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ORIGINAL ARTICLE

Male Endocrinology

# Expression of androgen receptor target genes in skeletal muscle

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We aimed to determine the mechanisms of the anabolic actions of androgens in skeletal muscle by investigating potential androgen receptor (AR)-regulated genes in *in vitro* and *in vivo* models. The expression of the myogenic regulatory factor *myogenin* was significantly decreased in skeletal muscle from testosterone-treated orchidectomized male mice compared to control orchidectomized males, and was increased in muscle from male AR knockout mice that lacked DNA binding activity (AR<sup>ΔZF2</sup>) versus wildtype mice, demonstrating that *myogenin* is repressed by the androgen/AR pathway. The ubiquitin ligase *Fbxo32* was repressed by 12 h dihydrotestosterone treatment in human skeletal muscle cell myoblasts, and *c-Myc* expression was decreased in testosterone-treated orchidectomized male muscle compared to control orchidectomized male muscle, and increased in AR<sup>ΔZF2</sup> muscle. The expression of a group of genes that regulate the transition from myoblast proliferation to differentiation, *Tceal7*, *p57<sup>Kip2</sup>*, *Igf2* and *calcineurin Aα*, was increased in AR<sup>ΔZF2</sup> muscle, and the expression of all but *p57<sup>Kip2</sup>* was also decreased in testosterone-treated orchidectomized male muscle compared to control orchidectomized male muscle. We conclude that in males, androgens act via the AR in part to promote peak muscle mass by maintaining myoblasts in the proliferative state and delaying the transition to differentiation during muscle growth and development, and by suppressing ubiquitin ligase-mediated atrophy pathways to preserve muscle mass in adult muscle.

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**Keywords:** androgen receptor; anabolic; atrophy; gene expression; skeletal muscle

## INTRODUCTION

There is considerable interest in identifying the mechanisms of androgen action in muscle as androgens are one of the few classes of drugs that are anabolic in muscle. However, they are not used widely because of their potential and known negative side effects.<sup>1,2</sup> Developing more targeted androgen-related therapies may enable their use in a wide range of disorders of low muscle mass including those associated with trauma, chronic disease and age-related sarcopenia.

Clinical studies show that testosterone treatment in hypogonadal<sup>3–5</sup> and eugonadal<sup>6,7</sup> men can increase skeletal muscle mass and strength. Furthermore, testosterone suppression can result in a reduction in muscle size and strength.<sup>8–10</sup> Androgens act through the androgen receptor (AR), predominantly via the genomic pathway, which involves DNA binding and transcriptional regulation of target genes.<sup>11</sup> To understand how androgens increase muscle mass, it is necessary to identify the genes regulated by androgens and the AR in muscle. Androgen-regulated genes have been identified from orchidectomy studies (in rodents)<sup>12,13</sup> and in a study comparing males and females;<sup>14</sup> however, the expression of these genes may be regulated by the combined actions of androgens and estrogens.

One of the questions regarding the anabolic actions of androgens in muscle is whether the mechanisms of action are the same during development as adulthood. Androgens, acting through the AR, are required in males to reach peak muscle mass in adulthood, which

may include effects on myogenic commitment<sup>15,16</sup> or anabolic/proliferation-related pathways.<sup>17,18</sup> Androgens are also required by adults to maintain muscle mass, which may occur via suppression of atrophy/protein turnover pathways.<sup>19</sup> There may or may not be an overlap between these gene regulatory pathways. Another issue is whether muscles of different fiber types or anatomical location respond to androgens in the same pattern. A recent review of the effects of androgen deprivation therapy (ADT) in men shows that in addition to the effects on muscle mass described above, upper limb strength is reduced more than lower limb strength,<sup>20</sup> suggesting anatomical differences in response. In contrast, there have been no demonstrated effects of ADT on muscle endurance,<sup>20</sup> suggesting that slow-twitch fibers, required for fatigue resistance, are less sensitive to androgen withdrawal. Testosterone can increase the cross-sectional area of both type I (slow) and type II (fast) twitch fibers, but with different dose sensitivities.<sup>21</sup> Consistent with previous findings of differential fiber-type effects of androgens in humans, we have shown similar fiber-type-specific effects in our AR<sup>ΔZF2</sup> mouse model.<sup>22</sup> This model has an exon 3 deletion that abolishes DNA binding activity, but retains ligand binding ability and potential non-genomic activity,<sup>23</sup> and shows that the AR is required for maximum contractile strength in male fast-twitch extensor digitorum longus (EDL) but not slow-twitch soleus (SOL) muscle; whereas, the AR reduces fatigue resistance in slow- but not fast-twitch muscle.<sup>24</sup> In rodents, different muscles also

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differ in their androgen responsiveness. The rodent levator ani (LA) muscle, which in males surrounds the rectum adjacent to the anus, is highly responsive to androgens compared to other skeletal muscles,<sup>25</sup> with orchidectomy in mice reducing LA mass by 85%.<sup>26</sup> However, the LA is not representative of most skeletal muscles, as hind-limb muscle mass is only reduced by 13%–16% following orchidectomy.<sup>26</sup> Thus, the LA muscle may provide a greater response to identify similar effects of smaller magnitude in other skeletal muscles, or may have a unique response that is not typical or representative of all muscles.

We have previously identified AR-regulated genes expressed in skeletal muscle using our AR<sup>ΔZF2</sup> mouse model.<sup>24</sup> Microarray analysis on AR<sup>ΔZF2</sup> gastrocnemius (GAST) muscle identified a suite of 96 differentially regulated genes, including genes involved in the polyamine biosynthesis pathway, such as *Odc1* (ornithine decarboxylase 1).<sup>24,27</sup> A subset of these genes were validated by quantitative real-time PCR in AR<sup>ΔZF2</sup> male muscle compared to wildtype (WT) and many, but not all, were regulated by androgens in adult male muscle, showing differential expression in orchidectomized WT males treated with testosterone compared to control orchidectomized males.<sup>24</sup> These genes may be direct AR target genes, or regulated as a secondary consequence of loss of AR DNA binding function. Another way to identify AR-regulated genes in muscle is to use the candidate gene approach. The myogenic regulatory factors (MRFs): *MyoD1*, *Myf5*, *Myf6* and *myogenin*, are critical for muscle development. *Fbxo32* (MAFbx/atrogin1), an important ubiquitin ligase, is another candidate as it induces muscle atrophy by increasing proteolysis,<sup>28</sup> and has been shown to be regulated by androgens in a model of glucocorticoid-induced muscle atrophy.<sup>29</sup> In addition, *c-Myc*, a potent regulator of proliferation in a number of systems, is regulated by androgens in the rat prostate gland<sup>30</sup> and has increased expression during hypertrophy in rat skeletal muscle.<sup>31</sup>

The aim of the current study is to identify and investigate the expression pattern of potential AR-regulated genes in skeletal muscle. One of the limitations of previous studies to identify AR-regulated genes has been the validity of the models used. *In vitro* studies have examined a number of different cell lines with different levels of AR expression and different proliferative status. *In vivo* studies have used different lengths of treatment and determined expression in different muscles that may not be representative of postural and locomotive muscles. To overcome these limitations, we used a combination of different models to examine gene expression in the current study. These include human skeletal muscle cell (SkMC) myocytes treated with the non-aromatizable androgen dihydrotestosterone (DHT) *in vitro* to identify direct androgen effects on gene expression, our previously described orchidectomy model<sup>24,26</sup> to identify androgen regulation of genes in adult muscle and our global AR<sup>ΔZF2</sup> model to identify genes dependent on the DNA binding-dependent actions of the AR during development and in adult muscle.<sup>24</sup> We also investigated gene expression in normal muscle homeostasis, by comparing gene expression during embryonic development, in sexually immature and adult mice and also in males compared to females. In addition, we also compared gene expression in the LA and hind-limb muscles of different fiber types to determine how representative the androgen-sensitive LA muscle is of the locomotor muscles, and if expression differs in slow- and fast-twitch muscles.

## MATERIALS AND METHODS

### Tissue culture

Human SkMC myocytes were cultured as myoblasts in skeletal muscle growth media, or as myotubes in differentiation media

containing Dulbecco's Modified Eagle's medium supplemented with 3% horse serum. For androgen treatment studies, SkMC were cultured as myoblasts or myotubes for 24 h, then media was replaced with media containing charcoal-stripped horse serum supplemented with vehicle (0.1% ethanol) or 10 nmol l<sup>-1</sup> DHT ( $n \geq 5$ ) for 12 or 24 h.

### Mice

To determine the effect of orchidectomy and testosterone replacement on gene expression, we used RNA from GAST muscle of WT C57BL/6 males aged 18–19 weeks that had undergone orchidectomy (orx) aged 8–9 weeks and been treated with control (orx control) ( $n = 9$ ) or testosterone-filled implants (orx + T) ( $n = 9$ ) for 10 weeks, as previously described.<sup>24,26</sup> Testosterone-filled implants delivered a supraphysiological dose, as previously described.<sup>26</sup>

To determine the effect of global deletion of the DNA binding-dependent actions of the AR, we used RNA from GAST muscle of WT ( $n = 6$ ) and AR<sup>ΔZF2</sup> ( $n = 6$ ) male littermates aged 9 weeks, on a congenic C57BL/6 background, as previously described.<sup>24</sup>

To examine gene expression during development, WT C57BL/6 mice were examined at embryonic day (E) 18.5 ( $n = 10$  males,  $n = 9$  females), in sexually immature mice, aged 4 weeks ( $n = 6$  males,  $n = 6$  females) and in adult mice, aged 12 weeks ( $n = 5$  males,  $n = 5$  females). Muscles including mixed fiber GAST, the fast-twitch EDL and the slow-twitch SOL were dissected from the hind-limbs of both males and females, and the LA was dissected from the perineum of males. For embryos, all hind-limb muscles were combined, due to their small size. Muscles were snap frozen in liquid nitrogen and then stored at -80°C for subsequent RNA extraction.

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.

### Gene expression

Total RNA isolation and cDNA synthesis were performed using standard methodology as described.<sup>32</sup> Quantitative real-time PCR was performed in duplicate using 500 ng cDNA and TaqMan gene expression assays (Applied Biosystems, Mulgrave, Victoria, Australia). C<sub>T</sub> values were normalized to a house keeping gene, either 18S or β-actin, and a reference cDNA control using the ΔΔC<sub>T</sub> method as described.<sup>24</sup> The following human and mouse expression assays were used: AR (Hs00907244\_m1) (Mm0138475\_m1), 18S (4319413E), *b-actin* (Mm00607939\_s1), *MyoD1* (Hs00159528\_m1) (Mm00440387\_m1), *Myf5* (Hs00929416\_g1) (Mm00435125\_m1), *myogenin* (Hs00231167\_m1) (Mm00446194\_m1), *Myf6* (Hs00231165\_m1) (Mm00435126\_m1), *Fbxo32* (Hs01041408\_m1) (Mm01207878\_m1), *c-Myc* (Hs00153408\_m1) (Mm00487804\_m1), *Tceal7* (Hs00385936\_m1) (Mm01300577\_g1), *Cdkn1c* (*p57<sup>Kip2</sup>*) (Hs00911652\_s1) (Mm00438170\_m1), *Igf2* (Hs00277496\_s1) (Mm00439564\_m1) and *calcineurin Aa* (Hs00174223\_m1) (Mm01317678\_m1).

### Statistical analysis

Student's *t*-test was used to compare the mean gene expression values of two groups (myoblast versus myotube, vehicle versus DHT, orx versus orx + T and WT versus AR<sup>ΔZF2</sup>), with Levene's test of homogeneity used to determine equal or unequal variance. To determine the effect of age and gender on gene expression, a two-way analysis of variance (ANOVA) was used. To determine the effect of muscle type on gene expression, the mean values of different muscle types were compared by one-way ANOVA with Tukey's or Tamhane's post hoc test (equal or unequal variance) using IBM SPSS version 19 (IBM

Corp., Armonk, NY, USA). A value of  $P < 0.05$  was used to identify significance.

## RESULTS

### The AR regulates its own gene expression

To determine if the AR gene is autologously regulated by androgens in muscle, we first examined regulation *in vitro* using the human myocyte cell line, SkMC, which we have previously shown expresses AR levels similar to human muscle.<sup>27</sup> AR mRNA was up-regulated in myotubes compared to myoblasts (Figure 1a). However, AR gene expression was not regulated by 12 or 24 h DHT treatment of either SkMC myoblasts or myotubes *in vitro* (Figure 1b).

In contrast, AR gene expression was repressed by androgens and the AR in muscle *in vivo*, with AR mRNA levels decreased in muscle from testosterone-treated orchidectomized males compared to control orchidectomized males (Figure 1c), and increased in AR<sup>ΔZF2</sup> males compared to WT male controls (Figure 1d). We have also previously shown that testosterone levels are modestly decreased in AR<sup>ΔZF2</sup> males compared to WT males.<sup>33</sup>

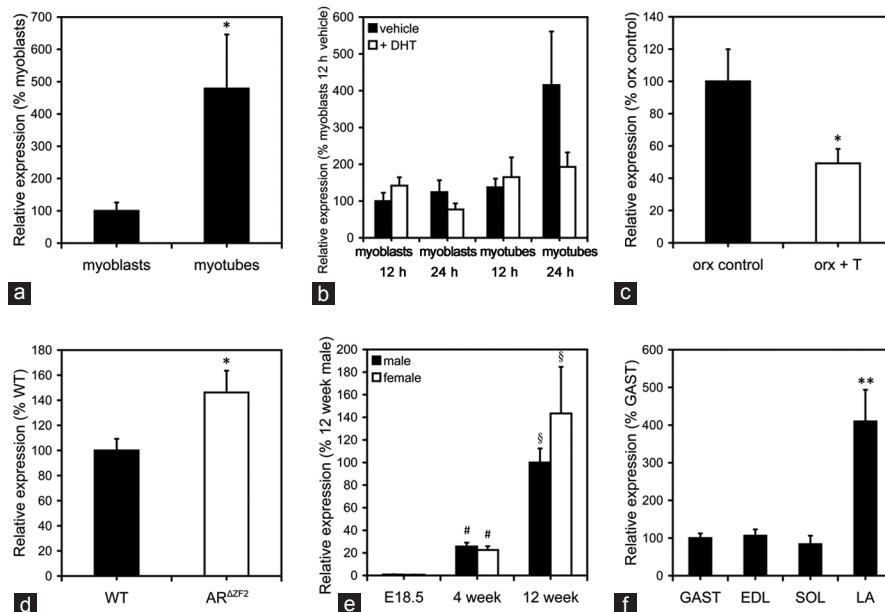
We examined the expression of the AR gene during normal muscle homeostasis, to determine if expression is regulated by developmental stage or gender. We compared AR expression in hind-limb muscles of WT C57BL/6 male and female mice from three different ages; during embryonic development (E18.5), in sexually immature mice aged 4 weeks and in adult mice aged 12 weeks. Two-way ANOVA, to determine if there was an effect of age or gender, showed that there was a significant effect of age but no effect of gender. The expression level of AR in muscle was very

low at E18.5; was higher in both males and females aged 4 weeks ( $P < 0.05$ ) and was highest in 12-week-old males and females ( $P < 0.05$ ) (Figure 1e).

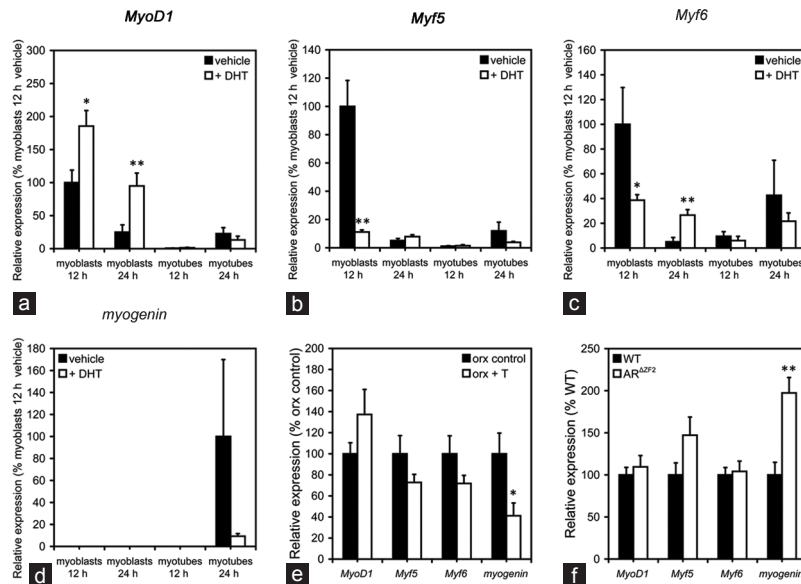
To determine if there was any difference in AR gene expression in muscles of three different fiber-types or anatomical location in males, we measured expression in three hind-limb muscles, the mixed fiber GAST, the fast-twitch EDL and the slow-twitch SOL, and in the highly androgen-sensitive perineal muscle, the LA. AR expression was higher in the LA compared to the hind-limb muscles, but there was no effect of fiber composition, with no difference between mixed, fast- or slow-twitch hind-limb muscles in males (Figure 1f).

### Investigating MRFs as AR-regulated genes

We examined the possible androgen regulation of the MRF genes *MyoD1*, *Myf5*, *Myf6* and *myogenin* in SkMC myoblasts and myotubes. *MyoD1* expression was increased by DHT in SkMC myoblasts following both 12 and 24 h of treatment (Figure 2a). In contrast, *Myf5* expression was decreased by DHT in myoblasts at 12 h (Figure 2b). *Myf6* expression was also decreased by DHT in myoblasts at 12 h, but increased in myoblasts by DHT at 24 h (Figure 2c). *Myogenin* expression was not regulated by DHT *in vitro* (Figure 2d). To determine if these genes were regulated by androgens in adult skeletal muscle, we examined their expression in orchidectomized males treated with or without testosterone. Despite the fact that *MyoD1*, *Myf5* and *Myf6* were regulated by DHT in SkMC myoblasts *in vitro*, they were not regulated by orchidectomy and testosterone *in vivo* (Figure 2e). In contrast, despite the fact that *myogenin* was not regulated *in vitro*, *in vivo* expression was decreased in muscle from testosterone-treated



**Figure 1:** Relative AR gene expression. AR gene expression in (a) SkMC cultured as myoblasts in proliferation media ( $n = 9$ ) or as myotubes in differentiation media ( $n = 10$ ) for 24 h, normalized to myoblasts,  $*P < 0.05$  vs SkMC myoblasts; (b) SkMC cultured in proliferation (myoblasts) or differentiation (myotubes) media and treated with 10 nmol l<sup>-1</sup> DHT or vehicle ( $n \geq 5$  per group) for 12 and 24 h, normalized to myoblast 12 h vehicle; (c) GAST muscle of 18- to 19-week-old male mice following orchidectomy with control implants (orx control) at 8–9 weeks or orchidectomy with testosterone implants (orx + T) ( $n = 9$  per group), normalized to orx control (model previously described<sup>26</sup>),  $*P < 0.05$  vs orx control; (d) GAST muscle of 9-week-old AR<sup>ΔZF2</sup> male mice and WT male littermates ( $n = 6$  per group), normalized to WT (model previously described<sup>24</sup>),  $*P < 0.05$  vs WT; (e) WT male and female mice at E18.5 (all hind-limb muscles) ( $n \geq 9$  per group), 4 weeks (GAST muscle) ( $n = 6$  per group) and 12 weeks (GAST muscle) ( $n = 5$  per group), normalized to 12 week males, # $P < 0.05$  vs E18.5 sex-matched control, § $P < 0.05$  vs 4 week sex-matched control and (f) 12-week-old male muscles (GAST, EDL, SOL, LA) ( $n = 5$  per group), normalized to GAST. \*\* $P < 0.001$  vs GAST, EDL, SOL. All data are mean  $\pm$  s.e.m. and one-way ANOVA, Tukey's post hoc test (age) and Student's *t*-test (sex) or Student's *t*-test (muscle) applied. AR: androgen receptor; DHT: dihydrotestosterone; EDL: extensor digitorum longus; GAST: gastrocnemius; s.e.m.: standard error of the mean; LA: levator ani; orx: orchidectomy; SOL: soleus; SkMC: skeletal muscle cells; WT: wildtype.



**Figure 2:** Expression patterns of MRFs. Gene expression in SkMC cultured in proliferation (myoblasts) or differentiation (myotubes) media and treated with 10 nmol l<sup>-1</sup> DHT or vehicle ( $n \geq 5$  per group) for 12 and 24 h, normalized to myoblast 12 h vehicle: (a) *MyoD1*, \* $P < 0.05$ , \*\* $P < 0.01$  vs vehicle, (b) *Myf5*, \*\* $P < 0.001$  vs vehicle, (c) *Myf6*, \* $P < 0.05$ , \*\* $P < 0.01$  vs vehicle and (d) *myogenin*. MRF expression in (e) 18- to 19-week-old male mice following orchidectomy with control implants (orx control) at 8–9 weeks or orchidectomy with testosterone implants (orx + T) ( $n = 9$  per group), normalized to orx control, \* $P < 0.05$  vs orx control and (f) 9-week-old AR<sup>ΔZF2</sup> male mice and WT male littermates ( $n = 6$  per group), normalized to WT, \*\* $P < 0.001$  vs WT. All data are mean  $\pm$  s.e.m., Student's *t*-test applied. AR: androgen receptor; DHT: dihydrotestosterone; MRFs: myogenic regulatory factors; orx: orchidectomy; SkMC: skeletal muscle cells; WT: wildtype.

orchidectomized males compared to control orchidectomized males (Figure 2e). Similarly, there was no difference in the expression of *MyoD1*, *Myf5* and *Myf6* in AR<sup>ΔZF2</sup> male muscle compared to control (Figure 2f), but *myogenin* mRNA levels were higher in AR<sup>ΔZF2</sup> muscle than WT controls (Figure 2f).

We examined the patterns of expression of the MRFs during development in males and females in different muscle types. There was no difference in expression prior to puberty (at E18.5 and 4 weeks of age), but in adults, levels of *MyoD1*, *Myf5*, *Myf6* and *myogenin* were all lower in males than in females (Figure 3a–3d).

In 12-week-old males, *MyoD1* expression was higher in LA muscle compared to the hind-limb muscles, and expression was also higher in the fast-twitch EDL and the mixed fiber GAST compared to the slow-twitch SOL (Figure 3e). *Myf5* expression was higher in the LA than in the hind-limb muscles (Figure 3f). *Myf5* expression was lower in the EDL than the SOL and also lower in the GAST compared to the SOL (Figure 3f). *Myf6* expression was higher in the LA compared to the hind-limb muscles, with no difference in expression between different hind-limb muscles (Figure 3g). *Myogenin* expression was also higher in the LA compared to the hind-limb muscles (Figure 3h), with expression being lower in the EDL than in the SOL (Figure 3h).

#### Investigating regulators of muscle atrophy and myoblast proliferation as AR-regulated genes

We next examined the androgen regulation of the atrophy regulator *Fbxo32* and the regulator of proliferation *c-Myc*. *Fbxo32* expression was decreased by DHT treatment in myoblasts, at both 12 and 24 h (Figure 4a), but although basal expression of *Fbxo32* was higher in myotubes, it was not regulated by DHT in these cells. *c-Myc* was not regulated by androgens *in vitro* (Figure 4b).

Despite the fact that *Fbxo32* was repressed by DHT in SkMC myoblasts, expression of *Fbxo32* in muscle was not altered by orchidectomy and testosterone treatment (Figure 4c) or AR<sup>ΔZF2</sup> (Figure 4d). In contrast, expression of *c-Myc* was decreased

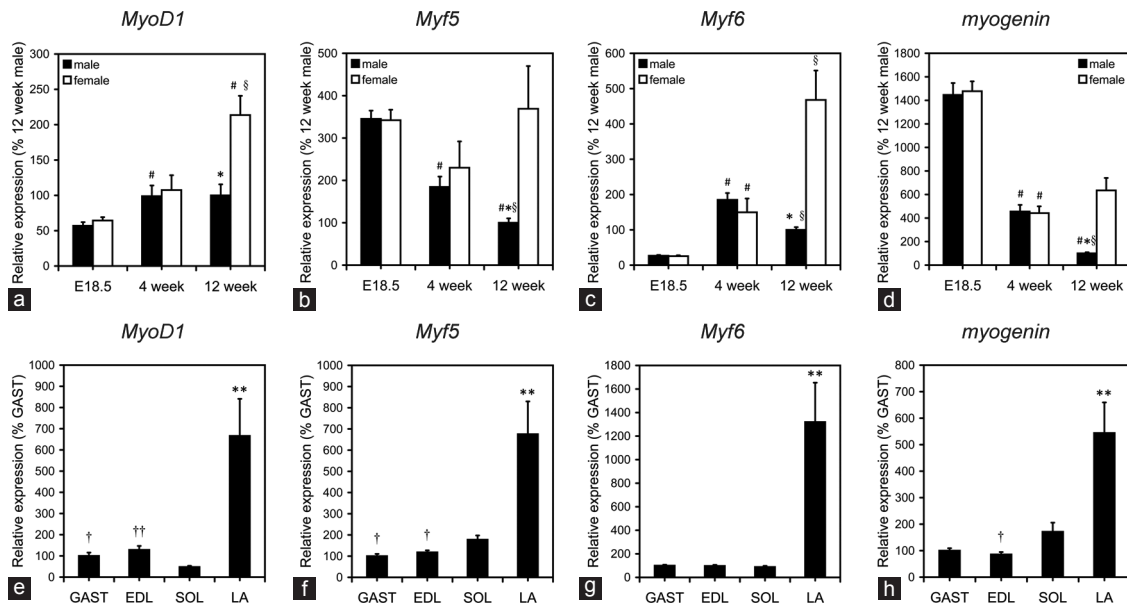
in muscle of testosterone-treated orchidectomized males compared to control orchidectomized males (Figure 4c), and increased in AR<sup>ΔZF2</sup> males compared to WT controls (Figure 4d), suggesting *c-Myc* is repressed by androgens via the AR.

Expression levels of *Fbxo32* in muscle were higher in adults compared to embryonic or 4-week-old mice consistent with the fact that adult muscle comprised predominantly post-proliferative myotubes or myofibers. There is no difference in expression between male and female prepubertally, but *Fbxo32* levels were lower in males than females at 12 weeks of age (Figure 5a). Despite the fact that muscle cell proliferation is higher prenatally than postnatally, levels of *c-Myc* in muscle were higher at 4 weeks of age compared to E18.5 consistent with the orchidectomy and AR<sup>ΔZF2</sup> data suggesting androgens repress *c-Myc* in adult muscle. *c-Myc* expression was lower in males compared to females at 12 weeks of age (Figure 5b).

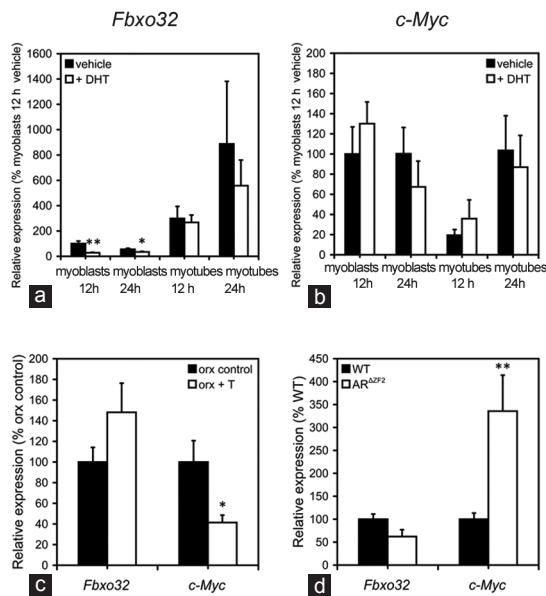
*Fbxo32* expression was lower in the LA compared to the EDL muscle (Figure 5c), but there was no significant difference between the LA and other hind-limb muscles. *c-Myc* expression was higher in the LA than in all the hind-limb muscles (Figure 5d).

#### Investigating regulators of myoblast differentiation as AR-regulated genes

We examined the expression of four genes that regulate the transition of muscle cells from the proliferative to the differentiated state, *Tceal7*<sup>34</sup>, *p57<sup>Kip2</sup>* (*p57*)<sup>35</sup>, *Igf2*<sup>36,37</sup> and *calcineurin Aa*.<sup>38</sup> We have previously shown that levels of these four genes are higher in AR<sup>ΔZF2</sup> males than in WT male controls, and that expression of *Tceal7*, *Igf2* and *calcineurin Aa*, but not *p57*, is decreased in muscle from testosterone-treated orchidectomized males compared to control orchidectomized males.<sup>24</sup> *Tceal7* was decreased in SkMC myoblasts following 12 h of DHT treatment, but increased following 24 h of DHT treatment (Figure 6a). *p57* expression was decreased following 12 h of DHT treatment in SkMC myoblasts (Figure 6b). There was no regulation of *Igf2* or *calcineurin Aa* expression by androgens *in vitro* (Figure 6c and 6d).



**Figure 3:** *In vivo* gene expression of MRFs. Gene expression in WT male and female mice at E18.5 (all hind-limb muscles) ( $n \geq 9$  per group), 4 weeks (GAST muscle) ( $n = 6$  per group) and 12 weeks (GAST muscle) ( $n = 5$  per group), normalized to 12 week males: (a) *MyoD1*,  $^{\#}P < 0.05$  vs E18.5 sex-matched control,  $^{\S}P < 0.05$  vs 4 week sex-matched control,  $^*P < 0.05$  vs age-matched group, (b) *Myf5*,  $^{\#}P < 0.05$  vs E18.5 sex-matched control,  $^{\S}P < 0.05$  vs 4 week sex-matched control,  $^*P < 0.05$  vs age-matched group, (c) *Myf6*,  $^{\#}P < 0.05$  vs E18.5 sex-matched control,  $^{\S}P < 0.05$  vs 4 week sex-matched control,  $^*P < 0.05$  vs age-matched group, (d) *myogenin*,  $^{\#}P < 0.05$  vs E18.5 sex-matched control,  $^{\S}P < 0.05$  vs 4 week sex-matched control,  $^*P < 0.05$  vs age-matched group. Gene expression in 12-week-old male mice muscle (GAST, EDL, SOL, LA) ( $n = 5$  per group), normalized to GAST, (e) *MyoD1*,  $^{†††}P < 0.001$  vs GAST, EDL, SOL,  $^{\dagger}P < 0.05$ ,  $^{††}P < 0.001$  vs SOL, (f) *Myf5*,  $^{***}P < 0.001$  vs GAST, EDL, SOL,  $^{\dagger}P < 0.05$  vs SOL, (g) *Myf6*,  $^{***}P < 0.001$  vs GAST, EDL, SOL and (h) *myogenin*,  $^{***}P < 0.001$  vs GAST, EDL, SOL,  $^{\dagger}P < 0.05$  vs SOL. All data are mean  $\pm$  s.e.m., one-way ANOVA, Tukey's *post hoc* test (age) and Student's *t*-test (sex) or Student's *t*-test (muscle) applied. EDL: extensor digitorum longus; GAST: gastrocnemius; s.e.m.: standard error of the mean; LA: levator ani; MRFs: myogenic regulatory factors; SOL: soleus; SkMC: skeletal muscle cells; WT: wildtype.



**Figure 4:** Relative *Fbxo32* and *c-Myc* gene expression. Gene expression in SkMC cultured in proliferation (myoblasts) or differentiation (myotubes) media and treated with 10 nmol l<sup>-1</sup> DHT ( $n \geq 8$  per group) or vehicle ( $n \geq 6$  per group) for 12 and 24 h, normalized to myoblasts 12 h vehicle: (a) *Fbxo32*,  $^*P < 0.05$ ,  $^{**}P < 0.01$  vs vehicle and (b) *c-Myc*. *Fbxo32/c-Myc* expression in (c) 18- to 19-week-old male mice following orchidectomy with control implants (orx control) at 8–9 weeks or orchidectomy with testosterone implants (orx + T) ( $n = 9$  per group), normalized to orx control,  $^*P < 0.05$  vs orx control and (d) 9-week-old AR<sup>ΔZF2</sup> male mice and WT male littermates ( $n = 6$  per group), normalized to WT,  $^{**}P < 0.01$  vs WT. All data are mean  $\pm$  s.e.m., Student's *t*-test applied. AR: androgen receptor; DHT: dihydrotestosterone; orx: orchidectomy; s.e.m.: standard error of the mean; SkMC: skeletal muscle cells; WT: wildtype.

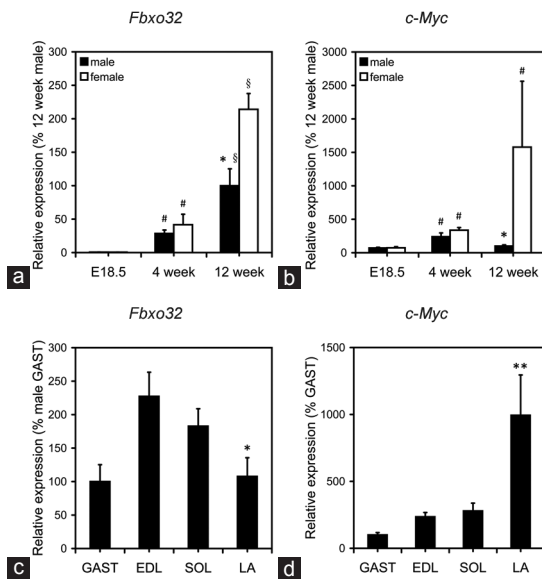
The expression of *Tceal7* decreased with age, with highest expression in embryonic muscle. In adults, *Tceal7* expression was lower in males compared to females at 12 weeks (Figure 7a). *p57* expression also decreased with age, and was higher in males than in females at 4 weeks of age and lower in males than in females at 12 weeks of age (Figure 7b). Similarly, the expression of *Igf2* also decreased with age and was lower in males than females at 4 and 12 weeks of age (Figure 7c). In contrast, expression levels of *calcineurin Aa* increased with age and were lower in males than females at 4 and 12 weeks of age (Figure 7d).

*Tceal7* expression was lower in the LA than in the SOL and the EDL (Figure 7e), whereas in contrast, *p57* was higher in the LA compared to the SOL, EDL and GAST (Figure 7f). *Igf2* was higher in the LA compared to the SOL, EDL and GAST (Figure 7g). *Calcineurin Aa* expression was higher in the LA than the SOL or GAST (Figure 7h).

## DISCUSSION

The aims of this study were to identify AR-regulated genes in skeletal muscle, to determine if the same genes are regulated during muscle growth and development as during maintenance of muscle mass in adulthood, to examine expression in normal muscle homeostasis in males and females, and to compare gene expression in different muscle types. All the genes we investigated, which included the MRFs as well as other genes known to control proliferation and/or transition to differentiation, showed regulation by androgens and the AR in at least one system, but regulation differed in the various model systems, highlighting the importance of examining multiple models.

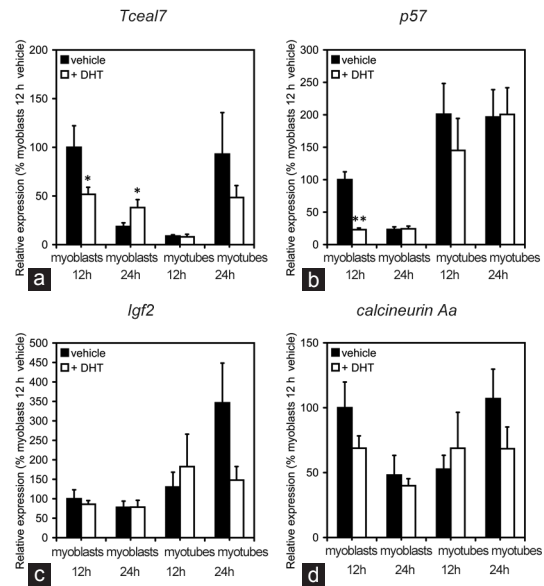
There was increased gene expression in adult AR<sup>ΔZF2</sup> male muscle compared to WT for three of the genes examined, namely the AR, *myogenin* and *c-Myc*. These genes are downregulated either directly



**Figure 5:** *In vivo* *Fbxo32* and *c-Myc* gene expression. Gene expression in WT male and female mice at E18.5 (all hind-limb muscles) ( $n \geq 9$  per group), 4 weeks (GAST muscle) ( $n = 6$  per group) and 12 weeks (GAST muscle) ( $n = 5$  per group), normalized to 12 week males: (a) *Fbxo32*,  $\#P < 0.05$  vs E18.5 sex-matched control,  $\S P < 0.05$  vs 4 week sex-matched control,  $*P < 0.05$  vs age-matched group and (b) *c-Myc*,  $\#P < 0.05$  vs E18.5 sex-matched control,  $*P < 0.05$  vs age-matched group. Gene expression in 12-week-old male mice muscle (GAST, EDL, SOL, LA) ( $n = 5$  per group): (c) *Fbxo32*,  $*P < 0.05$  vs EDL and (d) *c-Myc*,  $**P < 0.001$  vs GAST, EDL, SOL. All data are mean  $\pm$  s.e.m., one-way ANOVA, Tukey's *post hoc* test (age) and Student's *t*-test (sex) or Student's *t*-test (muscle) applied. EDL: extensor digitorum longus; GAST: gastrocnemius; s.e.m.: standard error of the mean; LA: levator ani; SOL: soleus; WT: wildtype.

or indirectly via the AR and these effects could either occur during development or in adulthood. In our previous study, we showed that *Tceal7*, *p57*, *Igf2* and *calcineurin Aa* were also expressed at higher levels in AR<sup>ΔZF2</sup> male muscle compared to WT.<sup>24</sup> Of these seven genes with elevated expression in the absence of AR DNA-binding activity, all but one, *p57*, were also repressed when orchidectomized WT males were treated with testosterone. The regulation of these genes by testosterone could potentially occur via the AR or the estrogen receptor (ER), but the fact that these genes were also differentially expressed in AR<sup>ΔZF2</sup> demonstrates that they must be regulated by the AR pathway. This demonstrates that the AR, *myogenin*, *c-Myc*, *Tceal7*, *Igf2* and *calcineurin Aa* genes are repressed by androgens and the AR in adult male muscle, and therefore these genes are involved in maintaining peak androgen-dependent muscle mass in adult males. In contrast, the fact that *p57* expression was only elevated in the AR<sup>ΔZF2</sup> model, but unaffected in testosterone-treated orchidectomized males, suggests that *p57* is only repressed by the AR during muscle growth and development, and that this repression is not required to maintain muscle mass during adulthood.

Four of the genes repressed by androgens and the AR regulate the transition of myoblasts from the proliferative to the terminally differentiated state. *Myogenin*, a MRF, is essential for muscle terminal differentiation<sup>39</sup> and promotes myotube formation. *Tceal7*, first identified as an ovarian tumor suppressor, also promotes the switch from myoblast proliferation to differentiation.<sup>34</sup> Similarly, *Igf2* and *calcineurin Aa*, are associated with myoblast differentiation.<sup>40-42</sup> During development, tissue growth is a balance of both the rate and number of rounds of cellular proliferation occurring prior to terminal differentiation. Our data suggest that during muscle development

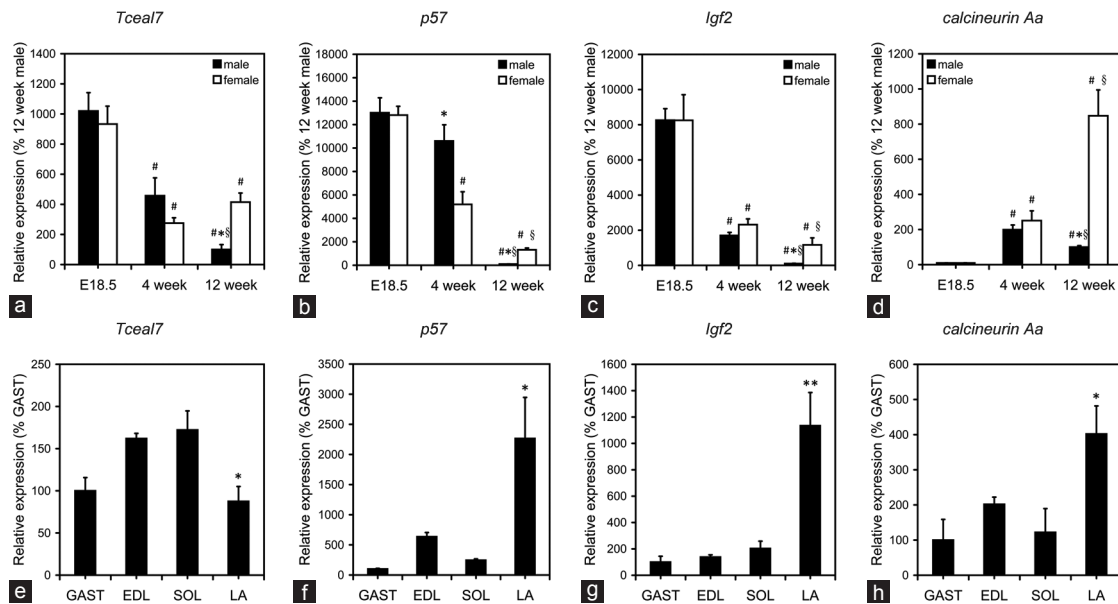


**Figure 6:** *In vitro* expression patterns of genes involved in the regulation of muscle cell proliferation to differentiation. Gene expression in SkMC cultured in proliferation (myoblasts) or differentiation (myotubes) media and treated with 10 nmol l<sup>-1</sup> DHT ( $n \geq 8$  per group) or vehicle ( $n \geq 6$  per group) for 12 and 24 h, normalized to myoblasts 12 h vehicle: (a) *Tceal7*,  $*P < 0.05$  vs vehicle, (b) *p57*,  $**P < 0.001$  vs vehicle, (c) *Igf2* and (d) *calcineurin Aa*. All data are mean  $\pm$  s.e.m., Student's *t*-test applied. DHT: dihydrotestosterone; s.e.m.: standard error of the mean; SkMC: skeletal muscle cells.

in males, the androgen/AR pathway maintains myoblasts in the proliferative state by delaying myotube formation. This would allow the formation of additional myoblasts prior to fusion into myofibers, resulting in an overall increase in muscle size and mass.

The one gene that does not fit this pattern of the negative regulation of AR-regulated genes delaying terminal myoblast differentiation is the proto-oncogene *c-Myc*, which controls cellular proliferation in many tissues including skeletal muscle.<sup>43,44</sup> Our data show that *c-Myc* expression is repressed by the androgen/AR pathway in muscle, which is consistent with studies showing that *c-Myc* is repressed by androgens in the ventral prostate.<sup>30,45</sup> However, *c-Myc* usually promotes cellular proliferation,<sup>44,46,47</sup> therefore the increase in *c-Myc* expression in AR<sup>ΔZF2</sup> and orchidectomized control males would be expected to increase muscle growth. Increased *c-Myc* expression has been associated with compensatory hypertrophy and  $\beta$ -agonist-induced hypertrophy.<sup>31</sup> Therefore, the increased *c-Myc* expression in orchidectomized control and AR<sup>ΔZF2</sup> males may be a compensatory mechanism attempting to stimulate muscle growth in the absence of androgens and the AR. The lack of hypertrophic response despite the increased *c-Myc* expression in AR<sup>ΔZF2</sup> and control orchidectomized males could therefore indicate that androgens regulate a downstream effector of *c-Myc*, and thus the elevated *c-Myc* is unable to stimulate proliferation in these muscles. It has been shown that increased *Tceal7* levels inhibit *c-Myc* transcriptional activity.<sup>48</sup> Therefore, the concomitant elevation of *Tceal7* expression in the AR<sup>ΔZF2</sup> and control orchidectomized males could be one mechanism for the lack of proliferative and hypertrophic effect of the increased *c-Myc* expression.

In adult males, the increased expression of *myogenin* in muscle of control orchidectomized males compared to testosterone-treated males is similar to the upregulation of *myogenin* that occurs during denervation-induced atrophy.<sup>49</sup> In addition to its role as an MRF



**Figure 7:** *In vivo* expression patterns of genes involved in the regulation of muscle cell proliferation to differentiation. Gene expression in WT male and female mice at E18.5 (all hind-limb muscles) ( $n \geq 9$  per group), 4 weeks (GAST muscle) ( $n = 6$  per group) and 12 weeks (GAST muscle) ( $n = 5$  per group), normalized to 12 week males. (a) *Tceal7*, (b) *p57*, (c) *Igf2* and (d) *calcineurin Aa*,  $^{\#}P < 0.05$  vs E18.5 sex-matched control,  $^{\$}P < 0.05$  vs 4 week sex-matched control,  $^{*}P < 0.05$  vs age-matched group. Gene expression in 12-week-old male mice muscle (GAST, EDL, SOL, LA) ( $n = 5$  per group), normalized to GAST. (e) *Tceal7*,  $^{*}P < 0.05$  vs EDL, SOL, (f) *p57*,  $^{*}P < 0.05$  vs GAST, EDL, SOL, (g) *Igf2*,  $^{**}P < 0.001$  vs GAST, EDL, SOL and (h) *calcineurin Aa*,  $^{*}P < 0.05$  vs GAST, SOL. All data are mean  $\pm$  s.e.m., one-way ANOVA, Tukey's *post hoc* test (age) and Student's *t*-test (sex) or Student's *t*-test (muscle) applied. EDL: extensor digitorum longus; GAST: gastrocnemius; s.e.m.: standard error of the mean; LA: levator ani; SOL: soleus; SkMC: skeletal muscle cells; WT: wildtype.

during development, myogenin controls neurogenic atrophy by upregulating the ubiquitin ligases *Fbxo32* and *MuRF1* that promote muscle protein ubiquitination and degradation.<sup>49,50</sup> Although we did not see any changes in *Fbxo32* expression *in vivo*, expression of *Fbxo32* was repressed by DHT in myoblasts *in vitro*. Other orchidectomy studies have shown upregulation of *Fbxo32* expression in muscle,<sup>13,51</sup> with increased muscle protein degradation during atrophy,<sup>52</sup> while androgen treatment increases muscle protein synthesis.<sup>53</sup> Therefore, it is possible that androgen withdrawal-dependent atrophy occurs in part via the same myogenin-dependent ubiquitin ligase pathways activated in neurogenic atrophy, due to the loss of *myogenin* and *Fbxo32* repression by the AR.

In addition to its role in muscle differentiation, calcineurin signaling also regulates fiber-type determination. Calcineurin signaling upregulates genes of the slow fiber phenotype,<sup>54</sup> with inhibition of calcineurin signaling inhibiting the induction of slow fiber phenotype.<sup>55</sup> The repression of *calcineurin Aa* by androgens and the AR in adult male muscle, and the lower expression in adult males than females, is therefore likely to maintain more fast-twitch and fewer slow-twitch muscle fibers in males. This is consistent with our previous data showing that the slow-twitch SOL muscle of female mice is more fatigue-resistant than males, and male AR <sup>$\Delta$ ZF2</sup> SOL fatigue resistance is equivalent to female.<sup>24</sup>

There was no consistent pattern between the *in vitro* androgen regulation of gene expression and the regulation observed *in vivo*. For example, of the genes repressed by androgens and the AR in the AR <sup>$\Delta$ ZF2</sup> and orchidectomy models, only one, *Tceal7*, also showed repression by DHT in SkMC myoblasts, and only after 12 h of treatment. This suggests that *Tceal7* is a direct target of the AR in myoblasts. As previously discussed, *Tceal7* represses myoblast proliferation and enhances differentiation into myotubes,<sup>34</sup> suggesting the AR may

regulate myoblast transition from proliferation to differentiation directly via *Tceal7*. A number of genes regulated by DHT *in vitro* showed no change in AR <sup>$\Delta$ ZF2</sup> or orchidectomy models, including *MyoD1*, *Myf5*, *Myf6* and *Fbxo32*. This could be due to a number of factors, including the genes being rapidly regulated by androgens and the AR but then reaching equilibrium in the *in vivo* models by the time they were examined, or the fact that these genes may have developmental effects only when myoblast cell numbers are highest whereas adult muscle is mostly composed of myofibers. In addition, SkMC may not be a good model for physiological myocytes, as they suffer the limitation of all *in vitro* models, having been selected for the ability for continuous proliferation *in vitro*, and thus may not retain the characteristics of *in vivo* muscle cells, despite the fact that they express the AR gene at similar levels to normal muscle.<sup>56</sup> A previous study using non-quantitative Western analysis of C2C12 cells stably transfected with the AR suggested testosterone may increase *myogenin* protein levels,<sup>57</sup> in contrast to the lack of effect of DHT on *myogenin* mRNA levels in SkMC, but the repression of *myogenin* by the androgen/AR pathway *in vivo* in our study. The variation in results *in vitro* may be due to the different methodologies and different cell lines examined. More generally, the observation of differences between results *in vitro* and *in vivo* emphasizes the importance of examining gene expression in multiple model systems to determine the expression and regulation of genes in normal physiology.

Expression levels of *myogenin*, *Tceal7*, *p57* and *Igf2* decreased with age, concomitant with the rise in serum testosterone levels that occurs in male mice during post-natal development, reaching a peak in adulthood.<sup>58</sup> Therefore, one of the factors contributing to the downregulation of these genes in adulthood could be an increase in the AR-mediated repression. In contrast, the levels of *calcineurin Aa* and the MRFs *MyoD1* and *Myf6* were relatively low in embryonic

mice, and increased with age. The fact that *Tceal7* expression is lower in adult LA muscle compared to other skeletal muscles could be because *AR* expression is higher in adult male LA muscle compared to other skeletal muscles, resulting in greater repression of *Tceal7* expression in the LA than other muscles.

We also examined gene expression in normal male and female muscle, and a number of genes that we showed were repressed by androgens in *AR<sup>AZF2</sup>* males and the orchidectomy model also had lower expression in adult males compared with females, including *myogenin*, *c-Myc*, *Tceal7* and *Igf2*. This supports the hypothesis that these genes are repressed by androgens during normal muscle homeostasis in adult males. There was no difference in the expression of other genes; therefore, factors other than androgens and the *AR* are likely to be more important for regulation of their expression. Gender differences in expression for genes not regulated in *AR<sup>AZF2</sup>* and orchidectomy models are most likely due to ER-mediated effects.

When comparing the expression pattern of genes in the LA compared to hind-limb skeletal muscles, the LA had the highest expression of all the *MRF* genes, as well as *c-Myc*, *p57*, *Igf2* and *calcineurin Aa*, but lower expression of *Fbxo32* and *Tceal7*. These data demonstrate that LA gene expression patterns are not representative of other skeletal muscle. Animal studies show that there is an increased density of *AR* in the myonuclei of the LA compared to other muscle such as the EDL,<sup>59,60</sup> and our study confirms that the *AR* gene is expressed at higher levels in the LA than all hind-limb muscles. These data may explain the differences in expression of *AR*-regulated genes in the LA compared to the other muscles. Therefore, our results suggest that LA muscle may not be a good representative model for studying androgen-dependent pathways of importance to developing therapies with effects on locomotor and postural muscles.

In conclusion, we have used a number of different *in vitro* and *in vivo* models to determine *AR*-regulated gene expression in skeletal muscle, and have demonstrated that the *AR*, *myogenin*, *c-Myc*, *Tceal7*, *Igf2* and *calcineurin Aa* are repressed by the androgens and the *AR*. Furthermore, this study identified gender differences in the expression of genes including *myogenin*, *c-Myc*, *Tceal7* and *Igf2*, and showed that LA gene expression patterns are not representative of other skeletal muscle. There is considerable overlap in the genes that are regulated by the *AR* throughout development, and by androgens in adult males. Our data suggest that androgens regulate the development of peak muscle mass in males via *AR*-mediated repression of *myogenin*, *c-Myc*, *Tceal7*, *p57*, *Igf2* and *calcineurin Aa* through delaying the transition of myoblasts from proliferation to differentiation. In addition, we propose that in adult male muscle, androgens and the *AR* repress *myogenin*, *Fbxo32*, *c-Myc*, *Tceal7*, *Igf2* and *calcineurin Aa* expression, resulting in suppression of myogenin/ubiquitin ligase-mediated atrophy pathways to preserve muscle mass and maintaining a tendency towards a more fast-twitch phenotype in male muscle. The development of strategies to target the activation of these molecules and signaling pathways specifically in skeletal muscle may therefore be useful in the search for anabolic therapies to treat disorders of low muscle mass.

#### AUTHOR CONTRIBUTIONS

KR performed data analysis and drafted the manuscript. NKLL performed all gene expression studies. JDZ participated in the coordination of the study. HEM conceived the study, participated in the design, coordination of the study and drafting of the manuscript.

#### COMPETING INTERESTS

The authors declare no competing interests.

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