Process or function
			Immune/inflammatory response				
C3	K02782	Complement component 3	1.61	0.59	2.7 decrease	0.051	Complement activation, alternative pathway; complement activation, classical pathway; inflammatory response
Pla2g7	AK005158	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	1.45	0.56	2.6 decrease	0.024	Inflammatory response; lipid catabolism
Cd24a	NM_009846	CD24a antigen	1.38	0.64	2.2 decrease	<0.001	Defense response
Lyzs	AV058500	Lysozyme	1.52	0.73	2.1 decrease	0.005	Carbohydrate metabolism; cell wall catabolism; cytolysis; defense response to bacteria
Igh-6	AI326478	Immunoglobulin heavy chain 6 (heavy chain of IgM)	1.40	0.86	1.6 decrease	0.046	Humoral defense mechanism (sensu Vertebrata); regulation of B-cell proliferation
Lzp-s	NM_013590	P lysozyme structural	1.34	0.82	1.6 decrease	0.023	Carbohydrate metabolism; cell wall catabolism; cytolysis; defense response to bacteria
Ccl9	AF128196	Chemokine (C-C motif) ligand 9	1.34	0.87	1.5 decrease	0.032	Chemotaxis; immune response; signal transduction
Cd28	AV313615	CD28 antigen	0.52	1.53	2.9 increase	<0.001	Immune response; positive regulation of T-cell proliferation
			Growth factor/signal transduction				
Cnksr1	BB739754	Connector enhancer of kinase suppressor of Ras 1	1.31	0.74	1.8 decrease	0.010	Ras protein signal transduction; rho protein signal transduction
Mchr1	BE647763	Melanin-concentrating hormone receptor 1	1.26	0.76	1.7 decrease	0.017	Neuropeptide signaling pathway
Dkk3	AK004853	Dickkopf homolog 3 (*Xenopus laevis*)	1.27	0.84	1.5 decrease	0.013	Wnt signaling pathway
Nog	NM_008711	Noggin	1.20	0.79	1.5 decrease	0.047	BMP antagonist
Sytl2	NM_031394	Synaptotagmin-like 2	0.63	1.39	2.2 increase	0.033	Rab27A effector molecule
Arrdc3	BG072824	Arrestin domain containing 3	0.67	1.28	1.9 increase	0.028	Receptor internalization
Fzd4	BF783030	Frizzled homolog 4 (*Drosophila*)	0.79	1.30	1.6 increase	<0.001	Wnt signaling pathway
Igf2	NM_010514	Insulin-like growth factor 2	0.83	1.26	1.5 increase	0.030	Growth factor
Tgfb2	BF144658	Transforming growth factor, beta 2	0.81	1.22	1.5 increase	<0.001	Growth factor
			Energy/metabolism				
Cbr2	BC010758	Carbonyl reductase 2	1.75	0.33	5.3 decrease	0.003	NADH oxidation; metabolism
Aldh1a1	NM_013467	Aldehyde dehydrogenase family 1, subfamily A1 (retinaldehyde dehydrogenase)	1.53	0.47	3.3 decrease	0.006	Metabolism; retinoic acid metabolism
Mpa2l	BM241485	Macrophage activation 2 like	1.51	0.51	3.0 decrease	<0.001	Metabolism

TABLE 3. (*continued*)

Gene	Accession no.	Name	Mean WT	Mean ARKO	Fold change in ARKO	*P*	Process or function
Mgst1	BI150149	Microsomal glutathione S-transferase 1	1.38	0.68	2.0 decrease	0.044	Glutathione metabolism
Bdh1	BF322712	3-Hydroxybutyrate dehydrogenase (heart, mitochondrial)	1.30	0.72	1.8 decrease	<0.001	Metabolism
Cyp27a1	NM_024264	Cytochrome P450, family 27, subfamily a, polypeptide 1	1.27	0.72	1.8 decrease	<0.001	Electron transport
Xlkd1	AV124537	Extracellular link domain-containing 1	1.42	0.77	1.8 decrease	0.019	Glycosaminoglycan catabolism
Neu2	AK009828	Neuraminidase 2	0.65	1.38	2.1 increase	0.013	Carbohydrate metabolism
Gstm2	NM_008183	Glutathione S-transferase, mu 2 (Gstb2)	0.72	1.23	1.7 increase	0.009	Metabolism
St3gal5	BB829192	Sialyltransferase 9 (CMP-NeuAc: lactosylceramide alpha-2,3-sialyltransferase) (GM3 synthase)	1.25	0.80	1.6 decrease	0.001	Ganglioside biosynthesis
Gamt	AF015887	Guanidinoacetate methyltransferase	0.77	1.25	1.6 increase	0.021	Creatine biosynthesis
Cell cycle							
Cdc2l6	NM_198164	Cell division cycle 2-like 6 (CDK8-like)	1.54	0.50	3.1 decrease	<0.001	Cell cycle regulation
Cdkn1c	NM_009876	Cyclin-dependent kinase inhibitor 1C (p57)	0.70	1.42	2.0 increase	0.046	Cell cycle regulation; cdk inhibitor
Sesn1	AV016566	Sestrin 1	0.66	1.29	2.0 increase	<0.001	Cell cycle regulation
Hipk2	AF170301	Homeodomain interacting protein kinase 2	1.29	0.77	1.7 decrease	<0.001	Negative cell cycle regulation
Gadd45a	NM_007836	Growth arrest and DNA-damage-inducible 45 alpha	0.75	1.23	1.6 increase	0.013	Cell cycle regulation
		Imprinted					
Xlr4	NM_021365	X-linked lymphocyte-regulated 4	1.04	0.69	1.5 decrease	0.016	Imprinted
Peg3	AB003040	Paternally expressed 3	0.68	1.28	1.9 increase	<0.001	Transcriptional regulation; imprinted
Rian	AB076245	RNA imprinted and accumulated in nucleus (snoRNA)	0.83	1.29	1.5 increase	0.055	Imprinted
		Transport					
BC011467	BC025823	cDNA sequence BC011467 (spinster-like protein)	1.62	0.59	2.7 decrease	0.063	Transport
Slc40a1	AF226613	Solute carrier family 40 (iron-regulated transporter), member 1 (ferroportin 1)	1.40	0.63	2.2 decrease	<0.001	Iron ion transport
Slc38a4	AK003626	Solute carrier family 38, member 4 (Na-dependent neutral amino acid transporter); (system A amino acid transporter 3)	1.23	0.73	1.7 decrease	<0.001	Amino acid transport; imprinted
Slc30a2	AW542735	Solute carrier family 30 (zinc transporter), member 2	1.28	0.80	1.6 decrease	0.003	Zinc ion transport
Atp1b4	NM_133690	ATPase, $(Na^+)/K^+$ transporting, beta 4 polypeptide	0.62	1.28	2.1 increase	0.055	Potassium/sodium ion transport

TABLE 3. (*continued*)

Gene	Accession no.	Name	Mean WT	Mean ARKO	Fold change in ARKO	*P*	Process or function
Atp1b2	BG261955	ATPase, Na^+/K^+ transporting, beta 2 polypeptide (AMOG)	0.79	1.25	1.6 increase	<0.001	Potassium/sodium ion transport; mTOR signaling pathway
Kcnc4	BC024837	Potassium voltage gated channel, Shaw-related subfamily, member 4 (Kv3.4)	0.77	1.24	1.6 increase	0.001	Potassium ion transport
Kcnu1	NM_008432	Potassium channel, subfamily U, member 1 (potassium large conductance pH-sensitive channel, subfamily M, alpha member 3)	0.80	1.30	1.6 increase	0.048	Potassium ion transport
		Miscellaneous					
Hmga2-ps1	AV377334	High mobility group AT-hook 2, pseudogene1	1.53	0.46	3.3 decrease	<0.001	
Crip3	AF367970	Cysteine-rich protein 3 (thymus LIM protein)	1.53	0.67	2.3 decrease	0.048	T-cell proliferation
Serpinb6a	NM_009254	Serine (or cysteine) proteinase inhibitor, clade B, member 6a	1.36	0.65	2.1 decrease	0.001	Serine-type endopeptidase inhibitor activity
Golph2	BC011152	Golgi phosphoprotein 2 (GP73)	1.28	0.66	1.9 decrease	0.013	
Zmynd17	AK014794	Zinc finger, MYND domain containing 17	1.34	0.70	1.9 decrease	0.047	
Phlda3	NM_013750	Pleckstrin homology-like domain, family A, member 3 (Tih1)	1.27	0.70	1.8 decrease	0.027	
Timp4	BI788452	Tissue inhibitor of metalloproteinase 4	1.33	0.75	1.8 decrease	0.003	Metalloendopeptidase inhibitor activity
Fkbp5	U16959	FK506 binding protein 5	1.28	0.73	1.7 decrease	0.035	Chaperone protein; known androgen responsive
Tceal7	BB378019	Transcription elongation factor A (SII)-like 7	0.39	1.80	4.6 increase	0.020	Translation elongation factor activity
9630055N22Rik	BB276950	Adult retina cDNA, RIKEN full-length enriched library, clone: A930001C09	0.40	1.54	3.9 increase	0.004	
Chac1	BC025169	Cation transport regulator-like 1	0.41	1.54	3.7 increase	0.003	
Zfp503	BB447914	Zinc finger protein 503	0.51	1.52	3.0 increase	0.013	
AI448196	BE865094	Expressed sequence AI448196	0.59	1.44	2.4 increase	0.001	
Bhlhb9	AK012577	Basic helix-loop-helix domain containing, class B9	0.58	1.36	2.3 increase	0.008	
BB001228	BG071655	Clone IMAGE:4507681, mRNA	0.62	1.44	2.3 increase	0.019	
BC021831	BC021831	cDNA clone MGC: 67258 IMAGE:6413648, complete cds	0.65	1.43	2.2 increase	0.014	
5430432N15Rik	AK017382	RIKEN cDNA 5430432N15	0.69	1.30	1.9 increase	0.017	
A930003A15Rik	BB522820	15 days embryo head cDNA, RIKEN full-length enriched library, clone: D930010E11	0.71	1.32	1.9 increase	0.023	

TABLE 3. (*continued*)

Gene	Accession no.	Name	Mean WT	Mean ARKO	Fold change in ARKO	*P*	Process or function
Otud1	BE687142	OTU domain containing 1	0.77	1.44	1.9 increase	0.021	
Tceal3	AI837848	Transcription elongation factor A (SII)-like 3	0.75	1.37	1.8 increase	0.048	Translation elongation factor activity
2210403K04Rik	AK008813	RIKEN cDNA 2010305C02	0.71	1.30	1.8 increase	0.001	
2610042L04Rik	BM195235	RIKEN cDNA 2610042L04	0.76	1.35	1.8 increase	0.005	
Angptl2	BG244279	Angiopoietin related protein 2	0.72	1.31	1.8 increase	<0.001	
Erdr1	AJ007909	Erythroid differentiation regulator 1	0.69	1.27	1.8 increase	0.029	
1110033L15Rik	AK011824	RIKEN cDNA 1110033L15	0.76	1.26	1.7 increase	0.008	
Frem2	BM201912	Fras1 related extracellular matrix protein 2	0.76	1.24	1.6 increase	0.006	Cell adhesion; cell communication
Klhl4	BB037068	Kelch-like 4 (*Drosophila*)	0.77	1.26	1.6 increase	0.016	
sm6	BE949068	LSM6 homolog, U6 small nuclear RNA associated	0.79	1.24	1.6 increase	<0.001	Nuclear mRNA splicing

5*B*). Expression of transcription elongation factor A (SII)-like 7 (*Tceal7*) was also increased in ARKO muscle (Fig. 5*C*). Growth factor genes insulin-like growth factor 2 (*Igf2*) and transforming growth factor β2 (*Tgfb2*) were up-regulated in ARKO muscle; however, there was no change in the expression of *Igf1* in muscle (Fig. 5*C*).

Altered gene expression in orchidectomized male muscle

We previously showed that 10 wk postorchidectomy, WT male mice have decreased muscle mass and strength, and this effect is prevented by testosterone supplementation (16). We therefore examined the expression of the androgen-responsive genes identified above in gastrocnemius from control orchidectomized (orx+C) and testosterone-treated orchidectomized (orx+T) males. The majority of genes showed a similar pattern of expression in the orx+C *vs.* orx+T groups to that observed in the ARKO males (**Fig. 6**). We also confirmed that *Igf1* expression was not altered by orchidectomy and testosterone treatment (data not shown). These results indicate that many of the same androgen-dependent pathways are regulated during androgen deprivation-dependent muscle atrophy as during androgen/AR-mediated muscle growth.

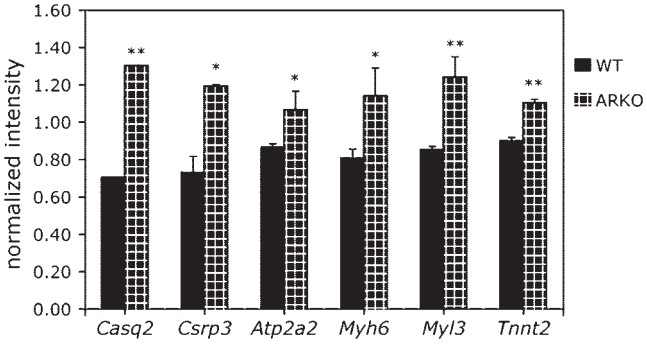

Figure 4. Microarray analysis of gene expression in ARKO muscle. Normalized intensity of signal from 9 wk WT and ARKO male gastrocnemius muscle, showing up-regulation of slow-twitch phenotype genes in the ARKO. Calsequestrin 2 (*Casq2*); cysteine and glycine-rich protein 3 (*Csrp3* or muscle LIM protein); ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2 (*Atp2a2*); myosin, heavy polypeptide 6, cardiac muscle α (*Myh6*); myosin, light polypeptide 3 (*Myl3*); and troponin T2, cardiac (*Tnnt2*). Data presented as mean ± SE; $n = 2$/group. $^*P < 0.05$, $^{**}P < 0.001$ *vs.* WT (Student's *t* test).

DISCUSSION

Our findings demonstrate that androgens play a normal physiological role in achieving peak muscle mass in males, but not females, as ARKO male mice, which have deletion of the genomic actions of the AR, have decreased muscle mass, but ARKO female mice have normal muscle mass. Androgens also act through the AR to increase strength but decrease fatigue resistance in male muscle. Genes associated with a number of regulatory pathways show altered expression in ARKO muscle and also orchidectomized male muscle, including genes involved in polyamine biosynthesis, Wnt signaling, regulation of fiber type, and modulation of muscle differentiation. These results indicate that the anabolic actions of androgens occur *via* multiple pathways in skeletal muscle.

The lack of effect of AR deletion on muscle mass in

TABLE 4. *Genes of interest showing no significant change in 9-wk-old ARKO vs.* WT male gastrocnemius muscle, from microarray analysis

Gene	Accession no.	Name	Mean WT	Mean ARKO
Akt1	BE952690	Thymoma viral proto-oncogene 1	0.91	1.11
Ar	BB553658	Androgen receptor	0.96	0.88
Cdkn1a	NM_007669	Cyclin-dependent kinase inhibitor 1A (p21)	0.89	0.96
Fbxo32	AF441120	F-box only protein 32	1.27	0.88
Foxo1	AI462296	Forkhead box O1	1.15	0.98
Foxo3	BM245920	Forkhead box O3	1.00	0.99
Gata2	NM_008090	GATA binding protein 2	1.02	0.98
Gsk3b	BQ173949	Glycogen synthase kinase 3β	0.93	0.96
Igf1	AF440694	Insulin-like growth factor 1	0.97	0.95
Igf1r	BE980124	Insulin-like growth factor I receptor	0.98	1.09
Igf2r	BG092290	Insulin-like growth factor 2 receptor	0.99	0.98
Myf5	NM_008656	Myogenic factor 5	0.91	1.07
Myf6	NM_008657	Myogenic factor 6 (MRF4)	1.03	1.00
Myod1	NM_010866	Myogenic differentiation 1	0.86	0.86
Myog	NM_031189	Myogenin	0.87	1.11
Pax7	AF254422	Paired box gene 7	1.00	0.98

females is strongly suggestive that androgens are not required to achieve peak muscle mass in sedentary females. Thus, although female muscle expresses the AR and is capable of an anabolic response when exogenous androgens are administered (5), our data suggest that androgens play little or no role in regulating normal female muscle mass. It remains to be demonstrated whether treatment of females with supraphysiological doses of androgens would activate the same pathways that we identified in the ARKO male muscle.

In males, androgens have been proposed to promote muscle hypertrophy through a number of different cellular pathways, including stimulating mesenchymal commitment into the myogenic lineage (18), promoting satellite cell or myoblast proliferation (11, 19), and increasing muscle protein synthesis (20). The LA muscle is one of the most androgen-dependent muscles (21), with satellite cells in the female undergoing apoptosis during perinatal development causing failure of the LA to develop in adult females (22, 23). The complete lack of development of the LA muscle in adult ARKO males indicates that these actions of androgens occur through the AR, during muscle development. Previous studies have suggested that androgens also regulate myofiber protein turnover (20). In microarray analysis of ARKO muscle, there was no change in expression of genes encoding the major muscle contractile proteins, nor was there any change in the expression of ubiquitin ligase genes up-regulated during muscle atrophy. However, there was a significant reduction in the expression of genes encoding cytoskeletal proteins. Therefore, our data suggest that androgens regulate muscle mass both through control of muscle cell commitment or proliferation and through regulation of muscle protein balance.

In ARKO males, fast-twitch muscle mass and force production were reduced proportionally, down to the level of WT females, indicating muscle strength is dependent on muscle mass, which is controlled by androgens in males. This decrease in force production from the EDL muscle was similar to the decrease that we previously observed in WT C57BL/6 males 10 wk postorchidectomy, compared to testosterone-treated orchidectomized mice (16). In contrast, the slow-twitch soleus muscle showed a significant increase in fatigue resistance in ARKO males, also equivalent to WT female soleus. Previous studies have shown that female muscles are more fatigue resistant than male (24), and our results indicate that this occurs through an AR-dependent effect. In ARKO male muscle, there was increased expression of genes characteristic of slow-twitch phenotype (Fig. 4). These data suggest that androgens promote the fast-twitch phenotype, favoring type II glycolytic fibers that produce higher force but are highly fatigable. Our previous orchidectomy study showed that testosterone treatment of orchidectomized males also paradoxically increased the fatigue resistance of the soleus muscle compared to control orchidectomized mice (16). This difference in findings could be caused by the effect of aromatization of testosterone to estradiol in our previous study, as supraphysiological doses of testosterone were used, or may reflect differences in the effect of loss of androgen action throughout development in the ARKO muscle *vs.* androgen deprivation in adult muscle in the orchidectomy study.

Microarray analysis ruled out known myogenic regulatory factors as being targets of the AR, and there was no change in the expression of other factors implicated in regulation of muscle atrophy and hypertrophy (25). Q-PCR showed that expression of *Ppp3ca* (calcineurin A) was increased in muscle from both ARKO and orchidectomized males, despite the fact that up-regulation of calcineurin signaling pathways has been implicated in IGF1-dependent muscle hypertrophy (26). Other studies do not support a role for calcineurin in muscle hypertrophy (27), and our data are more consistent with the demonstrated function of calcineurin A

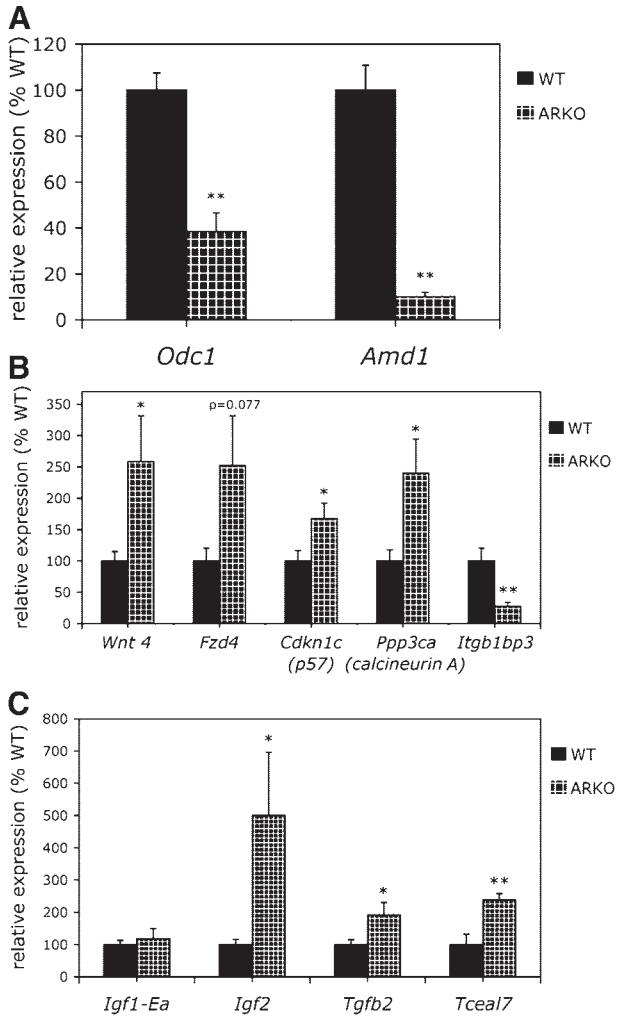

Figure 5. Quantitative real-time PCR analysis of gene expression in ARKO muscle. Relative gene expression (normalized to WT gastrocnemius) in 9 wk WT and ARKO male gastrocnemius muscle. *A*) *Odc1* and *Amd1*. *B*) *Wnt4*; frizzled 4 (*Fzd4*); cyclin dependent kinase inhibitor 1c (*Cdkn1c* or $p57^{Kip2}$); protein phosphatase 3, catalytic subunit α (*Ppp3ca* or *calcineurin A*); and integrin β1 binding protein 3 (*Itgb1bp3*). *C*) Insulin-like growth factor I-Ea (*Igf1-Ea*), insulin-like growth factor 2 (*Igf2*), transforming growth factor β2 (*Tgfb2*), and transcription elongation factor A (SII)-like 7 (*Tceal7*). Data presented as mean ± SE; $n = 12$/group. $^*P < 0.05$, $^{**}P < 0.001$ *vs.* WT (Student's *t* test).

as an inducer of slow-twitch fiber phenotype (27) than as a mediator of hypertrophy.

Our results suggest that one of the major regulatory mechanisms of androgens in skeletal muscle is regulation of polyamine biosynthesis. Q-PCR confirmed that there was a significant decrease in the expression of the genes encoding the rate-limiting polyamine biosynthetic enzymes *Odc1* and *Amd1* in ARKO males. Polyamines have been implicated in numerous cellular processes, including transcription, cell proliferation, and apoptosis (reviewed in ref. 28). Data suggest that polyamines play a role in muscle hypertrophy (29), although their mechanisms of action are still unknown. *Odc1* is androgen responsive in the murine kidney and is directly regulated by the AR (30). Expression of *Odc1* and *Amd1* was also decreased in muscle from orchidectomized males compared to orchidectomized males treated with testosterone. Thus, it is likely that one of the major pathways *via* which androgens increase or maintain muscle mass is through increased polyamine synthesis, *via* up-regulation of *Amd1* and *Odc1* expression.

Expression of the cell cycle regulatory gene *Cdkn1c* ($p57^{Kip2}$), which couples differentiation and cell cycle exit in myoblasts (31), was increased in ARKO muscle. This may limit the number of cycles of myoblast proliferation and cause premature differentiation, contributing to reduced muscle mass in ARKO muscle. Testosterone has been shown to have no effect on proliferation of porcine primary myoblasts but suppresses differentiation into myotubes (32). Therefore, the anabolic actions of androgens in muscle may occur in part *via* the AR repressing $p57^{Kip2}$ expression, thus maintaining myoblasts in the proliferative state and allowing prolonged muscle growth. The expression pattern of other genes in ARKO muscle is also consistent with the proposed mechanism that androgens maintain myoblasts in the proliferative state and delay differentiation. Itgb1bp3 is a negative regulator of muscle differentiation (33). Expression of *Itgb1bp3* is high in proliferating C2C12 myoblasts *in vitro,* and overexpression of *Itgb1bp3* prevents terminal myogenic differentiation (33). The expression of *Itgb1bp3* is decreased in ARKO muscle, consistent with a smaller, more differentiated muscle. Similarly, *Igf2* expression, which is increased in ARKO muscle compared with WT, is up-regulated in differentiated myotubes *in vitro* (34), and may drive terminal myogenic differentiation (35).

Wnt signaling through both canonical and noncanonical pathways occurs during embryonic muscle determination, and muscle regeneration and hypertrophy (36). Although the expression of *Wnt4* is up-regulated in muscles from myostatin-null mice, which exhibit fiber hyperplasia (37); *Wnt4* and one of its receptors, *Fzd4*, were also up-regulated in ARKO and orchidecto-

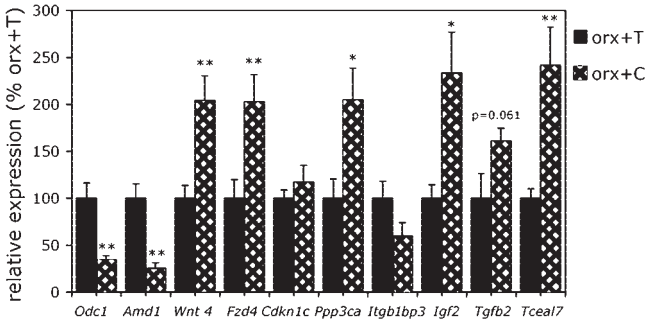

Figure 6. Quantitative real-time PCR analysis of gene expression in orchidectomized male muscle. Relative gene expression (normalized to testosterone-treated orchidectomized males gastrocnemius) in gastrocnemius muscle from testosterone-treated orchidectomized (orx+T) and control-orchidectomized (orx+C) males, 10 wk postorchidectomy and treatment (16): *Odc1*, *Amd1*, *Wnt4*, *Fzd4*, *Cdkn1c*, *Ppp3ca*, *Itgb1bp3*, *Igf2*, *Tgfb2*, and *Tceal7*. Data presented as mean ± SE; $n = 9$/group. $^*P < 0.05$, $^{**}P < 0.01$ *vs.* orx+T (Student's *t* test).

mized males, and the negative regulator *Dkk3* was down-regulated. This finding suggests that up-regulation of components of the Wnt signaling pathway is not sufficient to induce hypertrophy, but instead can also be associated with reduced muscle mass.

IGF1 has been a proposed mediator of the anabolic actions of androgens in muscle, as IGF1 is a potent muscle growth factor, and data suggest that androgens can regulate circulating or local muscle IGF1 levels (13, 38). However, serum IGF1 levels did not differ between WT and ARKO males, Q-PCR demonstrated that *Igf1* gene expression in ARKO muscle was normal, and expression was also not affected by orchidectomy. From microarray analysis, there was also no difference in the expression of the genes encoding the IGF binding proteins, or the IGF1 receptor. Therefore, it is likely that the anabolic actions of androgens in skeletal muscle are not mediated *via* modulation of IGF1 production or action.

The ARKO male muscle phenotype is likely to be caused by absence of androgen action during both embryonic development and in the peri- to postpubertal period, when androgen levels are high in males. We and others have previously investigated muscle responses to androgen deprivation or supplementation in adult males (3, 16). Orchidectomy causes androgen withdrawal-dependent muscle atrophy, whereas in our ARKO model, WT males have androgen-dependent muscle growth compared to the androgen-insensitive ARKO males. Therefore, different androgen-dependent pathways could potentially be regulated during skeletal muscle atrophy following orchidectomy, compared to ARKO muscle, which fails to develop peak muscle mass but has not undergone atrophy. However, our data demonstrate that a number of androgen-responsive genes show the same pattern of expression following orchidectomy as in ARKO muscle, suggesting conservation of gene pathways during androgen deprivation-dependent atrophy and androgen-stimulated growth.

Only one previous study has investigated AR-mediated actions in muscle (39), using an ARKO mouse model in which no AR protein is detectable (40). In contrast to our findings, these ARKO males showed down-regulation of slow-twitch markers and increased fast troponin T1. Muscle mass was not investigated (39); however, in another study the group reported that muscle mass is unchanged in these ARKO mice (41). These differing results may reflect the effects of genetic background (42), with the mice in the previous studies on a mixed genetic background, compared to the homogenous C57BL/6 background in our study. Alternatively, this could potentially reflect a difference between total ablation of AR-dependent signaling *vs.* loss of genomic signaling. Our genomic ARKO model retains the potential for nonclassical AR signaling, as the second zinc finger of the DNA binding domain is deleted inframe, and AR protein is still produced (14). *In vitro* studies have shown effects of testosterone on intracellular Ca^{2+} and ERK phosphorylation in myotubes, which occur too rapidly to involve DNA binding (reviewed in ref. 11). Although the physiological relevance of these nonclassical signaling pathways is unknown, our genomic ARKO model can be used to address this question further in the future. Given the consistency of our findings with the known actions of androgens on muscle in humans, we also believe our study provides a good model for elucidating the mechanisms of anabolic actions of androgens in skeletal muscle.

Our data provide the first direct evidence that androgens acting *via* the AR are not required for development of normal female muscle mass, but are required for normal male muscle mass, and contractile strength. Differences in fatigue resistance between male and female muscles are also controlled *via* the AR. Our results indicate that IGF1 does not mediate the anabolic actions of androgens in skeletal muscle. This study suggests that two of the major mechanisms mediating the anabolic actions of androgens are the regulation of genes encoding the polyamine biosynthetic enzymes and genes controlling progression of myoblast proliferation to differentiation. FJ

This research was supported by National Health and Medical Research Council (NHMRC) project grant 350334 and an Eva and Les Erdi research grant; H.E.M. was supported by NHMRC career development award 359226. We thank Ursula Lichtenberg (Institute for Genetics, University of Cologne, Cologne, Germany) for the CMV-cre mice.

REFERENCES

1. Mauras, N., Hayes, V., Welch, S., Rini, A., Helgeson, K., Dokler, M., Veldhuis, J. D., and Urban, R. J. (1998) Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. *J. Clin. Endocrinol. Metab.* **83,** 1886–1892
2. Schroeder, E. T., Terk, M., and Sattler, F. R. (2003) Androgen therapy improves muscle mass and strength but not muscle quality: results from two studies. *Am. J. Physiol. Endocrinol. Metab.* **285,** E16–E24
3. Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Bhasin, D., Berman, N., Chen, X., Yarasheski, K. E., Magliano, L., Dzekov, C., Dzekov, J., Bross, R., Phillips, J., Sinha-Hikim, I., Shen, R., and Storer, T. W. (2001) Testosterone dose-response relationships in healthy young men. *Am. J. Physiol. Endocrinol. Metab.* **281,** E1172–E1181
4. Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Mac, R. P., Lee, M., Yarasheski, K. E., Sinha-Hikim, I., Dzekov, C., Dzekov, J., Magliano, L., and Storer, T. W. (2005) Older men are as responsive as young men to the anabolic effects of graded doses of testosterone on the skeletal muscle. *J. Clin. Endocrinol. Metab.* **90,** 678–688
5. Franke, W. W., and Berendonk, B. (1997) Hormonal doping and androgenization of athletes: a secret program of the German Democratic Republic government. *Clin. Chem.* **43,** 1262–1279
6. MacLean, H. E., Warne, G. L., and Zajac, J. D. (1997) Localization of functional domains in the androgen receptor. *J. Steroid Biochem. Mol. Biol.* **62,** 233–242
7. Matsumoto, T., Takeyama, K., Sato, T., and Kato, S. (2005) Study of androgen receptor functions by genetic models. *J. Biochem. (Tokyo)* **138,** 105–110
8. Jones, M. E., Boon, W. C., Proietto, J., and Simpson, E. R. (2006) Of mice and men: the evolving phenotype of aromatase deficiency. *Trends Endocrinol. Metab.* **17,** 55–64

9. Matsumine, H., Hirato, K., Yanaihara, T., Tamada, T., and Yoshida, M. (1986) Aromatization by skeletal muscle. *J. Clin. Endocrinol. Metab.* **63,** 717–720
10. Glenmark, B., Nilsson, M., Gao, H., Gustafsson, J. A., Dahlman-Wright, K., and Westerblad, H. (2004) Difference in skeletal muscle function in males vs. females: role of estrogen receptor-β. *Am. J. Physiol. Endocrinol. Metab.* **287,** E1125–E1131
11. Chen, Y., Zajac, J. D., and MacLean, H. E. (2005) Androgen regulation of satellite cell function. *J. Endocrinol.* **186,** 21–31
12. Yang, L. Y., and Arnold, A. P. (2000) Interaction of BDNF and testosterone in the regulation of adult perineal motoneurons. *J. Neurobiol.* **44,** 308–319
13. Hobbs, C. J., Plymate, S. R., Rosen, C. J., and Adler, R. A. (1993) Testosterone administration increases insulin-like growth factor-I levels in normal men. *J. Clin. Endocrinol. Metab.* **77,** 776–779
14. Notini, A. J., Davey, R. A., McManus, J. F., Bate, K. L., and Zajac, J. D. (2005) Genomic actions of the androgen receptor are required for normal male sexual differentiation in a mouse model. *J. Mol. Endocrinol.* **35,** 547–555
15. MacLean, H. E., Chiu, W. M., Ma, C., McManus, J. F., Davey, R. A., Cameron, R., Notini, A. J., and Zajac, J. D. (2008) A floxed allele of the *androgen receptor* gene causes hyperandrogenization in male mice. *Physiol Genomics* 33: 133–137
16. Axell, A. M., MacLean, H. E., Plant, D. R., Harcourt, L. J., Davis, J. A., Jimenez, M., Handelsman, D. J., Lynch, G. S., and Zajac, J. D. (2006) Continuous testosterone administration prevents skeletal muscle atrophy and enhances resistance to fatigue in orchidectomized male mice. *Am. J. Physiol. Endocrinol. Metab.* **291,** E506–E516
17. Poortmans, A., and Wyndaele, J. J. (1998) M. levator ani in the rat: does it really lift the anus? *Anat. Rec.* **251,** 20–27
18. Singh, R., Artaza, J. N., Taylor, W. E., Gonzalez-Cadavid, N. F., and Bhasin, S. (2003) Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway. *Endocrinology* **144,** 5081–5088
19. Powers, M. L., and Florini, J. R. (1975) A direct effect of testosterone on muscle cells in tissue culture. *Endocrinology* **97,** 1043–1047
20. Sheffield-Moore, M. (2000) Androgens and the control of skeletal muscle protein synthesis. *Ann. Med.* **32,** 181–186
21. Venable, J. H. (1966) Morphology of the cells of normal, testosterone-deprived and testosterone-stimulated levator ani muscles. *Am. J. Anat.* **119,** 271–301
22. Forger, N. G., Hodges, L. L., Roberts, S. L., and Breedlove, S. M. (1992) Regulation of motoneuron death in the spinal nucleus of the bulbocavernosus. *J. Neurobiol.* **23,** 1192–1203
23. Joubert, Y., Tobin, C., and Lebart, M. C. (1994) Testosterone-induced masculinization of the rat levator ani muscle during puberty. *Dev. Biol.* **162,** 104–110
24. Fulco, C. S., Rock, P. B., Muza, S. R., Lammi, E., Cymerman, A., Butterfield, G., Moore, L. G., Braun, B., and Lewis, S. F. (1999) Slower fatigue and faster recovery of the adductor pollicis muscle in women matched for strength with men. *Acta Physiol. Scand.* **167,** 233–239
25. Glass, D. J. (2003) Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat. Cell Biol.* **5,** 87–90
26. Semsarian, C., Wu, M. J., Ju, Y. K., Marciniec, T., Yeoh, T., Allen, D. G., Harvey, R. P., and Graham, R. M. (1999) Skeletal muscle hypertrophy is mediated by a Ca^{2+}-dependent calcineurin signalling pathway. *Nature* **400,** 576–581
27. Serrano, A. L., Murgia, M., Pallafacchina, G., Calabria, E., Coniglio, P., Lomo, T., and Schiaffino, S. (2001) Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13108–13113
28. Childs, A. C., Mehta, D. J., and Gerner, E. W. (2003) Polyamine-dependent gene expression. *Cell. Mol. Life Sci.* **60,** 1394–1406
29. Abukhalaf, I. K., von Deutsch, D. A., Wineski, L. E., Silvestrov, N. A., Abera, S. A., Sahlu, S. W., and Potter, D. E. (2002) Effect of hindlimb suspension and clenbuterol treatment on polyamine levels in skeletal muscle. *Pharmacology* **65,** 145–154
30. Crozat, A., Palvimo, J. J., Julkunen, M., and Jänne, O. A. (1992) Comparison of androgen regulation of ornithine decarboxylase and S-adenosylmethionine decarboxylase gene expression in rodent kidney and accessory sex organs. *Endocrinology* **130,** 1131–1144
31. Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W., and Elledge, S. J. (1999) p21(^{CIP1}) and p57(^{KIP2}) control muscle differentiation at the myogenin step. *Genes Dev.* **13,** 213–224
32. Doumit, M. E., Cook, D. R., and Merkel, R. A. (1996) Testosterone up-regulates androgen receptors and decreases differentiation of porcine myogenic satellite cells *in vitro*. *Endocrinology* **137,** 1385–1394
33. Li, J., Mayne, R., and Wu, C. (1999) A novel muscle-specific β1 integrin binding protein (MIBP) that modulates myogenic differentiation. *J. Cell Biol.* **147,** 1391–1398
34. Tomczak, K. K., Marinescu, V. D., Ramoni, M. F., Sanoudou, D., Montanaro, F., Han, M., Kunkel, L. M., Kohane, I. S., and Beggs, A. H. (2004) Expression profiling and identification of novel genes involved in myogenic differentiation. *FASEB J.* **18,** 403–405
35. Schmid, C., Steiner, T., and Froesch, E. R. (1983) Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF I and IGF II) in primary cultures of chicken embryonic cells. *FEBS Lett.* **161,** 117–121
36. Armstrong, D. D., and Esser, K. A. (2005) Wnt/β-catenin signaling activates growth-control genes during overload-induced skeletal muscle hypertrophy. *Am. J. Physiol. Cell Physiol.* **289,** C853–C859
37. Steelman, C. A., Recknor, J. C., Nettleton, D., and Reecy, J. M. (2006) Transcriptional profiling of myostatin-knockout mice implicates Wnt signaling in postnatal skeletal muscle growth and hypertrophy. *FASEB J.* **20,** 580–582
38. Xu, T., Shen, Y., Pink, H., Triantafillou, J., Stimpson, S. A., Turnbull, P., and Han, B. (2004) Phosphorylation of $p70^{s6}$ kinase is implicated in androgen-induced levator ani muscle anabolism in castrated rats. *J. Steroid Biochem. Mol. Biol.* **92,** 447–454
39. Altuwaijri, S., Lee, D. K., Chuang, K. H., Ting, H. J., Yang, Z., Xu, Q., Tsai, M. Y., Yeh, S., Hanchett, L. A., Chang, H. C., and Chang, C. (2004) Androgen receptor regulates expression of skeletal muscle-specific proteins and muscle cell types. *Endocrine* **25,** 27–32
40. Yeh, S., Tsai, M. Y., Xu, Q., Mu, X. M., Lardy, H., Huang, K. E., Lin, H., Yeh, S. D., Altuwaijri, S., Zhou, X., Xing, L., Boyce, B. F., Hung, M. C., Zhang, S., Gan, L., and Chang, C. (2002) Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13498–13503
41. Lin, H. Y., Xu, Q., Yeh, S., Wang, R. S., Sparks, J. D., and Chang, C. (2005) Insulin and leptin resistance with hyperleptinemia in mice lacking androgen receptor. *Diabetes* **54,** 1717–1725
42. Davey, R. A., and MacLean, H. E. (2006) Current and future approaches using genetically modified mice in endocrine research. *Am. J. Physiol. Endocrinol. Metab.* **291,** E429–E438

Received for publication January 16, 2008.
Accepted for publication March 13, 2008.