Muscle-specific androgen receptor deletion shows limited actions in myoblasts but not in myofibers in different muscles *in vivo*

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Abstract

The aim of this study was to investigate the direct muscle cell-mediated actions of androgens by comparing two different mouse lines. The cre-loxP system was used to delete the DNA-binding activity of the androgen receptor (AR) in mature myofibers (MCK mAR^{ΔZF2}) in one model and the DNA-binding activity of the AR in both proliferating myoblasts and myofibers (α -actin mAR^{ΔZF2}) in another model. We found that hind-limb muscle mass was normal in MCK mAR^{ΔZF2} mice and that relative mass of only some hind-limb muscles was reduced in α -actin mAR^{ΔZF2} mice. This suggests that myoblasts and myofibers are not the major cellular targets mediating the anabolic actions of androgens on male muscle during growth and development. Levator ani muscle mass was decreased in both mouse lines, demonstrating that there is a myofiber-specific effect in this unique androgen-dependent muscle. We found that the pattern of expression of genes including *c-myc*, *Fzd4* and *Igf2* is associated with androgen-dependent changes in muscle mass; therefore, these genes are likely to be mediators of anabolic actions of androgens. Further research is required to identify the major targets of androgen actions in muscle, which are likely to include indirect actions via other tissues.

Key Words

- ▶ androgen receptor
- ▶ muscle
- ▶ myoblasts
- ▶ myofibers

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Introduction

Androgens significantly increase skeletal muscle mass; however, due to their potential negative side-effects (Kaufman & Vermeulen 2005), they are not widely used therapeutically. There is a dose-dependent response of muscle to exogenously administered testosterone in young and elderly men, with increases in muscle size, strength and fiber hypertrophy (Bhasin *et al.* 2001, Sinha-Hikim *et al.* 2002), and testosterone treatment in frail elderly men with low-borderline testosterone can prevent loss of limb strength, and increase lean body mass and decrease fat mass (Srinivas-Shankar

et al. 2010). Because of these anabolic actions, there is increasing interest in the development of selective androgen receptor (AR) modulators (SARMs) that are anabolic for muscle and bone in the absence of actions in the reproductive tract (Gao *et al.* 2005, Allan *et al.* 2008), including studies investigating the target cells of SARM actions in muscle (Dubois *et al.* 2015). Development of more targeted therapies for increasing muscle mass will be accelerated by a greater understanding of the mechanisms of androgen actions in skeletal muscle.

Androgens can increase muscle mass, either via exogenous treatment or during development, to achieve peak muscle mass in males vs females (Bhasin *et al.* 1996, Snyder *et al.* 1999, Wang *et al.* 2000, Basaria & Dobs 2001). These actions are mediated via the AR, as we have shown that male mice with deletion of the second zinc finger (ZF2) of the DNA-binding domain (AR^{ΔZF2}), causing deletion of the DNA binding-dependent actions of the AR (Pang *et al.* 2011), have reduced muscle mass (MacLean *et al.* 2008b). However, it is not known if these anabolic actions occur predominantly during development or postpuberty. Androgens are also required to increase muscle mass in adult males. Androgen withdrawal-dependent muscle atrophy has been demonstrated in both normal men and men with prostate cancer undergoing androgen ablation therapy (Mauras *et al.* 1998, Basaria *et al.* 2002), with a similar decrease in muscle mass occurring following orchidectomy in male mice (Axell *et al.* 2006). Because testosterone levels decline in aging males, this may be one mechanism contributing to age-related sarcopenia.

One of the unanswered questions regarding androgen actions in muscle is whether the AR-regulated pathways are similar or different in androgen-induced muscle growth during development, androgen-dependent muscle hypertrophy in adulthood and androgen withdrawal-dependent atrophy in adult males. Androgens can drive the commitment of mesenchymal cells into the myogenic lineage (Singh *et al.* 2003, 2009), and is likely to be one action occurring in males during the development of peak muscle mass. However, the relevance of this action to androgen-dependent muscle hypertrophy in mature muscle is less clear. Adult skeletal muscle fibers are terminally differentiated, and muscle repair and hypertrophy can occur either through an increase in the number of myonuclei, via activation of satellite cells (muscle stem cells) to proliferate as myoblasts, differentiate into myotubes and fuse with myofibers, or via an increase in protein content of postproliferative myofibers. While some studies have shown that androgens increase myoblast proliferation rate and/or delay differentiation (Powers & Florini 1975, Doumit *et al.* 1996, Sinha-Hikim *et al.* 2006, Diel *et al.* 2008), others suggest the predominant actions are on protein accretion in myofibers (Ferrando *et al.* 1998, 2002, Chen *et al.* 2008). Maximal hypertrophic response to muscle overloading requires myofiber AR, but also involves other target cells (Ferry *et al.* 2014). The relative contribution of these mechanisms in different muscle cell types, as well as the AR target genes mediating these actions (Rana *et al.* 2014), remains to be determined.

We have previously shown that global AR^{ΔZF2} male mice have a 20–25% decrease in hind-limb muscle mass, as well as a reduction in maximum tetanic force in fast-twitch muscles (MacLean *et al.* 2008b). This is in contrast to AR-null AR knockout (ARKO) models, which have very mild reductions in muscle mass (Ophoff *et al.* 2009a). A myofiber-specific AR-null ARKO mouse model has little change in the mass of hind-limb muscles, similar to their global ARKO model, but a reduction in the mass of the highly androgen-sensitive perineal muscle, the levator ani (LA) (Ophoff *et al.* 2009a,b, Pang *et al.* 2011). A muscle-specific ARKO mouse line with deletion of the AR in both myoblasts and myofibers also has no reduction in hind-limb muscle mass, but reduced LA muscle mass (Chambon *et al.* 2010). This deletion is associated with loss of hind-limb muscle strength, despite the normal muscle mass (Chambon *et al.* 2010). A satellite cell-specific ARKO model also shows no reduction in hind-limb muscle mass, but a decrease in LA mass (Dubois *et al.* 2014, 2015). The phenotypic differences between global AR-null models and our global AR^{ΔZF2} mice may arise through different methodologies used to assess muscle mass, through different genetic backgrounds, or may be because our model has deletion of DNA binding-dependent actions, but retains non-DNA binding-dependent actions (Pang *et al.* 2011). Thus, it is possible that deletion of DNA binding-dependent AR actions has a greater effect on muscle anabolism than total loss of AR signaling, and suggests that our AR^{ΔZF2} model is useful for identifying the mechanisms underlying the anabolic actions of androgens in muscle. Therefore, to identify the direct muscle cell-mediated actions of androgens, we have compared two different mouse lines, one in which the DNA-binding activity of the AR is deleted only in mature myofibers (MCK mAR^{ΔZF2}), and the other in which the AR is deleted in both proliferating myoblasts and myofibers (α-actin mAR^{ΔZF2}).

Materials and methods

Mice

The exon 3 floxed (AR^{lox}) mouse line (Supplementary Fig. 1A, see section on supplementary data given at the end of this article) was generated as described (Notini *et al.* 2005). Deletion of exon 3 causes an in-frame deletion of the ZF2 of the DNA-binding domain, and loss of DNA binding-dependent AR signaling (Notini *et al.* 2005, Pang *et al.* 2011). Muscle creatine kinase (MCK) mAR^{ΔZF2} mice were generated using the MCK-cre line (Bruning *et al.* 1998)

(a kind gift of C Ronald Kahn, Joslin Diabetes Center, Boston, MA, USA), which uses the MCK promoter to drive cre expression in postproliferative myofibers. α -actin mAR Δ ZF2 mice were generated using our previously described α -actin cre line (Kaczmarczyk *et al.* 2003), which uses the human α -skeletal actin promoter to drive cre expression in myoblasts. All mouse lines were back-crossed to a congenic C57BL/6 background (>10 generations back-cross), and control littermates (AR^{lox} hemizygous males, cre heterozygous males and wildtype (WT) males) were used for both lines. We also analyzed gene expression in gastrocnemius muscle from our previously described global AR Δ ZF2 and androgen deprivation models: WT and global AR Δ ZF2,

9 weeks of age, $n \geq 24$, and WT C57BL/6 orchidectomized males treated $\pm$ testosterone for 10 weeks, 8 weeks of age, $n \geq 8$ (Axell *et al.* 2006, MacLean *et al.* 2008b). Mice were housed in a conventional facility, and standard chow and water provided *ad libitum*. Studies were performed with the approval of the Austin Health Animal Ethics Committee.

Voluntary physical activity and food intake

Voluntary physical activity was measured in mice aged 9–10 weeks ($n = 7$ –9/genotype) using computer-linked running wheels (Lafayette Instruments, Lafayette, IN, USA), as described (Rana *et al.* 2011), with the number of

Figure 1

(A) Body mass ($n \geq 19$ /genotype), (B) serum testosterone levels ($n = 12$ /genotype) and (C) voluntary physical activity ($n = 8$ –9/genotype) in WT, cre transgenic heterozygous (cre), AR^{lox} hemizygous (AR^{lox}) and mAR Δ ZF2 male littermates of the MCK mAR Δ ZF2 and α -actin mAR Δ ZF2 lines. Mean $\pm$ S.E.M., * $P < 0.05$ vs WT, cre heterozygous and AR^{lox} hemizygous males, one-way ANOVA and Tukey's *post hoc* test.

wheel revolutions per day determined from the average of readings over 4 days. Food was weighed daily during the wheel-running experiment to determine the average food intake (g/day).

Tissue collection

Blood was collected by cardiac puncture from mice anesthetized using inhaled isoflurane (Baxter, Deerfield, IL, USA). After clotting on ice, serum was separated by centrifugation, 2×8 min at $2100g$, and stored at -20°C before analysis. Following blood collection, mice were killed by cervical dislocation and tissues, including heart, kidney, liver, brain, testis, seminal vesicles, extensor digitorum longus (EDL) muscle, tibialis anterior (TA) muscle, soleus (SOL) muscle, gastrocnemius (GAST) muscle, LA muscle, subcutaneous fat, retroperitoneal fat and gonadal fat, were immediately dissected, weighed and snap-frozen in liquid nitrogen, or fixed in 4% paraformaldehyde. Wet weight of tissues was determined to an accuracy of 0.1 mg, from the mean mass bilaterally. Body and tissue mass were measured in 12 week $\text{mAR}^{\Delta\text{ZF2}}$ male mice and male littermate controls.

cDNA synthesis, reverse transcriptase PCR and quantitative real-time PCR

Total RNA isolation and cDNA synthesis were performed as described (MacLean *et al.* 2008a). To detect the expression of normal and exon 3-deleted *AR* genes, reverse transcriptase (RT)-PCR was performed on 500 ng cDNA using primers flanking exon 3 (Notini *et al.* 2005) (Supplementary Fig. 1A). Quantitative real-time PCR

(Q-PCR) was performed in duplicate using 500 ng cDNA, TaqMan gene expression assays (Applied Biosystems) and calculating relative expression compared with a reference muscle cDNA control using the $\Delta\Delta\text{C}_\text{T}$ method, as described previously (MacLean *et al.* 2008b).

Histology

Freshly dissected tissues were fixed in 4% paraformaldehyde in PBS, for 24 h at 4°C , washed in PBS and stored in 70% ethanol before paraffin processing and embedding. Six micron serial sections were cut, and stained with hematoxylin and eosin (H&E) using standard protocols. Fiber cross-sectional area (CSA) was calculated as described previously (Axell *et al.* 2006), with CSA dichotomized into groups of $100\mu\text{m}^2$ (100–200, 200–300, 300–400, 400–500, 500–600, 600–700, 700–800, 800–900, 900–1000 and $>1000\mu\text{m}^2$). LA sections from two $\text{mAR}^{\Delta\text{ZF2}}$ males and three WT littermate males, from the two lines, were examined, counting a minimum of 400 fibers per animal and calculating the mean number in each CSA category for both genotypes.

Western analysis

Protein isolation and polyacrylamide gel electrophoresis were performed as described (Pang *et al.* 2011), using $30\mu\text{g}$ protein sample per lane and PVDF membranes. The intensity of all proteins was normalized to α -tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) detected on the same membrane to control for loading. Antihuman myosin heavy chain (MHC) (slow) monoclonal antibody (mAb) (A4.951) (Cho *et al.* 1993)

Table 1 Fat mass in 12-week-old male littermates from the MCK $\text{mAR}^{\Delta\text{ZF2}}$ and α -actin $\text{mAR}^{\Delta\text{ZF2}}$ lines.

	MCK $\text{mAR}^{\Delta\text{ZF2}}$			α -actin $\text{mAR}^{\Delta\text{ZF2}}$		
	WT	AR^{lox}	$\text{mAR}^{\Delta\text{ZF2}}$	WT	AR^{lox}	$\text{mAR}^{\Delta\text{ZF2}}$
Subcutaneous fat (mg)	107.7 ± 3.8 (32)	$128.1 \pm 5.7^*$ (32)	$131.4 \pm 5.2^*$ (20)	110.7 ± 8.5 (20)	127.6 ± 9.6 (24)	124.2 ± 8.2 (27)
Subcutaneous fat/body weight (mg/g)	3.74 ± 0.13 (32)	$4.41 \pm 0.16^*$ (32)	$4.55 \pm 0.15^*$ (20)	3.88 ± 0.29 (20)	4.38 ± 0.32 (24)	4.36 ± 0.27 (27)
Retroperitoneal fat (mg)	31.5 ± 2.1 (36)	$44.7 \pm 3.9^*$ (32)	$51.6 \pm 5.4^*$ (20)	35.8 ± 5.1 (18)	45.8 ± 5.5 (22)	43.6 ± 4.7 (24)
Retroperitoneal fat/body weight (mg/g)	1.07 ± 0.07 (22)	$1.52 \pm 0.12^*$ (32)	$1.76 \pm 0.17^*$ (20)	1.25 ± 0.17 (18)	1.56 ± 0.18 (22)	1.51 ± 0.15 (24)
Gonadal fat (mg)	164.7 ± 7.6 (22)	$206.0 \pm 12.0^*$ (25)	$235.3 \pm 14.5^{**}$ (17)	167.3 ± 12.2 (16)	185.3 ± 14.0 (14)	$214.0 \pm 14.7^*$ (19)
Gonadal fat/body weight (mg/g)	5.78 ± 0.25 (22)	$7.08 \pm 0.34^*$ (25)	$8.09 \pm 0.41^{**}$ (17)	5.93 ± 0.40 (16)	6.53 ± 0.42 (14)	$7.50 \pm 0.045^*$ (19)

Mean $\pm$ S.E.M., sample size in parentheses, $*P < 0.05$, $**P < 0.001$ vs WT (one-way ANOVA, Tukey's or Tamhane's *post hoc* test).

(Developmental Studies Hybridoma Bank) was used at 1:800, and anti-AR Ab (C-19) (Santa Cruz Biotechnology), anti-c-met (R&D systems) and anti-Pax7 (Developmental Studies Hybridoma Bank) were used at 1:1000.

Testosterone assay

Serum testosterone was measured using the DPC Coat-A-Count assay (Siemens Medical Solutions Diagnostics, Doncaster, Victoria, Australia), which has a sensitivity of 0.14 nM, intra-assay coefficient of variation range of 4–11%, interassay coefficient of variation range of 5.9–12% and high specificity for testosterone (percentage cross-reactivity with other androgens: androstenedione

0.5%, 5 α -dihydrotestosterone $\leq$ 3.4%, 19-hydroxyandrostenedione 2.0% and 11 β -hydroxytestosterone 0.8%). Mouse serum samples were extracted in hexane:ethyl acetate (17:3) and assayed, as described previously (MacLean *et al.* 2008a).

Statistical analysis

For comparison of means for two groups, unpaired Student's *t*-test was performed. For comparison of means for more than two groups, data were analyzed by one-way ANOVA with Tukey's *post hoc* test, or Tamhane's *post hoc* test if Levene's test indicated unequal variance within groups. Fiber distributions were analyzed by χ^2 analysis. All analyses were performed using SPSS 16.0.

Figure 2

Muscle mass in WT, AR^{lox} hemizygous (AR^{lox}) and mAR^{ΔZF2} male littermates of the (A, B, C, D, E and K, L, M, N, O) MCK-mAR^{ΔZF2} and (F, G, H, I, J and P, Q, R, S, T) α-actin-mAR^{ΔZF2} lines. (A and F) Extensor digitorum longus (EDL), (B and G) tibialis anterior (TA), (C and H) soleus (SOL), (D and I) gastrocnemius (GAST), (E and J) levator ani (LA), (K and P) EDL mass/heart mass, (L and Q) TA mass/heart mass (M and R) SOL mass/heart mass, (N and S) GAST mass/heart mass, (O and T) LA mass/heart mass. Mean $\pm$ S.E.M., **P* < 0.05, ***P* < 0.001 vs WT; †*P* < 0.05, ††*P* < 0.001 vs AR^{lox} hemizygous, one-way ANOVA and Tukey's or Tamhane's *post hoc* test.

Results

Generation of myofiber-specific and myoblast/myofiber-specific AR^{ΔZF2} males

Myofiber-specific mAR^{ΔZF2} mice were generated using the MCK-cre line (Bruning *et al.* 1998), with cre expression under the control of the MCK promoter, which is only expressed in postproliferative myofibers. Myoblast/myofiber-specific mAR^{ΔZF2} mice were generated using the α -actin-cre line (Kaczmarczyk *et al.* 2003). The human skeletal α -actin promoter is expressed in myoblasts and myofibers (Schwander *et al.* 2003), and results in deletion of AR in both myoblasts and myofibers.

The degree of AR gene deletion in muscle from both mAR^{ΔZF2} lines was 93–99% in different hind-limb muscles, as determined by remaining AR gene expression (Supplementary Fig. 1B). To test the tissue specificity of deletion, we used RT-PCR to detect the deleted AR^{ΔZF2} allele. In both mAR^{ΔZF2} lines, the deleted allele was present in muscle, but could also be amplified from brain and adipose tissue of α -actin mAR^{ΔZF2} males (Supplementary Fig. 1D). To determine the degree of deletion in nonmuscle tissues, we also measured full-length AR mRNA expression, and found a three-fold increase in AR mRNA in liver of both mAR^{ΔZF2} lines; in α -actin mAR^{ΔZF2} mice, AR expression was reduced by 45% in brain ($P < 0.001$) and 60% in subcutaneous fat; however, the latter did not reach statistical significance ($P = 0.09$) (Supplementary Fig. 1E).

Tissue mass in mAR^{ΔZF2} males

For both the myofiber-specific MCK mAR^{ΔZF2} line and the myoblast/myofiber-specific α -actin mAR^{ΔZF2} line, mAR^{ΔZF2} males were examined at 12 weeks of age, and compared

with WT ($X^{+/Y}$), cre transgenic heterozygous and AR^{lox} hemizygous (AR^{lox/Y}) male littermates of each line. There was no difference between WT and cre transgenic heterozygotes for any tissue mass examined, including muscle mass (data not shown), so cre values have not been included in results to simplify presentation. In both mAR^{ΔZF2} lines, there was no difference in body mass to controls (Fig. 1A). We measured the mass of androgen-dependent tissues as a biological read-out of androgen action in mAR^{ΔZF2} mice, because we previously showed that AR^{lox} hemizygous males have a phenotype of hyperandrogenization characterized by an increase in the mass of androgen-dependent tissues including kidney and seminal vesicles (MacLean *et al.* 2008a). As expected, kidney and seminal vesicle mass was increased in both mAR^{ΔZF2} lines and AR^{lox} males compared with WT, but there was no significant difference between the mAR^{ΔZF2} and AR^{lox} groups (Supplementary Table 1). Both the α -actin cre and MCK-cre lines express cre in the heart as well as skeletal muscle; however, cardiac mass was normal in mAR^{ΔZF2} males of both lines (Supplementary Table 1), in keeping with our data that global AR^{ΔZF2} males have normal cardiac mass (MacLean *et al.* 2008b).

The mass of subcutaneous, retroperitoneal and gonadal fat depots was also determined (Table 1), as we previously showed that global AR^{ΔZF2} males have increased adiposity (Rana *et al.* 2011). In the MCK mAR^{ΔZF2} line, there was a significant increase in subcutaneous, retroperitoneal and gonadal fat pad mass in both mAR^{ΔZF2} and AR^{lox} males vs WT (Table 1), but no difference between AR^{lox} and mAR^{ΔZF2} males. A significant increase was also observed in α -actin mAR^{ΔZF2} gonadal fat vs WT, and a similar increase in mean fat mass values was also observed in other fat depots from the AR^{lox} and mAR^{ΔZF2} males of the α -actin line, although

Figure 3

(A) Levator ani muscle cross-sections from WT, cre transgenic heterozygous (cre), AR^{lox} hemizygous (lox) and mAR^{ΔZF2} male littermates of the MCK mAR^{ΔZF2} and α -actin mAR^{ΔZF2} lines (H&E stain, bar = 100 μ m). (B) Distribution of levator ani muscle fiber cross-sectional area from WT and MCK-mAR^{ΔZF2} male littermates ($n = 2$ –3/group), mean $\pm$ S.E.M., ** $P < 0.001$ vs WT, χ^2 analysis.

the smaller sample sizes used in this line meant that the study was not sufficiently powered to determine if differences were significant. The increase in fat mass is likely to be due to the floxed AR allele containing the *neomycin* selection cassette or AR deletion in brain or fat of α -actin $mAR^{\Delta ZF2}$ mice, rather than muscle-specific AR deletion.

Serum analysis and voluntary physical activity

Serum testosterone levels were increased 2.9-fold in MCK $mAR^{\Delta ZF2}$ males compared with all littermate controls (Fig. 1B), but α -actin $mAR^{\Delta ZF2}$ mice had normal testosterone levels. There was no effect of muscle-specific AR deletion

on voluntary physical activity, which was normal in $mAR^{\Delta ZF2}$ males of both lines compared with both WT and AR^{lox} controls (Fig. 1C). For both lines, food intake was also normal in $mAR^{\Delta ZF2}$ males (data not shown).

$mAR^{\Delta ZF2}$ males have reduced muscle mass and fiber area

We determined the muscle mass of fast-twitch (EDL and TA), slow-twitch (SOL) and mixed-fiber (GAST) hind-limb muscles, and the highly androgen-dependent LA muscle in the perineum. For the MCK $mAR^{\Delta ZF2}$ line, muscle mass was significantly decreased in both $mAR^{\Delta ZF2}$ and AR^{lox} males compared with WT for the EDL and TA (Fig. 2A and B), SOL mass was reduced in $mAR^{\Delta ZF2}$ vs WT (Fig. 2C)

Figure 4

Q-PCR showing expression of myogenic regulatory factors (A) *myoD1*, (B) *myf5*, (C) *myogenin* and (D) *myf6* in gastrocnemius (GAST) and levator ani (LA) muscles from WT and $AR^{\Delta ZF2}$ male littermates of the MCK $mAR^{\Delta ZF2}$ ($n=11-13$ /group), α -actin $mAR^{\Delta ZF2}$ ($n=11-13$ /group) and global $AR^{\Delta ZF2}$ lines ($n=11$ /group). Mean $\pm$ S.E.M., * $P < 0.05$, ** $P < 0.001$ vs WT control, Student's *t*-test.

and there was no change in GAST mass (Fig. 2D). For the α -actin mAR^{ΔZF2} line, there was no change in absolute muscle mass for any of the hind-limb muscles examined (Fig. 2E, G, H and I). In contrast, LA muscle mass was reduced by ~50% in both MCK mAR^{ΔZF2} and α -actin mAR^{ΔZF2} males compared with respective WT and AR^{lox} controls (Fig. 2E and J).

To control for variations in mouse size, we measured the ratio of muscle mass to heart mass, which is not dependent on DNA-binding activity of the AR (Supplementary Table 1 and (MacLean *et al.* 2008b)). In the MCK mAR^{ΔZF2} line, relative muscle mass was reduced in both MCK mAR^{ΔZF2} and AR^{lox} controls compared with WT for hind-limb muscles, but no difference between mAR^{ΔZF2} and AR^{lox} (Fig. 2K, L, M and N), showing no effect of AR deletion. In the α -actin mAR^{ΔZF2} line, relative hind-limb muscle mass was significantly lower in mAR^{ΔZF2} compared with both WT and AR^{lox} controls in the TA (16% decrease vs WT, 10% decrease vs AR^{lox}) and SOL (12.5% decrease vs WT, 13% decrease vs AR^{lox}) (Fig. 2P, Q, R, S and T). Thus, deletion of the AR in both myoblasts and myofibers causes only a modest reduction in relative muscle mass in some hind-limb muscles, which does not occur with deletion in myofibers alone.

There was a marked reduction in LA muscle fiber size in mAR^{ΔZF2} males of both lines (Fig. 3A), with a significant shift in distribution to smaller fibers in mAR^{ΔZF2} LA compared with WT males ($\chi^2=721$, $df=9$, $P<0.001$) (Fig. 3B). There was no difference in the ratio of LA nuclei to fibers between mAR^{ΔZF2}, WT and AR^{lox} controls for either the α -actin mAR^{ΔZF2} or MCK mAR^{ΔZF2} line (data not shown). Global AR^{ΔZF2} muscle has a switch to a more slow muscle phenotype (MacLean *et al.* 2008b). Western

analysis of mAR^{ΔZF2} GAST muscle showed a 30% decrease in fast MHC levels in α -actin mAR^{ΔZF2} but not in MCK mAR^{ΔZF2} GAST (Supplementary Fig. 2A and E), and no change in slow MHC levels in either line (Supplementary Fig. 2B and E), suggesting no consistent switch to a slow phenotype. There was also no consistent change in the levels of two satellite cell markers, Pax7 and c-met (Supplementary Fig. 2C, D and E).

Expression of muscle regulatory factors is regulated by AR deletion

To determine if the AR in muscle regulates the myogenic regulatory factors (*myoD1*, *myf5*, *myf6* and *myogenin*), we measured their gene expression in GAST and LA from both mAR^{ΔZF2} lines. Expression was also examined in GAST from global AR^{ΔZF2} males (MacLean *et al.* 2008b) (LA does not develop in global AR^{ΔZF2}) and in GAST from WT orchidectomized (orx) males treated for 10 weeks with control or testosterone-filled implants (Axell *et al.* 2006) (Fig. 4). GAST muscle was analyzed as it is a mixed-fiber muscle, so expression changes occurring in either fast- or slow-twitch fibers can be detected, and LA was analyzed because it is a uniquely AR-dependent muscle. Expression of *myoD1* was decreased by 25–30% in GAST from both mAR^{ΔZF2} lines but was not altered in global AR^{ΔZF2} GAST; however, there was no difference in *myoD1* expression in the LA of both mAR^{ΔZF2} lines (Fig. 4A). In contrast, *myf5* expression was unchanged in GAST from mAR^{ΔZF2} and global AR^{ΔZF2} males, but increased in LA from both mAR^{ΔZF2} lines (Fig. 4B). *Myogenin* expression was elevated in GAST and LA muscles from both mAR^{ΔZF2} lines, and also in global AR^{ΔZF2} GAST, but decreased in orchidectomized

Figure 5

Q-PCR showing expression of *c-myc*, *Fbxo32* and *Igf1* in (A, B and C) gastrocnemius (GAST) muscle and (D and E) levator ani (LA) muscle from WT and AR^{ΔZF2} male littermates of the (A and D) MCK mAR^{ΔZF2} ($n=11$ –13/group) (B and E) α -actin mAR^{ΔZF2} ($n=11$ –13/group) and (C) global AR^{ΔZF2} lines ($n=11$ /group). Mean $\pm$ s.e.m., * $P<0.05$ vs WT control, Student's *t*-test.

Figure 6

Q-PCR showing expression of putative AR target genes *Odc1*, *Amd1*, *Wnt4*, *Fzd4*, *p57*, *Igf2*, *Tceal7*, *Itgb1bp3*, *Tgfb1*, *Tgfb2* and *calcineurin Aa* in (A and B) gastrocnemius (GAST) muscle and (C and D) levator ani (LA) muscle from WT and AR Δ ZF2 male littermates of the (A and B) MCK mAR Δ ZF2 and (C and D) α -actin mAR Δ ZF2 lines ($n=9-13$ /group). Mean $\pm$ s.e.m., * $P < 0.05$, ** $P < 0.001$ vs WT control, Student's t -test.

males treated with testosterone (Fig. 4C). Expression of *myf6* was not altered by AR deletion (Fig. 4D).

Expression of other potential AR target genes

We examined the expression of other factors that may be important regulatory factors in muscle. Levels of *c-myc*, which was upregulated in global AR Δ ZF2 GAST (Fig. 5C), were normal in GAST muscle from both mAR Δ ZF2 lines (Fig. 5A and B), but increased 1.81-fold in the LA of MCK mAR Δ ZF2 males (Fig. 5D), and mean expression was 3.49-fold higher in α -actin mAR Δ ZF2 LA compared with WT (Fig. 5E). *Fbxo32* (*MAFbx/atrogen1*) expression was not different between mAR Δ ZF2 or global AR Δ ZF2 males and their respective controls (Fig. 5). In contrast, *Igf1* was normal in α -actin mAR Δ ZF2 muscles (Fig. 5B and E), but increased in both GAST and LA from MCK mAR Δ ZF2 males (Fig. 5A and D). Because *Igf1* expression was increased only in MCK mAR Δ ZF2 but not in α -actin mAR Δ ZF2 males, we cannot rule out the possibility that this could be a result of estradiol receptor activation, due to the aromatization of the elevated levels of testosterone present only in MCK mAR Δ ZF2 males (Fig. 1B).

For genes we previously showed were differentially expressed in GAST muscle from global AR Δ ZF2 males and orchidectomized WT males (MacLean *et al.* 2008b), expression of the polyamine biosynthetic gene *Amd1* was decreased in mAR Δ ZF2 muscles from both lines (Fig. 6A, B, C and D); in contrast, *Odc1* expression was only decreased in GAST but not in LA muscle from both mAR Δ ZF2 lines. *p57* was increased in MCK mAR Δ ZF2 GAST muscle but not in α -actin mAR Δ ZF2 muscles, and *Igf2* was increased in LA

but not in GAST of both lines. *Tceal7* showed variable expression, as did expression of *Tgfb1* and *Tgfb2* (Fig. 6). Expression of *integrin β 1 binding protein 3* (*Itgb1bp3*) was decreased in GAST from both mAR Δ ZF2 lines (Fig. 6A and B), but was increased in LA from both lines (Fig. 6C and D). *Calcineurin Aa* (*Ppp3ca*) was increased in both GAST and LA muscle from both mAR Δ ZF2 lines (Fig. 6).

Discussion

Androgens can have strong anabolic effects in skeletal muscle; however, the major target cell mediating these actions has not been elucidated. We previously showed that global deletion of the DNA binding-dependent actions of the AR in male mice (global AR Δ ZF2) causes a 15–20% reduction in hind-limb skeletal muscle mass (MacLean *et al.* 2008b). However, in this study, we now show that deletion of AR DNA binding-dependent actions in myofibers (MCK mAR Δ ZF2) or in myoblasts and myofibers (α -actin mAR Δ ZF2) has little effect on hind-limb muscle mass. This suggests that myoblasts and myofibers are not the major cellular targets mediating the anabolic actions of androgens on male muscle during growth and development. The highly androgen-dependent LA muscle has a 50% reduction in mass in both mAR Δ ZF2 lines, associated with decreased fiber cross-sectional area. Although the LA is used widely as a model muscle to assess the anabolic actions of androgens and SARMs, our results show that it differs in both the androgen dependence of its mass as well as the pattern of AR target gene expression compared with hind-limb muscles, as we have previously shown (Rana *et al.* 2014). These facts raise

questions regarding the relevance of results obtained from the LA for therapies that will ultimately be required to increase mass and strength in clinically significant limb and postural muscles.

The primary cellular targets of androgen action in skeletal muscle remain unresolved (Kadi 2008). Different studies have suggested that androgens act on satellite cells or myoblasts to increase the number of myonuclei (Mulvaney *et al.* 1988, Joubert *et al.* 1994, Joubert & Tobin 1995) or act on myofibers to increase protein content (Sheffield-Moore *et al.* 1999, Ferrando *et al.* 2002, Chen *et al.* 2008, Ophoff *et al.* 2009b). However, despite the fact that the AR is deleted by $\geq 93\%$ in hind-limb muscles of both mAR^{ΔZF2} lines in our study, muscle mass is virtually normal with only relative muscle mass decreased in α -actin mAR^{ΔZF2} TA and soleus, but all other hind-limb muscles not different to AR^{lox} controls. This suggests a modest muscle-specific effect of deletion of the AR in myoblasts but not in myofibers, to increase muscle mass in only specific hind-limb muscles. It is also possible that hind-limb muscle mass is altered in α -actin AR^{ΔZF2} males in part due to the effects of nonspecific AR deletion of AR in the brain, where AR expression is decreased by 45%, or subcutaneous fat (decreased by 60%). The minor changes in muscle mass are in contrast to our global AR^{ΔZF2} mice, in which muscle mass was decreased in all hind-limb muscles (MacLean *et al.* 2008b) and indicates that the anabolic actions of androgens must occur through

target cells other than myoblasts and myofibers, either in mesenchymal precursors, or other tissues such as the CNS.

The highly androgen-dependent LA muscle was reduced by 50% in both mAR^{ΔZF2} lines, showing androgen actions on myofibers in the LA only, which must regulate protein content and contribute to half the mass. The fact that the LA fails to develop at all in the global AR^{ΔZF2} (MacLean *et al.* 2008b), or in mice with AR deleted in undifferentiated embryonic perineal mesenchymal cells (Ipulan *et al.* 2014), is highly suggestive that androgens also act in the early muscle cell lineage, either in commitment to the myogenic lineage (Singh *et al.* 2003) or on early muscle satellite cells in this muscle. AR-positive fibroblasts are present in LA, and androgen actions on these fibroblasts could also contribute to muscle development and maintenance of muscle mass (Dubois *et al.* 2015).

We examined a number of potential AR target genes to determine if their expression would change associated with androgen/AR-dependent changes in muscle mass in the LA (decreased mass) and not the GAST (no change in mass). Of the myogenic regulatory factors, *myogenin* showed increased expression in LA but was also increased in the GAST of both mAR^{ΔZF2} lines and global AR^{ΔZF2}. *Myogenin* drives terminal differentiation of myoblasts into myotubes (Hasty *et al.* 1993, Nabeshima *et al.* 1993, Patapoutian *et al.* 1995, Rawls *et al.* 1995), and we previously showed that *myogenin* is repressed by androgens *in vitro* and *in vivo* (Rana *et al.* 2014). The current data

Table 2 Gene expression in muscle in MCK mAR^{ΔZF2} and α -actin mAR^{ΔZF2} lines, global AR^{ΔZF2} (MacLean *et al.* 2008b), and testosterone-treated orchidectomy models (Axell *et al.* 2006, Rana *et al.* 2011).

Gene	Function in muscle	mAR ^{ΔZF2} LA, not GAST	Regulated in global AR ^{ΔZF2} GAST	Orchidectomy + testosterone GAST
<i>Myf5</i>	Myogenic commitment	↑	↔	↔
<i>c-myc</i>	Cell proliferation, satellite cell activation	↑	↑	↓
<i>Fzd4</i>	Myogenic differentiation, satellite cell activation	↑	↑	↓
<i>Igf2</i>	Myoblast differentiation	↑	↑	↓
<i>Itgb1bp3</i>	Myoblast differentiation (–ve)	↑ (↓ in GAST)	↓	↔
<i>Tgfb1</i>	Myoblast differentiation (–ve), satellite cell activation (–ve)	↓ (↑ in GAST)	Not determined	Not determined
		mAR ^{ΔZF2} LA and GAST	Global AR ^{ΔZF2} GAST	Orchidectomy + testosterone GAST
<i>Myogenin</i>	Terminal myoblast differentiation	↑	↑	↓
<i>Wnt4</i>	Myogenic differentiation, satellite cell activation	↑	↑	↓
<i>Calcineurin A</i>	Myoblast differentiation	↑	↑	↓
<i>Amd1</i>	Polyamine biosynthesis	↓	↓	↑
		mAR ^{ΔZF2} GAST, not LA	Global AR ^{ΔZF2} GAST	Orchidectomy + testosterone GAST
<i>Odc1</i>	Polyamine biosynthesis, myoblast proliferation	↓	↓	↑
<i>p57</i>	Myoblast differentiation	↑ (MCK only)	↑	↔

demonstrate that androgens act via the AR in myofibers to repress *myogenin* expression. However, the fact that this loss of repression occurred in the mAR^{ΔZF2} GAST muscles, while GAST mass was normal, indicates that this change in expression is not sufficient to impact on muscle mass in the absence of other changes (Rana *et al.* 2014). In contrast, *Myf5* was upregulated in the LA muscle of both mAR^{ΔZF2} lines but not in GAST muscle, suggesting that this regulation is specific to the LA. *Myf5* may be one of the genes mediating the androgen effects on muscle mass in the LA, but the fact that it is not upregulated in global AR^{ΔZF2} hind-limb muscle suggests that this regulatory pathway in the LA is not widely applicable to or representative of all skeletal muscle (Rana *et al.* 2014).

We identified a group of genes that showed a consistent pattern of regulation by androgens and the AR in muscles that had decreased mass (Table 2, Supplementary Fig. 3). To rule out the confounding effects of the elevated serum testosterone in the MCK mAR^{ΔZF2} males, or off-target AR deletion in α -actin mAR^{ΔZF2} males, we have focused only on genes that were altered in both mAR^{ΔZF2} models and/or global AR^{ΔZF2} mice (MacLean *et al.* 2008b). A group of genes were upregulated only in LA but not in GAST of both mAR^{ΔZF2} lines including *myf5*, *c-myc*, *Fzd4*, *Igf2* and *Itgb1bp3*; *Tgfb1* was decreased in LA but not in GAST. Of these genes, *c-myc*, *Fzd4* and *Igf2* were also upregulated in global AR^{ΔZF2} GAST, in which muscle mass is reduced by 19% (MacLean *et al.* 2008b) (Table 2). *c-myc*, *Fzd4* and *Igf2* were also downregulated in GAST muscle from orchidectomized males treated with testosterone (Table 2), in which hind-limb muscle mass differs by 18–27% (Axell *et al.* 2006). This suggests that all these genes are repressed by the androgen/AR pathway in skeletal muscle associated with androgen-dependent regulation of muscle mass. Therefore, these genes are possible mediators of the anabolic actions of androgens in these muscles, through control of cell proliferation (*c-myc*) (Kelly *et al.* 1983, Falcone *et al.* 1985), regulation of myoblast differentiation (*Igf2*) (Wilson & Rotwein 2006, Schiaffino & Mammucari 2011) and myogenic differentiation or activation of satellite cells (*Fzd4*) (Rudnicki & Williams 2015).

Another group of genes was consistently regulated by androgens and the AR in both GAST and LA muscles. *Myogenin*, *Wnt4* and *calcineurin A* were upregulated in both mAR^{ΔZF2} lines and global AR^{ΔZF2}, and downregulated in testosterone-treated orchidectomized male mice (MacLean *et al.* 2008b). Conversely, *Amd1* was downregulated in both mAR^{ΔZF2} GAST and LA and in global AR^{ΔZF2} mice, and upregulated in testosterone-treated orchidectomized

males (Table 2). The fact that these genes were consistently regulated by deletion of the AR in all muscles of both mAR^{ΔZF2} lines and global AR^{ΔZF2} mice, and by androgens in the testosterone-treated orchidectomy model, suggests that they are regulated by the androgen-AR pathway in myofibers. However, because GAST muscle mass was not altered by muscle-specific AR deletion, the contribution of these genes to regulation of muscle mass must be minimal. Similarly, the altered regulation of genes such as *Odc1* and *p57* in GAST but not in LA of mAR^{ΔZF2} lines (Table 2) suggests not only that there is differential regulation of these genes in different muscles, but also that these changes in expression does not alter muscle mass.

To address whether the same genes are regulated by androgens/AR in the development of peak muscle mass, and the maintenance of muscle mass that prevents androgen withdrawal-dependent atrophy in adult males, we compared the genes dysregulated by AR deletion vs orchidectomy/testosterone treatment. Of the genes that were associated with decreased LA mass in both mAR^{ΔZF2} lines, *c-myc*, *Fzd4* and *Igf2* were also regulated by testosterone treatment of orchidectomized males, whereas *Myf5* and *Itgb1bp3* were not (Table 2). This shows that there is some overlap in the pathways that androgens/AR regulate to achieve peak muscle mass and to maintain mass in adulthood, but also other genes are only altered when the AR is deleted throughout development, but not following androgen withdrawal/replacement in adulthood. The genes that are regulated by androgens and the AR in all models may provide the best targets for therapeutic targeting, to maximize the possibility of increasing muscle mass.

One of the most striking findings of all muscle-specific ARKO models is their relative lack of effect on muscle mass. Most mARKO models have focused on the LA muscle because it is atypical in its androgen-responsiveness and shows a large reduction in muscle mass, whereas hind-limb muscle mass is normal or near-normal (Ophoff *et al.* 2009b, Chambon *et al.* 2010, Dubois *et al.* 2014). Our mAR^{ΔZF2} lines also show a similar lack of hind-limb muscle phenotype, despite the fact that global AR^{ΔZF2} males have a 15–20% decrease in hind-limb muscle mass (MacLean *et al.* 2008b). The phenotypic differences between the models may arise through different methodologies used to assess muscle mass, mixed genetic backgrounds of the AR-null mARKO models and also the fact that our model has deletion of DNA binding-dependent actions, but retains potential DNA binding-independent actions, because mutant AR protein is expressed. However, the relative lack of effect on muscle mass of AR deletion in satellite cells, myoblasts

and/or myofibers in all the muscle-specific models is strongly suggestive that the major target of androgen action is in other target tissues. Androgen effects on other tissues that could contribute to regulation of muscle mass could include actions in the CNS. We previously showed that global $AR^{\Delta F2}$ male mice have a 70% reduction in voluntary physical activity (Rana *et al.* 2011). The fact that the two $mAR^{\Delta F2}$ lines have normal voluntary activity demonstrates that this effect is not due to androgens acting through the AR in myoblasts or myofibers, and is, therefore, more likely to be mediated through the AR in other tissues. AR-null CNS-ARKO male mice have increased locomotor activity (cage movement), a measure of incidental ambulatory activity (Raskin *et al.* 2009). In contrast, voluntary physical activity (wheel-running) is decreased in our global $AR^{\Delta F2}$ model. Incidental ambulatory and voluntary physical activities have opposing regulation by estradiol in aromatase knockout mice (Hill *et al.* 2007); therefore, it is possible that they are also regulated in an opposing manner by the AR in the CNS.

In conclusion, our mouse models show that androgens acting through the DNA-binding actions of the AR do not play a major role in myoblasts or myofibers to regulate skeletal muscle mass. While muscle-specific AR deletion causes a 50% reduction in LA muscle mass, relative hind-limb muscle mass is only decreased in the TA and SOL of α -actin $mAR^{\Delta F2}$ but not in MCK $mAR^{\Delta F2}$ males, indicating only a very modest action through myoblasts in some hind-limb muscles. We found that the pattern of expression of genes including *c-myc*, *Fzd4* and *Igf2* is associated with androgen-dependent changes in muscle mass; therefore, these genes are likely to be mediators of anabolic actions of androgens. However, the differential effects of AR deletion on muscle mass and gene expression in the LA compared with hind-limb muscles suggests that the LA is not a good muscle model to study general mechanisms of androgen actions in skeletal muscle. Further research is required to identify the major cellular and molecular targets of androgen actions in muscle, which are likely to include indirect actions via other tissues.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0320>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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