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Citation for published version:

Welsh, M, Saunders, PTK, Atanassova, N, Sharpe, RM & Smith, LB 2009, 'Androgen action via testicular peritubular myoid cells is essential for male fertility', *The FASEB Journal*, vol. 23, no. 12, pp. 4218-4130. <https://doi.org/10.1096/fj.09-138347>

Digital Object Identifier (DOI):

[10.1096/fj.09-138347](https://doi.org/10.1096/fj.09-138347)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

The FASEB Journal

Publisher Rights Statement:

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Androgen action *via* testicular peritubular myoid cells is essential for male fertility

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ABSTRACT Androgens are essential for normal spermatogenesis and male fertility, but how androgens exert this effect remains uncertain. Androgen receptors (ARs) are expressed in several testicular cell types, but continuing uncertainty exists over which cell type mediates androgen control of spermatogenesis. Androgen signaling *via* Sertoli cells (SCs) is essential for complete spermatogenesis, but the role for androgen signaling *via* peritubular myoid (PTM) cells is contentious. To address this controversy, we generated PTM-specific AR-knockout (PTM-ARKO) mice in which gross reproductive development was normal, but all PTM-ARKO males were azoospermic and infertile. Testis weight was reduced beyond puberty, and in adulthood there was an 86% reduction in germ cells, compared with wild-type littermates. These changes were not explained by any deficits in testosterone, luteinizing hormone, or follicle-stimulating hormone concentrations. SC function was impaired in PTM-ARKO males, indicated by reduced seminiferous tubule fluid production and reduced expression of some androgen-dependent SC genes. Androgen action *via* PTM cells is therefore essential for normal testis function, spermatogenesis, and fertility in males. This study also provides the first direct evidence for the importance of androgen-driven stromal-epithelial interactions underpinning the regulation of spermatogenesis; PTM-ARKO mice will enable identification of the new molecular pathways involved.—Welsh, M., Saunders, P. T. K., Atanassova, N., Sharpe, R. M., Smith, L. B. Androgen action *via* testicular peritubular myoid cells is essential for male fertility. *FASEB J.* 23, 4218–4230 (2009). www.fasebj.org

Key Words: spermatogenesis • stromal-epithelial interactions • testosterone • steroid receptors

TESTOSTERONE IS ESSENTIAL for normal male fertility, controlling both the initiation and maintenance of spermatogenesis (1, 2). However, limited progress has been made in understanding how these processes are effected. This ignorance has hindered the development of new male contraceptives and treatments for male infertility, which is the most common cause of couple infertility (3).

Testosterone is produced in the testes by the Leydig cells (LCs) and binds to the androgen receptor (AR) to

modulate gene transcription in target cells (4). AR is expressed in several testicular cell types, but continuing uncertainty exists over which of these cell types mediates androgen control of spermatogenesis. Germ cells (GCs) lacking AR mature normally (5); therefore, testosterone is believed to regulate spermatogenesis *via* AR expression in testicular somatic cells, namely the Sertoli cells (SCs), the peritubular myoid (PTM) cells, or LCs themselves. SCs have long been considered the prime candidates because of their intimate contact with the developing GCs (6). This theory has been investigated directly by ablating AR in SCs (SCARKO mice) using the Cre/lox system, and it was shown that these mice are infertile because the GCs fail to complete meiosis (7, 8). However, in other androgen-dependent tissues, such as the prostate gland, AR signaling *via* the stromal cells has a profound influence on organ development and epithelial cell function (9, 10). If the same stromal-epithelial interactions apply in the testis, we would expect the stromal PTM cells to play a pivotal role in mediating effects of androgens on epithelial SC function and spermatogenesis.

PTM cells surround the seminiferous tubules and express AR from fetal life through to adulthood, unlike the SCs, which only become androgen responsive postnatally (11). Despite many years of research, surprisingly little is known about the role for PTM cells in male fertility. It has been shown *in vivo* that loss of PTM cells results in abnormal spermatogenesis (12), and 2 decades ago it was postulated that PTM cells might mediate androgenic control of spermatogenesis (13) as they interact intimately with SCs, for example, in laying down the basement membrane that surrounds the seminiferous tubules (14, 15). Furthermore, *in vitro*

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doi: 10.1096/fj.09-138347

evidence shows that androgens can stimulate PTM cells to secrete factors that modulate SC function and thus GC development (13, 16). Unfortunately, these PTM-derived secretory factors remain unidentified (16, 17). More recently, Cre/lox technology was used in mice to delete AR from PTM cells, and although this resulted in reduced sperm production, it did not result in male infertility (18). This result initially suggested that androgen action *via* PTM cells was not essential for spermatogenesis. However, an independent study (19) later reported that the SM22-Cre line used in these experiments to ablate AR functioned poorly in the testis, whereas, in contrast, Cre recombinase was highly expressed in the testis in smMHC-Cre mice. This raises the possibility that the mild phenotype reported by Zhang *et al.* in their PTM cell AR knockout (KO) (18) might not truly reflect the importance of androgen action *via* PTM cells.

The aim of our studies, therefore, was to compare the ability to ablate AR signaling *via* testicular PTM cells using both of these Cre mouse lines to provide definitive evidence for the role for AR signaling *via* PTM cells in male fertility.

MATERIALS AND METHODS

Breeding of transgenic mice

Mice in which the AR has been selectively ablated from the PTM cells were generated using Cre/loxP technology. PTM cells are smooth muscle cells; therefore, to delete AR from these cells, male mice heterozygous for Cre recombinase under the control of a smooth muscle myosin heavy chain (MH) (20) or a smooth muscle 22 α (SM) as used by Zhang *et al.* (18), on an identical strain background (21) promoter were mated to female mice homozygous for a floxed AR (8). The Cre-positive (AR^{lox} -positive) male offspring from these matings are termed MHAR KO (later termed PTM-ARKO) and SMAR KO, respectively, whereas the Cre-negative, AR^{lox} -positive littermates were used as controls (termed WT). Mouse nomenclature is summarized in **Table 1**. Cre-positive, AR^{lox} -negative mice were also generated by mating MH or SM Cre-positive stud males to C57BL/6J females to confirm that expression of Cre alone did not induce a phenotype. Sex and genotype ratios were all identified at the expected Mendelian ratios. Complete androgen receptor knockout (ARKO) mice were generated as published previously (22). All mice were bred under standard conditions of care and use under licensed approval from the UK Home Office.

PCR genotyping of mice

Stud male MH or SM mice were identified by genotyping from ear or tail DNA for the presence of Cre using standard PCR (<http://jaxmice.jax.org/pub/cgi/protocols/protocols.sh?objtype=>

protocol&protocol_id=288). Females homozygous for AR^{lox} were identified using primers for AR exon 2 (7); these primers revealed bands of 1142, 1072, and 612 bp, relating to mice with a floxed, WT, or excised allele of the AR, respectively. All MHAR and SMAR male offspring were genotyped for the presence of Cre using the primers detailed above; all male offspring were hemizygous for X-linked AR^{lox} .

Evaluation of fertility

To investigate fertility, postnatal day (d)50 and 100 MHAR KO and WT males were each housed with an adult C57BL/6J female for 4 days. This process was repeated with 3 subsequent WT females per male. Female mice were monitored for 25 days for litters to be born.

Recovery of reproductive tissues

Male mice were culled at various postnatal ages (d12–140) by inhalation of carbon dioxide and subsequent cervical dislocation. Body weight and anogenital distance were measured, and mice were examined for any gross abnormalities of the reproductive system. Testes, seminal vesicles, and ventral prostates were removed from the mice and weighed, whereas epididymides and vas deferens were recovered but not weighed. To collect fetal testes, dams were culled at embryonic day (e)17.5, and fetuses were recovered, decapitated, and placed in ice-cold PBS (Sigma, Poole, UK). Testes were collected from male fetuses and weighed. Tissues were either snap-frozen for subsequent RNA analysis or fixed in Bouin's fixative for 6 h. Bouin's-fixed tissues were processed and embedded in paraffin wax, and 5- μ m sections were used for histological analysis as reported previously (23). Sections of testis and epididymis were stained with hematoxylin and eosin using standard protocols and examined for histological abnormalities.

Determination of testicular cell composition

Standard stereological techniques involving point counting of cell nuclei were used as described (8) to determine the nuclear volume per testis of SCs, GCs, and the relative volumes of interstitium, seminiferous epithelium, and seminiferous tubule lumen. In brief, cross sections of testes from 4 to 6 MHAR KO or WT mice at d12, 21, 50, and 100 were examined using a Leitz 363 plan apo objective lens ($\times 63$) fitted to a Leitz Laborlux microscope and a 121-point eyepiece graticule (Leica Microsystems, Wetzlar, Germany). For each animal, 32–64 microscopic fields were counted, and values for percent nuclear volume were converted to absolute nuclear volumes per testis by reference to testis volume (=weight). SC and LC nuclear size and seminiferous tubule diameter were determined using an Olympus Optical BH-2 microscope (Olympus Optical Co., Tokyo, Japan) fitted with a Prior automatic stage (Prior Scientific Instruments, Cambridge, UK) and Image-Pro Plus (version 4.5.1) with Stereologer-Pro 5 plug-in software (Media Cybernetics, Bethesda, MD, USA). Data were used to determine the relative contribution of seminiferous epithelium and lumen to the testis

TABLE 1. Nomenclature of mouse strains

Cre line	Target	Knockout name
SM [as used by Zhang <i>et al.</i> (18)]	Blood vessel smooth muscle	SMAR KO
MH	Peritubular myoid cells	MHAR KO/PTM-ARKO

volume, the average seminiferous tubule diameter, the nuclear volumes of and number of SCs and LCs per testis at d100, and the nuclear volume per testis of each type of GC.

Immunohistochemical analysis

In addition to PCR genotyping, mice were examined by immunohistochemistry for the presence of *Cre* and absence of AR in PTM cells, identified by immunoreexpression of smooth muscle actin (SMA). AR expression in SCs and LCs was confirmed by fluorescent costaining of AR with WT-1 or 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β HSD), respectively. Single colorimetric immunohistochemistry was also performed for desmin, to demonstrate the smooth muscle layer; laminin, to highlight the basement membrane; WT-1, to demonstrate the location of SC nuclei; β -tubulin isotype III (Tubb3) expression in SCs; and luteinizing hormone (LH) receptor expression in LCs.

Sections were deparaffinized, rehydrated, and antigen retrieved before blocking of nonspecific binding sites, as detailed previously (23). Sections were incubated overnight at 4°C with the primary antibody diluted accordingly (Table 2). Immunostaining was detected using the secondary antibody and detection system specified in Table 2. Diaminobenzidine immunostained slides were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Histolab, Gothenburg, Sweden), and images were captured using a Provis microscope (Olympus) equipped with a Kodak DCS330 camera (Eastman Kodak, Rochester, NY, USA). Fluorescent immunostained sections were mounted in Mowiol mounting medium (Calbiochem, San Diego, CA, USA), and fluorescent images were captured using a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn, UK). To ensure reproducibility of results, representative testes from ≥ 3 animals at each age were used, and sections from PTM-ARKO and WT littermates were processed in parallel on the same slide on ≥ 2 occasions. Appropriate negative controls were included to ensure that any staining observed was specific. The antibody for Cre recombinase results in GC staining, even in mice genotyped as *Cre*-negative; this staining was considered nonspecific. All other antibodies used showed only minor nonspecific staining.

Hormone analysis

Immediately after culling, blood was collected from mice by cardiac puncture. Sera were separated and stored at -20°C

until assayed. Follicle-stimulating hormone (FSH), LH, and testosterone were measured using previously published assays (24, 25). Intratesticular testosterone concentrations were measured as published previously (26). All samples from each mouse were run in a single assay for each hormone, and the within-assay coefficients of variation were all <10%.

RNA extraction and reverse transcription

RNA was isolated from frozen testes from MHAR KO or WT mice using the RNeasy Mini extraction kit with RNase-free DNase on the column digestion kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. For quantitative RT-PCR, 5 ng of luciferase mRNA (Promega Corp., Madison, WI) was added to each testis sample before RNA extraction as an external standard (27). RNA was quantified on a Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Scientific, San Jose, CA, USA). Random hexamer primed cDNA was prepared using the Applied Biosystems TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Determination of deletion of AR exon 2

RT-PCR was performed using BioMix Red *Taq* polymerase (Bioline, London, UK) on cDNA synthesized from testes from PTM-ARKO mice and WT littermates using primers for AR exons 1 and 3 (forward AAGCAGGTAGCTCTGGGACA; reverse CGTTTCTGCTGGCACATAGA); these primers revealed bands of 765 and 613 bp, relating to mice with a WT and or excised exon 2 allele of AR, respectively. A weak central band was also identified; this was confirmed to be an artifact by repeating the RT-PCR for AR exon 2 using gel-purified combined AR exon 2 PCR products from ARKO and WT mice as a template (*i.e.*, no genomic DNA was included in the reaction, yet the central band was present).

Quantitative analysis of gene expression

Quantitative PCR was performed for the genes listed in Table 3, using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the Roche Universal ProbeLibrary (Roche, Welwyn, UK), according to the manufacturer's instructions. Expression of each gene was related to luciferase, an external positive control, and all genes were expressed per testis.

TABLE 2. Immunohistochemistry antibody details

Antibody	Antibody source	Dilution	Detection system
3 β HSD/AR			
3 β HSD	Santa Cruz Technology Inc. (Santa Cruz, CA, USA)	1:4000	Tyramide 488
AR	Santa Cruz	1:50	Goat anti-rabbit Alexa 546
Cre/SMA			
Cre	Abcam (Cambridge, UK)	1:5000	Tyramide 488
SMA	Sigma-Aldrich (St. Louis, MO, USA)	1:500	Goat anti-mouse Alexa 633
Cre/SMA/AR			
Cre/SMA	Abcam	1:5000	Tyramide 488
Cre	Sigma	1:500	Goat anti-mouse Alexa 633
SMA	Santa Cruz	1:1000	Tyramide 546
Desmin	Dako (Cambridge, UK)	1:400	Goat anti-mouse biotinylated + DAB
Laminin	Abcam	1:100	Goat anti-rabbit biotinylated + DAB
LH receptor	Santa Cruz	1:200	Tyramide 488
Tubb3	Sigma	1:2000	Goat anti-mouse biotinylated + DAB
WT-1	Dako	1:1000	Goat anti-mouse biotinylated + DAB

DAB, diaminobenzidine.

TABLE 3. *TaqMan primer details*

Gene	Forward primer	Reverse primer
<i>ABP</i>	TTGTACCCGAAAAGTCACTCAGG	ATCTCTCCCTTGGGGCTTTA
<i>Eppin</i>	GCACGTGCCAAGTCTTCAT	CCTCTGTTCAGGTGAGACTGC
<i>Rhox5 (Pem)</i>	AAATGAGCCAGTTGCTGAGG	ATCTGCCTACCCCCAGGAT
<i>Tubb3</i>	GGCAACTATGTAGGGGACTCAG	CCTGGGCACATACTTGTGAG
<i>WT-1</i> (all isoforms)	CACCAAAGGAGACACACAGG	GGGAAAACCTTTCGCTGACAA

Statistical analysis

Data were analyzed using GraphPad Prism (version 5; GraphPad Software Inc., San Diego, CA, USA) using a

1-tailed unpaired *t* test or a 1-way ANOVA, followed by Bonferroni *post hoc* tests. Values are expressed as means \pm SE. Normality was confirmed using the Kolmogorov-Smirnov normality test.

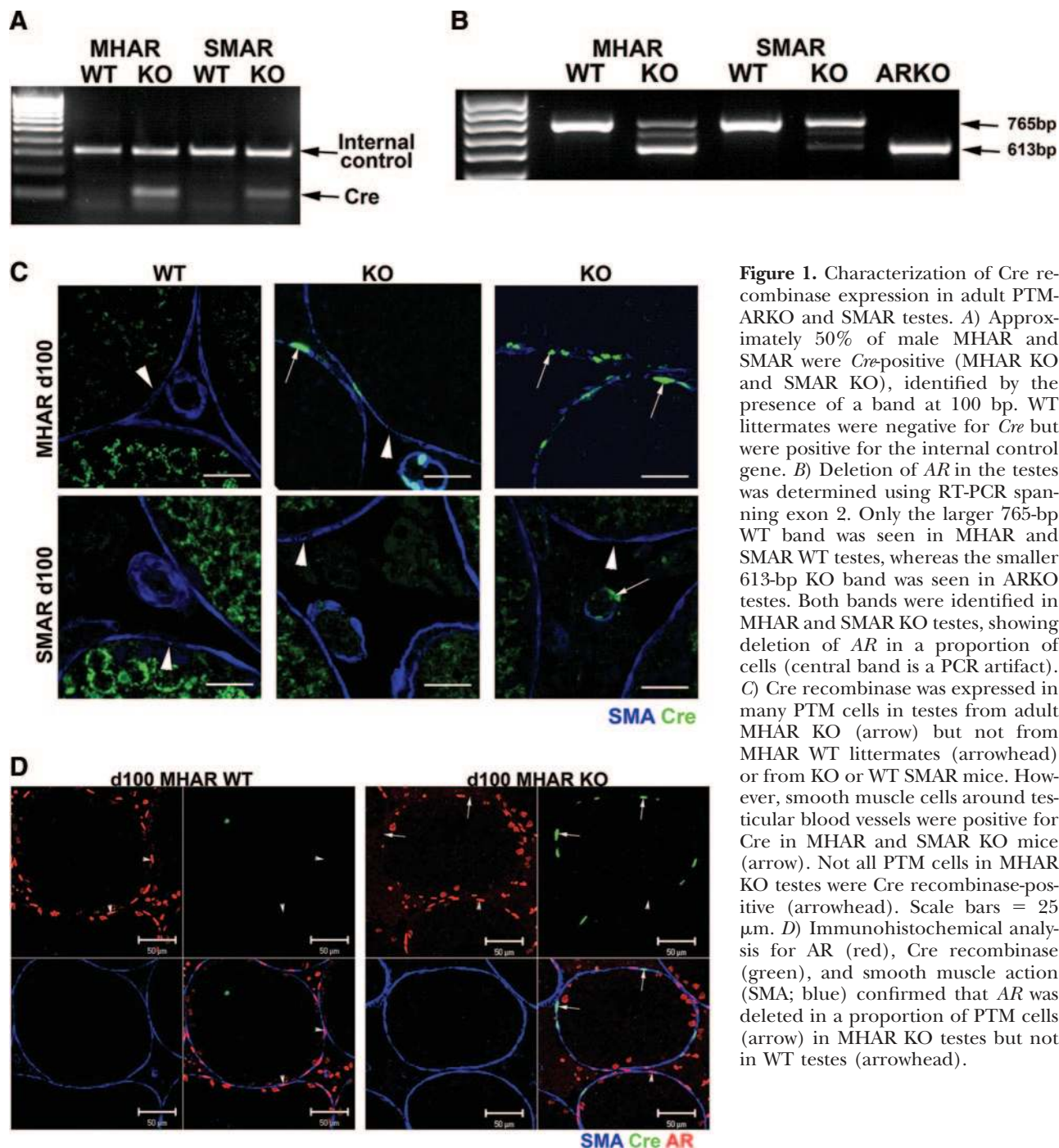


Figure 1. Characterization of Cre recombinase expression in adult PTM-ARKO and SMAR testes. *A*) Approximately 50% of male MHAR and SMAR were Cre-positive (MHAR KO and SMAR KO), identified by the presence of a band at 100 bp. WT littermates were negative for Cre but were positive for the internal control gene. *B*) Deletion of AR in the testes was determined using RT-PCR spanning exon 2. Only the larger 765-bp WT band was seen in MHAR and SMAR WT testes, whereas the smaller 613-bp KO band was seen in ARKO testes. Both bands were identified in MHAR and SMAR KO testes, showing deletion of AR in a proportion of cells (central band is a PCR artifact). *C*) Cre recombinase was expressed in many PTM cells in testes from adult MHAR KO (arrow) but not from MHAR WT littermates (arrowhead) or from KO or WT SMAR mice. However, smooth muscle cells around testicular blood vessels were positive for Cre in MHAR and SMAR KO mice (arrow). Not all PTM cells in MHAR KO testes were Cre recombinase-positive (arrowhead). Scale bars = 25 μ m. *D*) Immunohistochemical analysis for AR (red), Cre recombinase (green), and smooth muscle action (SMA; blue) confirmed that AR was deleted in a proportion of PTM cells (arrow) in MHAR KO testes but not in WT testes (arrowhead).

RESULTS

Generation and characterization of mice with deletion of AR in PTM cells

No reproductive phenotype was identified at d100 in *Cre*-positive, *AR^{fllox}*-negative male mice from both MH and SM lines, showing that expression of *Cre* alone had no effect (data not shown). All MHAR and SMAR males were hemizygous for X-linked *AR^{fllox}*, whereas ~50% of these males also carried the *Cre* transgene (Fig. 1A, KO); the *AR^{fllox}*-positive *Cre*-negative male littermates were used as WT controls.

To confirm that Cre recombinase was functioning in these mice, we assayed for the deletion of exon 2 of the *AR* in testes from MHAR and SMAR males by RT-PCR. MHAR and SMAR WT testes only expressed the full-length (WT) band whereas ARKO testes only expressed the smaller KO band, confirming that exon 2 of the *AR* had been deleted from all cells (Fig. 1B). Testes from MHAR and SMAR KOs expressed both the WT and the KO band, as expected, demonstrating that *AR* had been deleted only from a proportion of cells within the testis (Fig. 1B). Note that the weak central band was confirmed to be a PCR artifact (data not shown).

Immunohistochemical analysis was therefore undertaken to identify which cells in the testes expressed Cre recombinase. MHAR WTs showed no specific testicular Cre recombinase immunoreactivity at d100, whereas both the PTM cells and the smooth muscle cells around the blood vessels stained positively for Cre recombinase in MHAR KO mice (Fig. 1C). Variation was seen in the proportion of PTM cells staining positively for Cre recombinase both between and within different mice (Fig. 1C). The average proportion of Cre recombinase-positive PTM cells was 40% (ranging between 24 and 82%). SMAR WTs also showed no specific testicular Cre recombinase immunoreactivity at d100, whereas the smooth muscle cells around the blood vessels immunostained for Cre recombinase in SMAR KO mice (Fig. 1C). No Cre recombinase immunostaining was seen in PTM cells in any SMAR *Cre*-positive KO mice (Fig. 1C). Immunohistochemical analysis for AR, costained with SMA and Cre recombinase (Fig. 1D), showed that, as expected, AR and Cre recombinase were never coexpressed in the same cell (Fig. 1D). AR was detected in all PTM cell nuclei in MHAR WT (Fig. 1D), SMAR WT (data not shown), and SMAR KO (data not shown) testes at d100. Conversely, AR was absent from many, but not all, PTM cells in MHAR KO testes (Fig. 1D). AR was also absent from the blood vessel smooth muscle cells in both MHAR KO and SMAR KO testes (data not shown).

As we have conclusively shown that the PTM cells do not express Cre recombinase and continue to express AR in *Cre*-positive SMAR mice, further analysis was only undertaken in MHAR mice. From this point, MHAR KO mice will be referred to as PTM-ARKO.

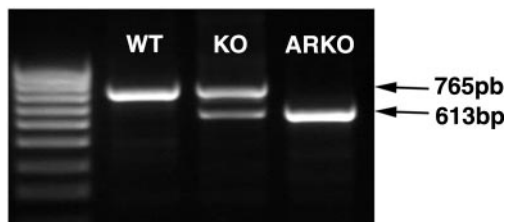


Figure 2. Deletion of *AR* in the testes was determined using RT-PCR spanning exon 2. Only the larger 765-bp WT band was seen in WT testes, whereas only the smaller 613-bp KO band was seen in ARKO testes. Both bands were identified in PTM-ARKO testes at e17.5, showing deletion of *AR* in a proportion of testicular cells in fetal life.

Onset of Cre recombinase expression in PTM-ARKO testes

PTM-ARKO testes at e17.5–d100 were assayed for the deletion of exon 2 of the *AR* by RT-PCR of cDNA, as used in Fig. 1B. Testes from MHAR WT littermate mice at all ages expressed only the full-length (WT) band, whereas testes from PTM-ARKO mice at e17.5 expressed both the WT and the smaller KO band (Fig. 2). Therefore, *AR* has been deleted from PTM cells around the age at which *AR* is normally first expressed.

Characterization of the reproductive system in PTM-ARKO mice

Body weight was not significantly different between PTM-ARKO and WT males at any age (data not shown). Male PTM-ARKO mice showed normal external sexual development, anogenital distance, and penis length (data not shown). In contrast to ARKO mice (8), the reproductive tract formed normally in PTM-ARKO males (Fig. 3A); however, seminal vesicle and ventral prostate weights were reduced at d100, compared with those in the WT littermates (data not shown). In PTM-ARKOs, testes were normally descended in adults but were obviously smaller than those in the WT littermates (Fig. 3A and inset). Testis weight was significantly reduced in PTM-ARKO mice from d15 onward, but not at d12 (Fig. 3A). Note that expression of *Cre* alone, without *AR^{fllox}* expression, had no effect on testis weight at d100 (Fig. 3A).

PTM-ARKO mice are infertile

WT males sired litters in 72% of matings (26 of 36), whereas no PTM-ARKO males sired any litters (0 of 30). Consistent with this result, only a few sperm were observed in the epididymides from PTM-ARKOs at d100, although cell debris was apparent in the epididymal lumens (Fig. 4).

Postnatal testis morphology and cell numbers

No dramatic testicular phenotype was observed in PTM-ARKO males at d12 (Fig. 5A), but at d21, the

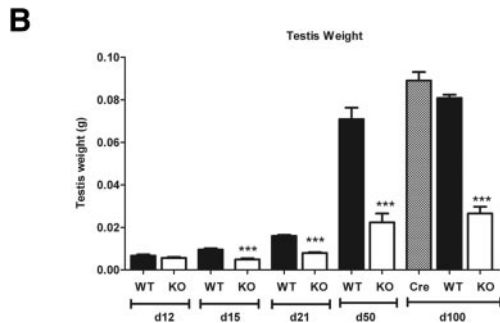
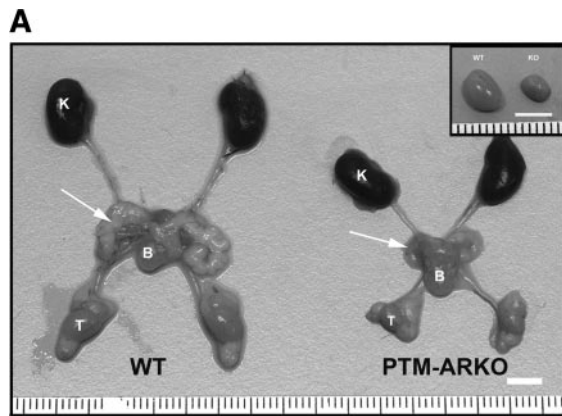


Figure 3. Gross morphology of the reproductive system from PTM-ARKO mice. *A*) Urogenital tract from males at d100 in which the testes (T; inset) and seminal vesicles (arrow) are obviously smaller in the PTM-ARKO than in the WT mice. *B*) Quantification of testis weight in PTM-ARKO mice from d12 to 100. Testis weight is not reduced in *Cre*-positive, *AR*^{lox}-negative mice (*Cre*). Values are means \pm SE; $n = 6$ –14 mice. K, kidney; B, bladder. *** $P < 0.001$ vs. WT. Scale bars = 50 mm.

seminiferous epithelium appeared disorganized compared with that of WT males, with cells often observed abnormally in the middle of the tubules (Fig. 5A). This disorganization remained apparent at d50 and 100 (Fig. 5A). At all ages, a layer of peritubular cells was observed surrounding the seminiferous tubules in the PTM-ARKO and WT testes (Fig. 5A). Stereological quantification demonstrated a reduction in seminiferous tubule lumen volume in PTM-ARKO testes at all ages examined (Fig. 5B) and a reduction in seminiferous tubule diameter at d50 and 100 (data not shown), as was obvious on gross inspection (Fig. 5A).

The total volume of GCs was normal at d12 in PTM-ARKOs but was significantly reduced at d21–100, compared with that in WT littermates (Fig. 6); unlike in

SCARKO mice, this was due to a reduction in the number of all types of GCs (Table 4). A progressive loss of spermatogonia was observed in PTM-ARKO mice with age, which reached significance at d100 (Table 4). A significant reduction in spermatocyte number was already apparent at d21 and persisted throughout adulthood; this reduction was more dramatic than that seen in adult SCARKO mice (Table 4). The number of round spermatids was also significantly reduced in PTM-ARKO testes at d21–100 (data not shown). This was the most advanced stage of GC development in WT testes at d21; however, the number of elongated spermatids (and thus the total number of spermatids) was also reduced in adult PTM-ARKO testes (Table 4). In addition, there was a reduction in the number of GCs supported by each SC in the PTM-ARKO testis at d21–100, as seen in SCARKO mice (Table 4).

PTM cell protein expression in PTM-ARKO testes

Smooth muscle protein expression was examined to investigate the direct effect of PTM-ARKO on the PTM cells. A reduction in desmin expression was noted in PTM cells, whereas no change in desmin immunorexpression was observed in the smooth muscle cells surrounding the blood vessels in PTM-ARKOs (Fig. 7A). This reduction was progressive with very little desmin immunorexpression in PTM cells in PTM-ARKO testes at d100 (Fig. 7A). SMA immunorexpression was slightly reduced (data not shown). PTM cells contribute to the seminiferous tubule basement membrane by producing laminin (15), immunorexpression of which was disrupted in PTM-ARKO testes, with a less well defined “ring” of laminin evident in the basement membrane compared with that in WT testes (Fig. 7B). This disruption was progressive and became more apparent at d50–100 (Fig. 7B).

Effect of PTM-ARKO on cell-cell communication in the testes: SC function

SC number (WT: $3.594 \pm 0.3739 \times 10^6$, KO: $2.668 \pm 0.3564 \times 10^6$; $P = 0.1$) and nuclear size (WT: $337.5 \pm 54.30 \mu\text{m}^3$, KO: $351.1 \pm 26.10 \mu\text{m}^3$; $P = 0.8$) were not significantly altered in the PTM-ARKO mice at d100. Expression of immature markers such as cytokeratin and anti-Müllerian hormone was lost in SCs from PTM-ARKO mice (data not shown), whereas maturation markers such as GATA1 and p27kip were activated and maintained through to adulthood as

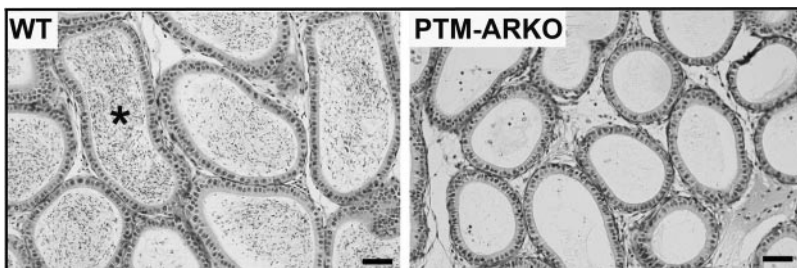


Figure 4. Elongate sperm were rarely seen in the caput epididymis from PTM-ARKO adult mice, unlike in WT adults (asterisk). Cell debris was observed in the lumen of PTM-ARKO epididymides. Scale bars = 50 μm .

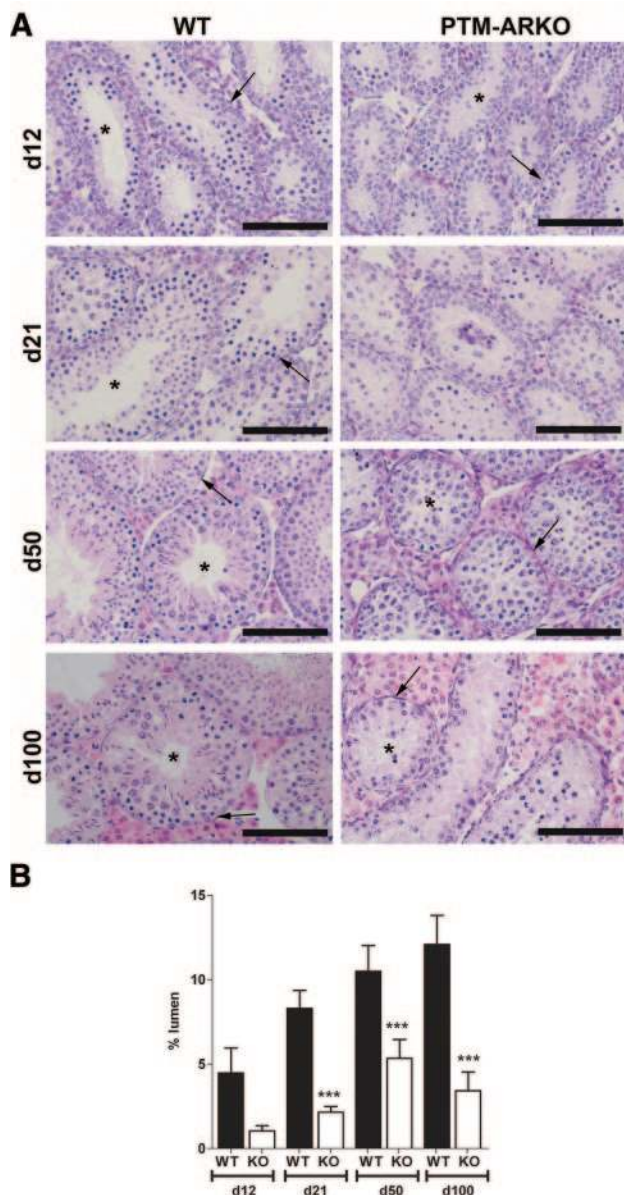


Figure 5. Histological comparison of PTM-ARKO and WT testes at d12–100. *A*) PTM-ARKO testes look comparable to WT testes at d12, whereas at d21 seminiferous tubules appear smaller in PTM-ARKO testes, with fewer GCs. Unlike in WT testes, spermatogenesis was disrupted and incomplete in PTM-ARKO testes at d50 and 100. Lumens (asterisks) were smaller/absent in the seminiferous tubules in PTM-ARKO testes at all ages, unlike in WT testes. PTM cells (arrow) were seen surrounding seminiferous tubules at all ages in both WT and PTM-ARKO testes. *B*) Percentage of the testis occupied by seminiferous tubule lumen was significantly reduced from d21 in PTM-ARKO testes compared with WT testes. Values are means \pm SE; $n = 4$ –6 mice. *** $P < 0.001$ vs. WT littermates. Scale bars = 50 μ m.

normal (data not shown). As observed in WT littermates, AR was expressed in PTM-ARKO SCs at d12–100, (d12–50, data not shown; d100, Fig. 1*D*). Expression of SC-specific genes previously shown to be androgen dependent (*Rhox5*, *Eppin*, and *Tubb3*) (28) and androgen independent [androgen-binding protein (ABP) (29) and Wilms' tumor-1 (*WT-1*) (30)] were examined by quanti-

tative RT-PCR. This analysis demonstrated that relative expression of *Rhox5*, *Eppin*, *Tubb3*, and *WT-1* were all significantly decreased in PTM-ARKO testes at d100, whereas ABP was not significantly altered compared with that in WT littermates (Fig. 8*A*). However, both *WT-1* and *Tubb3* protein could be detected in SCs from PTM-ARKO WT and KO mice at d100 by immunohistochemistry (Fig. 8*B*, *C*). Serum FSH concentrations were significantly increased (WT: 18.11 ± 1.386 ng/ml, KO: 25.91 ± 0.6913 ng/ml; $P = 0.0005$) at d100 in PTM-ARKO males compared with WT males. SC orientation in PTM-ARKOs became progressively disorganized with SC nuclei no longer located at the base of the seminiferous tubules at d100, as in WT testes, but instead often located in the middle of tubules (Fig. 8*C*).

Effect of PTM-ARKO on LC function and hormone profiles

PTM-ARKO males displayed normal AR expression in LCs, identified by costaining for AR and 3β HSD (Fig. 9*A*). An apparent increase in the size and/or number of LCs was noted in the PTM-ARKO testis at d100, compared with that in WT littermates (Fig. 9*A*). However, quantification revealed no change in LC size ($P = 0.34$) or number ($P = 0.35$) in PTM-ARKO mice at d100, compared with WT mice and the apparent increase in LCs observed histologically was due to the reduced seminiferous tubule volume. Serum testosterone concentrations were not significantly altered in PTM-ARKO mice at any age examined compared with WT mice (Fig. 9*B*). In contrast, serum LH and intratesticular testosterone concentrations (expressed per 100 mg of testis and per testis, respectively) were increased in adult PTM-ARKO males (Fig. 9*B*). LH receptor expression immunoreaction was readily detectable in PTM-ARKO testes at d100, as observed in WT mice (Fig. 9*C*).

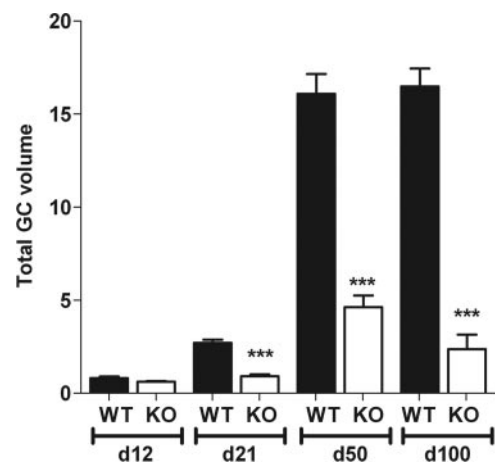


Figure 6. GC volume in PTM-ARKO and WT testes. Total GC nuclear volume per testis was significantly reduced in PTM-ARKO testes from d21 onward, compared with that in WT testes. Values are means \pm SE; $n = 4$ –6 mice. *** $P < 0.001$ vs. WT.

TABLE 4. GC composition of the testes of WT and PTM-ARKO mice at d21, 50 and 100–140

Age	Strain	Genotype	Average volume (mm ³ /testis)			GC volume (mm ³ /SCL)z
			Spermatogonia	Spermatocyte	Spermatid	
d21	PTMARKO	WT	0.5 ± 0.1	1.8 ± 0.2	NA	2.7 ± 0.5
	PTMARKO	KO	0.4 ± 0.4 (80%)	0.5 ± 0.1 (28%)*	NA	1.0 ± 0.2 (37%)*
d50	PTMARKO	WT	0.8 ± 0.1	6.4 ± 0.5	8.9 ± 1.1	15.9 ± 1.1
	PTMARKO	KO	0.6 ± 0.1 (75%)	3.1 ± 0.6 (48%)*	0.9 ± 0.3 (10%)*	6.2 ± 1.8 (39%)*
d100–140	PTMARKO (d100)	WT	0.8 ± 0.1	6.4 ± 0.5	9.3 ± 0.7	12 ± 0.9
	PTMARKO (d100)	KO	0.3 ± 0.04 (38%)	1.5 ± 0.5 (23%)*	0.6 ± 0.3 (6%)*	3.1 ± 0.8 (26%)*
	SCARKO (d140)	WT	1.2 ± 0.3	11.8 ± 1.4	16.4 ± 1.9	18.1 ± 2.1
	SCARKO (d140)	KO	0.7 ± 0.1 (58%)	5.8 ± 0.7 (49%)*	0.3 ± 0.1 (2%)*	4.9 ± 0.7 (27%)*

Data are expressed as average nuclear volume, indicative of total GC volume per testis. Values are means ± SE for 4–8 mice. Values in PTM-ARKOs as a percentage of the WT are indicated in parentheses. NA, not available. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. WT.

DISCUSSION

We have investigated the effect of two different smooth muscle-driven Cre lines (SM22-Cre and smMHC-Cre) on cell-specific ablation of testicular AR from mouse testes. We show that cross-breeding AR^{fllox} female mice with the SM22-Cre line does not alter AR expression in testicular PTM cells, whereas the smMHC-Cre line does delete AR from a proportion of PTM cells; both lines deleted AR from smooth muscle cells surrounding testicular blood vessels. This differential effectiveness of these Cre lines is consistent with the demonstration that Cre recombinase is poorly expressed in testes from SM22-Cre mice (19) yet highly expressed in testes from smMHC-Cre mice (19) and explains the very different testicular phenotypes we observed when these mouse lines were used to ablate AR. Namely, using the SM22-Cre line (*i.e.*, deleting AR from blood vessels) did not affect fertility, as reported previously using the same Cre line (18), whereas using the smMHC-Cre line (resulting in PTM-ARKO mice) abolished sperm production and fertility, despite the fact that not all PTM cells are AR-negative. Note that any potential confounding effects resulting from additional ablation of AR in the blood vessels in PTM-ARKO mice can be accounted for by the phenotype induced by the blood vessel-specific SM22-Cre line (18). Our results are therefore the first *in vivo* demonstration that androgen action on PTM cells is essential for normal spermatogenesis and fertility.

In contrast with ARKO mice (8), reproductive tract formation and testes descent were normal in PTM-ARKO males. However, the testes were almost 70% smaller in PTM-ARKO adult males than in their WT littermates, with almost no sperm production. Interestingly, at d12 there was no reduction in testis weight in PTM-ARKO males, and the testes were histologically comparable to WT testes with no reduction in GCs. Cre recombinase was expressed in the PTM cells from e17.5; this finding may imply that PTM-mediated AR signaling is not essential before puberty. However, in

our PTM-ARKO mice, AR is not deleted from all PTM cells. Therefore, the lack of an obvious phenotype at d12 may reflect patchy AR deletion and the fact that the remaining AR-positive PTM cells are sufficient to enable normal early testis development; this theory requires further investigation. Despite the patchy AR deletion from PTM cells, all PTM-ARKO males were infertile, and spermatogenesis was severely impaired. This finding demonstrates a crucial role for PTM-dependent AR signaling in male fertility.

PTM-ARKO mice were azoospermic with a significant reduction in all types of GCs. This reduction in GCs in PTM-ARKO mice became progressively worse from d21 onward, with almost no elongate spermatids in adults. Indeed, the reduction in both spermatocyte and spermatid number is greater than that reported in SCARKO mice (8). Because of the structure of the seminiferous epithelium and the presence of inter-SC tight junctions, any effect on postmeiotic GCs must be mediated *via* the SCs; this result therefore suggests that the reduction in postmeiotic GCs in PTM-ARKO mice is likely to be mediated *via* interactions with and effects on SCs. As in ARKO (8), spermatogonial numbers were reduced in PTM-ARKO mice, unlike in SCARKO mice in which spermatogonial numbers are normal and there is a specific block in GC maturation at meiosis (8). Our data suggest that spermatogonial number may be regulated by androgens acting *via* PTM cells; this is perhaps not surprising because PTM cells are in intimate contact with these basally located GCs. In their studies on SM22-AR^{-/-} mice, Zhang *et al.* (18) reported a 25% reduction in GC number, but no change in GC ratio, therefore confirming that the 85% reduction in GCs and spermatogonial loss in our PTM-ARKOs is due to ablation of AR from PTM cells not from the smooth muscle surrounding the blood vessels. This reduction in spermatogonia in PTM-ARKO mice may be due to alterations to the basement membrane and so reduced attachment. The reduction in GCs in PTM-ARKO mice is similar to that reported in rats with reduced testicular

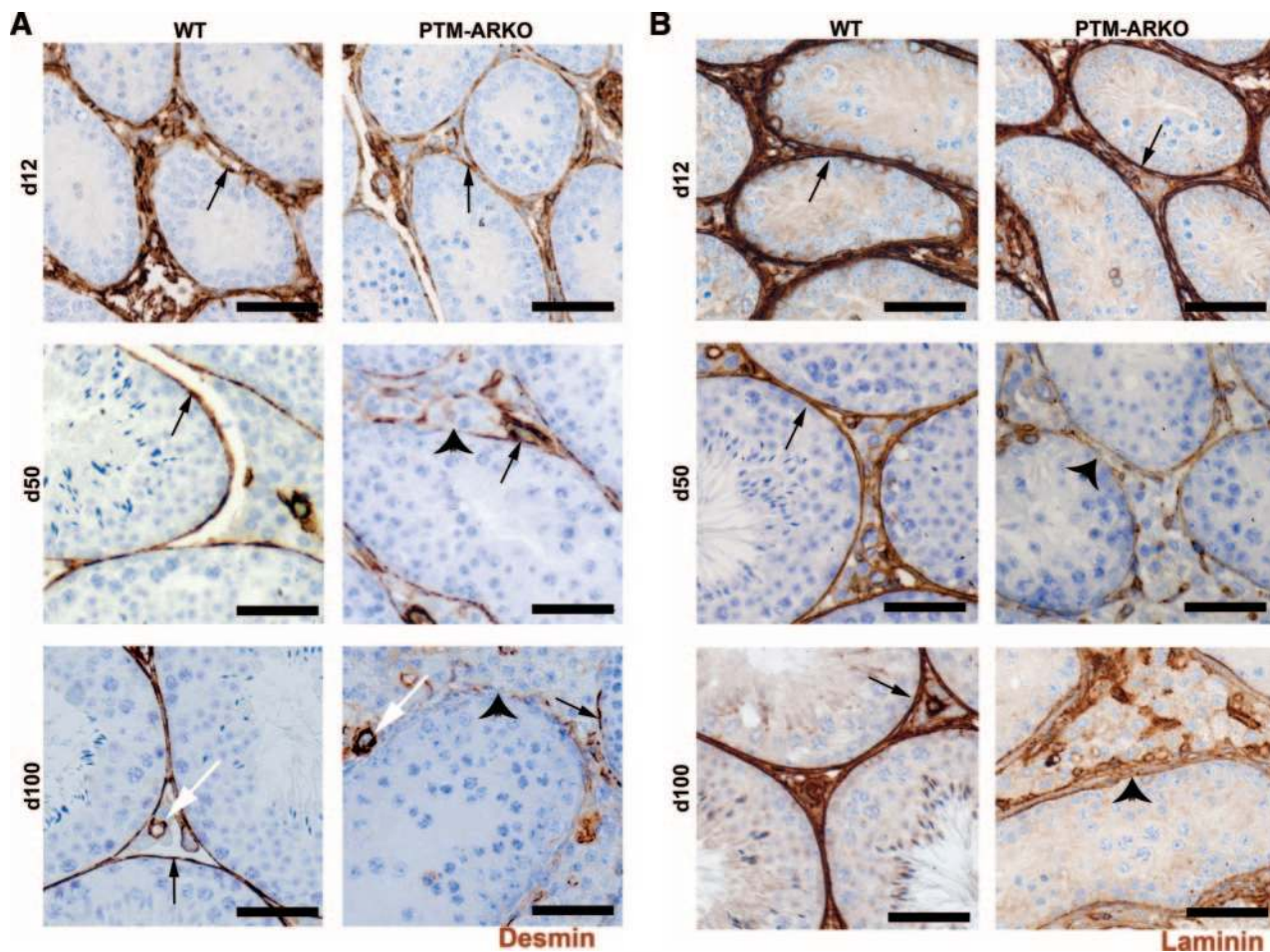


Figure 7. Evaluation of PTM cell protein expression in WT and PTM-ARKO males. *A*) Desmin (immunostained brown) is expressed in PTM cells (black arrow) in WT testes at d12–100, but expression was reduced in the PTM-ARKO testes at all ages (arrowhead). Desmin expression around the blood vessels was not altered (white arrow). *B*) Demarcation of the basement membrane by immunostaining for laminin (brown). Note that laminin forms a defined ring at the basement membrane of seminiferous tubules in WT testes (arrow) but is less defined around PTM-ARKO tubules (arrowhead). WT and KO testes were stained together on the same slides. Scale bars = 50 μ m.

androgen concentrations (1, 31). This finding suggests that the phenotype reported in these androgen-deficient rats may be due in part to PTM cell-mediated effects and not just to altered androgen-dependent SC signaling, which has previously been the primary focus. Although our results in PTM-ARKO mice demonstrate an essential role for AR signaling *via* PTM cells in GC maturation, it is also important to consider what effect this has on other cell types, namely the SCs (*via* stromal-epithelial interactions) and LCs.

It has long been recognized that stromal-epithelial interactions play a vital role in mediating androgen action throughout the male reproductive tract, but there is little *in vivo* evidence for this role in the testis. In mice, SC proliferation occurs during late fetal and early neonatal life (32) and is believed to be mediated in part by androgens, possibly acting *via* PTM cells because the SC number is dramatically reduced in ARKO mice but only slightly reduced in SCARKO mice (27). However, in our studies, no reduction in SC number was found in PTM-ARKO mice at d100, unlike in ARKOs (33). This finding could suggest that SC

number is not determined by AR signaling *via* PTM cells. However, as AR was not ablated from all PTM cells in our PTM-ARKOs, residual PTM AR signaling may have been sufficient to allow normal SC proliferation; this theory merits further investigation. Based on several key criteria, SCs mature normally in PTM-ARKO testes and express AR, suggesting that these processes are not dependent on AR signaling *via* PTM cells and that any changes in SC function cannot be attributed to any gross alteration in maturation or AR expression in SCs themselves. However, in PTM-ARKO testes, each SC supported fewer GCs than in WT littermates and seminiferous tubule diameter and lumen volume were both reduced, similar to the phenotype reported in ARKO and SCARKO testes (8). The presence of a lumen reflects production of seminiferous tubule fluid by SCs, which has previously been shown to be dependent on FSH (34, 35) during puberty and on androgens later in life (6, 8). Thus, our results demonstrate that SC function is altered in PTM-ARKO testes, even though the SCs continue to express AR and FSH levels are elevated. This important new finding highlights an

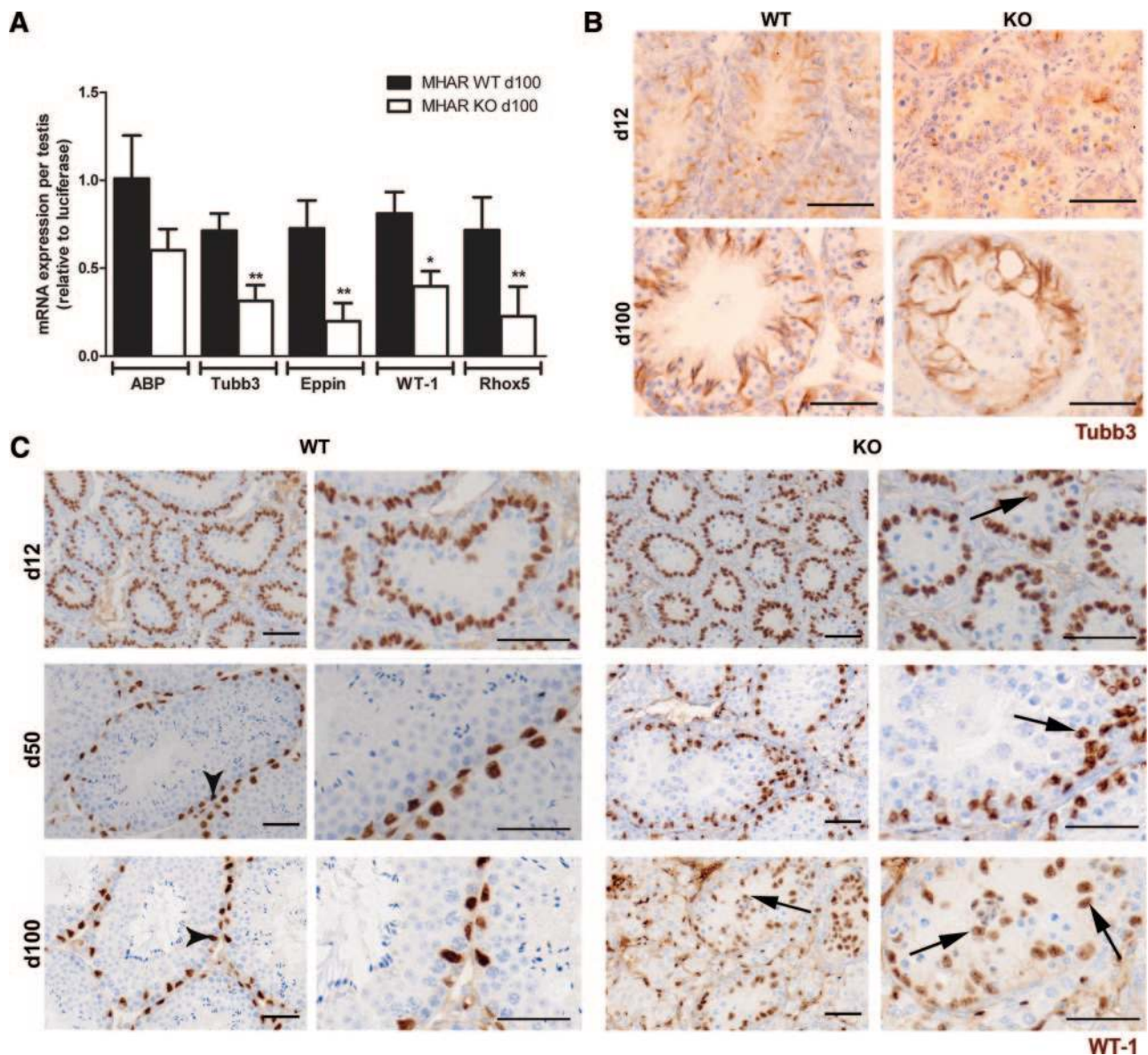


Figure 8. Evaluation of SC function in WT and PTM-ARKO males. *A*) Relative expression of SC genes was examined by quantitative RT-PCR in WT and PTM-ARKO testes at d100. *B*) Expression of Tubb3 protein (brown) in SCs in both PTM-ARKO and WT littermates at d12 and 100. The apparent altered pattern of expression of Tubb3 in PTM-ARKO mice at d100 is probably due to reduction in the GC complement. *C*) WT-1 staining (brown) was used to identify SC nuclei and highlighted their disorganization in PTM-ARKO males (arrow), compared with WT males (arrowhead). Values are means \pm SE; $n = 5$ mice. Scale bars = 50 μ m.

indirect role (*via* PTM cells) for AR signaling in mediating SC function and may offer some explanation for the reduced GC development and infertility seen in PTM-ARKO mice. For example, reduced seminiferous tubule fluid production will affect nutrient transport to the GCs (6) and would impair spermatogenesis. This new *in vivo* evidence for androgen-dependent stromal (PTM)-epithelial (SC) interactions in the testis parallels the androgen-driven mechanisms reported to mediate development and function of the rest of the male reproductive tract (9, 10).

Despite many years of study, exactly how androgens regulate spermatogenesis is not known. Studies in SCARKO mice have identified changes in SC gene expression, which could affect spermatogenesis (27,

28). Reductions in expression of three of these androgen-dependent SC-specific genes, *Rhox5*, *Tubb3*, and *Eppin*, were identified in PTM-ARKO testes at d100, providing further evidence for altered SC function. Conversely, expression of *ABP*, another SC-specific gene, was not significantly altered in PTM-ARKO testes, unlike in SCARKO testes in which *ABP* was increased (27). These results suggest that genes previously reported to be dependent on androgen action in SCs (27, 28), may also be affected by androgens acting *via* PTM cells and that a complex dynamic exists between PTM and SCs that may affect their ability to respond to hormones. This observation highlights possible mechanisms *via* which spermatogenesis could be affected in PTM-ARKO mice. These changes in gene expression

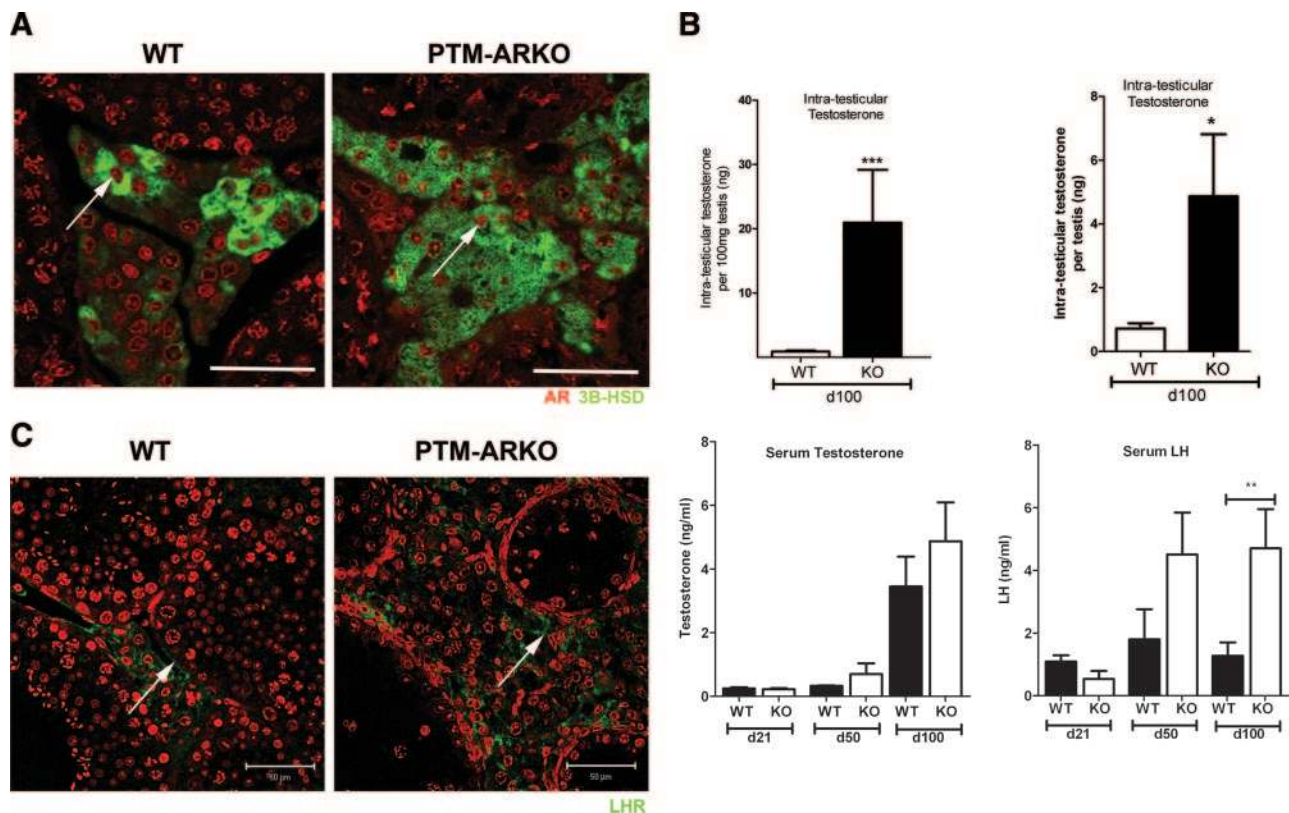


Figure 9. Evaluation of LC function in WT and PTM-ARKO males. *A*) LCs (arrow, immunostained green for 3β HSD) immunostained positively for AR (red) in PTM-ARKO and WT males. *B*) Serum testosterone concentrations were not significantly different in PTM-ARKO males, compared with WT males, whereas serum LH and intratesticular testosterone concentrations were both significantly increased in PTM-ARKO males at d100. *C*) LCs (arrow) in the PTM-ARKO express the LH receptor (LHR; immunostained green, counterstained red) at d100, similar to WT control males. Values are means \pm SE; $n = 8$ –18 mice. $**P < 0.01$ vs. WT littermates. Scale bars = 50 μ m.

provide further *in vivo* evidence that altered AR signaling *via* the stromal PTM cells has “knock-on” effects on the function of the epithelial SCs and thereafter on GC development. *WT-1* gene expression was also reduced in PTM-ARKO testes, yet *WT-1* protein could still be detected by immunohistochemistry. This result merits further investigation into the implications on SC function; however, it has been shown that *WT-1* heterozygous KO mice are fertile (36), suggesting that this reduction in *WT-1* mRNA in PTM-ARKO mice may not affect fertility.

PTM cells in mice are normally arranged in a single layer surrounding the seminiferous tubules, but in ARKO mice, PTM cells form multiple layers (8). PTM cell layering was normal in both PTM-ARKO and SCARKO testes (8), which could suggest that neither PTM nor SC AR signaling regulates PTM cell layering. However, because AR has not been ablated from all PTM cells in our PTM-ARKO mice, the residual PTM AR signaling could be sufficient to maintain PTM cells in a normal single layer. It is believed that this single layer of PTM cells contract to help with transport of mature sperm through the seminiferous tubules (37). In PTM-ARKO testes, a progressive decrease was noted in the immunorexpression of desmin and SMA in PTM cells but not in the smooth muscle cells surrounding the blood vessels. This reduction in smooth muscle

protein expression in PTM cells may reflect an androgen-dependent loss of a smooth muscle phenotype, resulting in an impaired ability to contract. This possible androgen dependency of PTM cell contractility has been reported previously *in vitro* (38) and merits further study.

PTM cells are also involved in laying down the basement membrane, which not only gives the seminiferous tubule structural support but is also thought to be important for communication with and effects on SCs. Disruption to the basement membrane surrounding the seminiferous tubules in PTM-ARKOs is likely to impair cell communication particularly with the neighboring SCs and spermatogonia. This finding may have implications for maintaining spermatogonia within their niche attached to the basement membrane, which permits their movement laterally along the basement membrane (reviewed in ref. 39) and merits further investigation in PTM-ARKO testes. The seminiferous epithelium appeared disorganized in PTM-ARKO testes, with SC nuclei often displaced from their normal basal location, suggesting that these SCs may also be losing their attachment to the damaged basement membrane, similar to that reported in SCARKO testes (40). This disorganization and apparent lack of polarity observed in PTM-ARKO testes is likely to have implications for SC function and is probably due to altered

signaling from the PTM cells as well as the reduction in spermatogonial number, as it has been shown that the presence of GCs plays a role in maintaining SC size and organization within seminiferous tubules (41, 42). These data highlight an important role for androgen signaling *via* PTM cells in maintaining normal seminiferous tubule structure and function and may offer a possible mechanism for impaired GC development and infertility in PTM-ARKOs.

LC AR expression was normal in PTM-ARKO testes, implying that any effect on LC function must be due to deletion of AR from PTM cells and/or the smooth muscle cells around the blood vessels. LC number per testis was also unchanged in PTM-ARKO testes at d100, compared with WT testes. This result is in contrast to the decrease in LC number seen in ARKO, SCARKO, and FSHRKO testes in adulthood (43, 44) and is in agreement with the belief that SCs and not the PTM cells regulate LC number *via* both FSHR and AR signaling (43, 44). It is less clear what regulates adult LC size, with a reduction reported in ARKO testes (43), an increase in SCARKO testes (43), and no change in PTM-ARKO testes. The primary function of the LC is to produce testosterone and in PTM-ARKO adult males serum testosterone concentrations were unaltered whereas intratesticular testosterone (ITT) and serum LH were both elevated. This elevation was not due to any obvious deficiencies in LHR expression in LCs but is indicative of compensatory LC failure, which may be the result of impaired transport of the testicular testosterone out into the bloodstream and so reduced negative feedback to the pituitary. The hormone profile reported here in PTM-ARKO mice is similar to that reported in ARKO mice, whereas no increase in serum LH or testosterone levels was observed in SCARKO adult mice (43). Furthermore, no change in serum testosterone or LH was reported by Zhang *et al.* (18) with SM22-Cre to ablate AR expression. Taken together, these results suggest that the changes in hormone concentrations reported here in PTM-ARKO mice are specifically due to ablating AR from the PTM cells and not to deletion of AR from the blood vessel smooth muscle cells. High ITT is associated with spermatogonial arrest (45); therefore, the high ITT in PTM-ARKO mice might also offer some explanation for the reduced GC numbers.

CONCLUSIONS

We have successfully generated a PTM-ARKO mouse model that demonstrates the essential role *in vivo* for AR signaling *via* the PTM cells in normal spermatogenesis and male fertility. This is mediated in part *via* altered SC function, thus providing new *in vivo* evidence for androgen-driven stromal-epithelial interactions in the testis and their vital role in normal male fertility. This mouse model provides a unique tool to identify the underlying molecular mechanisms of androgen action *via* the PTM cells in spermatogenesis,

which could provide insight for the development of new male contraceptives and treatments for male infertility. EJ

We are grateful to Karel De Gendt and Guido Verhoeven (Catholic University of Leuven, Leuven, Belgium) for providing the AR^{fllox} mice, The Jackson Laboratory (Bar Harbor, ME, USA) for SM22-Cre mice, and Michael Kotlikoff (Cornell University, Ithaca, NY, USA) for smMHC-Cre mice. We thank the UK Medical Research Council for funding (WBS U.1276.00.002.0003.01). We also thank Ian Swanston, Mark Fiskien, Lindsey Moffat, Lindsay Rutherford, Nancy Nelson, and the members of the histology department for technical assistance.

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Received for publication June 5, 2009.
Accepted for publication July 30, 2009.