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New Insights into the Role of Androgens in Wolffian Duct Stabilization in Male and Female Rodents

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Androgen-mediated wolffian duct (WD) development is programmed between embryonic d 15.5 (e15.5) and 17.5 in male rats, and WD differentiation has been shown to be more susceptible to reduced androgen action than is its initial stabilization. We investigated regulation of these events by comparing fetal WD development at e15.5–postnatal d0 in male and female androgen receptor knockout mice, and in rats treated from e14.5 with flutamide (100 mg/kg/d) plus di-n(butyl) phthalate (500 mg/kg/d) to block both androgen action and production, testosterone propionate (20 mg/kg/d) to masculinize females, or vehicle control. In normal females, WD regression occurred by e15.5 in mice and e18.5 in rats, associated with a lack of epithelial cell proliferation and increased apoptosis, disintegration of the basement membrane, and reduced epithelial cell height. Exposure to testosterone masculinized female rats including stabilization and partial differentiation of WDs. Genetic or chemical ablation of androgen action in males prevented masculinization and induced WD regression via similar processes to those in normal females, except this occurred 2–3 d later than in females. These findings provide the first evidence that androgens may not be the only factor involved in determining WD fate. Other factors may promote survival of the WD in males or actively promote WD regression in females, suggesting sexually dimorphic differences in the preprogrammed setup of the WD. **(*Endocrinology* 150: 2472–2480, 2009)**

Before sex determination and differentiation of gonads into either testes or ovaries, male and female fetuses have an identical urogenital system (1, 2), with reproductive target tissues reported to express the androgen receptor (AR) in both sexes (3). After functional differentiation of the fetal testis, the Leydig cells secrete testosterone (2, 4, 5). Testicular androgens bind to and activate the AR, which in turn drives masculinization, critical features of which include stabilization and differentiation of the wolffian duct (WD), prostate formation and expansion of the anogenital distance (AGD) (2, 6–8). Once the WD has been stabilized in males, it differentiates to form its adult derivatives, the epididymis, vas deferens, and seminal vesicles (9–11). Conversely, in females the ovary does not produce testosterone at this time, and so the WD degenerates (1, 2, 6). This is thought to be due to the lack of available ligand in the female rather than an inability to respond to androgens (12).

Previous studies have investigated androgen-dependent differentiation of the reproductive tract by examining the impact of blocking fetal androgen action either genetically (13) or chemically, using AR antagonists such as flutamide (11, 14) or compounds such as Di(*n*-butyl) phthalate (DBP), which reduces fetal testicular testosterone production (15–18). Recently, we discovered that the critical window for androgen action in ensuring both initial WD stabilization and later differentiation is between embryonic d 15.5 (e15.5) and e17.5 in rats (14). This window is just after the onset of fetal testicular testosterone production, and surprisingly several days either before the peak in testicular testosterone levels per testis (4, 19) or morphological differentiation of the WD (11, 14). These studies demonstrated that WD differentiation is more susceptible to reduced androgen action than is its initial stabilization (11, 14). However, in these studies it is possible that the treatment regime may not block AR-mediated signaling events completely because the flutamide was administered to the pregnant mother rather than directly to the fetus (11, 14), and endogenous testosterone levels are high in male fetuses during the last week of gestation, especially in the testis and WD (20). Therefore, it is possible that WD stabilization may proceed in the presence of lower levels of androgen action than that re-

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Abbreviations: AGD, Anogenital distance; AR, androgen receptor; ARKO, androgen receptor knockout; CAIS, complete androgen insensitivity syndrome; DBP, Di(*n*-butyl) phthalate; e, embryonic day; DBP plus F, flutamide (100 mg/kg^{-1}) plus Di(*n*-butyl) phthalate (500 mg/kg^{-1}); MD, müllerian duct; pnd0, postnatal d 0; TP, testosterone propionate; WD, wolffian duct; WT, wild type.

quired for its differentiation or that WD stabilization and differentiation may be regulated by different mechanisms and/or additional factors.

The present study sought to gain additional insight into WD development by comparing normal WD stabilization in males with WD regression in males in which androgen action was chemically or genetically ablated and with females in which androgens are naturally absent (2, 21). Furthermore, because fetal urogenital tissues in the female express AR (3), we administered exogenous testosterone to pregnant dams to "masculinize" their female fetuses and examined the impact on WD stabilization/development. We demonstrate that exogenous testosterone can rescue and partially differentiate the WD in females, but, surprisingly, ablation of androgen action in males leads to WD regression 2–3 d later than in normal females.

Materials and Methods

In vivo rat studies

Wistar rats were bred and maintained in our own animal house under standard conditions according to United Kingdom Home Office guidelines. Animals had access *ad libitum* to water and a soy-free breeding diet (SDS, Dundee, UK). Time matings were established, and the presence of a vaginal plug was taken as evidence of mating; this was defined as e0.5. A total of 38 pregnant dams were used for this study with dams randomly allocated to treatment groups. The natural regression studies were undertaken in rats that had not been dosed with any treatment (n = 9 litters). Other "treated" dams were dosed daily between 0830 and 1000 h according to maternal body weight with: 1) testosterone propionate (TP) at 20 mg/kg^{-1} by sc injection in 0.4 ml/kg^{-1} corn oil between e14.5 and 21.5 (n = 10); or 2) DBP (500 mg/kg^{-1}) plus flutamide (100 mg/kg^{-1}) (DBP plus F) by oral gavage in 1 ml/kg^{-1} corn oil/2.5% dimethylsulfoxide between e14.5 and 21.5 (n = 6 litters). Control dams (n = 10 litters) were gavaged daily with the vehicle alone (1 ml/kg^{-1} corn oil/2.5% dimethylsulfoxide). Dams were dosed from e14.5 until the day before cull. This window of exposure was selected to begin before the onset of androgen production at e15.5 (4) and encompass the period of fetal male reproductive development (e15.5–e21.5) (12). The doses of flutamide and DBP were selected based on results previously reported, highlighting their impact on male reproductive tract development (11, 22–24). The dose of TP selected was based on previous results showing that doses above 1 mg TP (per rat) increased female fetal testosterone by 80% and increased female AGD without a high incidence of toxicological effects (25). However, it is worth noting that TP is aromatizable, so it is possible that some of the testosterone injected into the pregnant dam will be converted into estradiol in the placenta (26), therefore, the exact dose of testosterone that the fetus was exposed to cannot be defined. Dams were checked daily for signs of toxicity, and dam weights were recorded daily throughout the dosing regime. Male and female offspring were subsequently evaluated during fetal life (e16.5–e21.5). Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation, and pups were recovered, decapitated, and placed in ice-cold PBS (Sigma-Aldrich Corp., St. Louis, MO).

Before recovery of reproductive tracts, AGD was measured in fetal (e21.5) males and females using digital calipers (Faithfull Tools, Kent, UK) because AGD reflects the degree of masculinization of the animal (reviewed in Ref. 7). The urogenital sinus (prospective prostate) and gonads with the attached WDs were collected from control and treated male and female fetuses, examined with a Leica MZ6 dissecting microscope, and photographed using a Leica ICA camera (Leica Microsystems GmbH, Wetzlar, Germany) to enable gross morphological evaluation.

Androgen receptor knockout (ARKO) mice

Female mice heterozygous for the X-linked hypoxanthine phosphoribosyltransferase-Cre transgene (27) were mated to male ARflox mice (28) to produce females carrying one deleted allele and one wild-type (WT) allele of the X-linked AR gene. These females were subsequently mated to produce ARKO males and control littermates. Genotype was established by PCR. The presence of the Cre recombinase transgene was determined using primers GATCGCTGCCAGGATATACG and AGGCCAGGTATCTCTGACCA. Genotyping of ARKO mice was completed using primers GCTGATCATAGGCCTCTCTC and TGCCCTGAAAGCAGTCCTCT, which generate amplicons of 1072 and 680 bp for WT and ARKO, respectively.

Dams (n = 9) were killed by inhalation of carbon dioxide and subsequent cervical dislocation, and pups were recovered, decapitated, and placed in ice-cold PBS. Gonads with the attached WDs were collected from male and female fetuses (e15.5 and e16.5) or from neonates on day of birth [postnatal d 0 (pnd0), n = 2 litters], and examined with a Leica MZ6 dissecting microscope and photographed using a Leica ICA camera to enable gross morphological evaluation.

Tissue fixation

WDs were fixed in Bouin's for 1 h before being transferred into 70% ethanol and processed for 17.5 h in an automated Leica TP1050 processor. WDs were then embedded in paraffin wax, sectioned (5 μm), and floated onto slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma-Aldrich) and dried overnight at 50 C before histological analysis (see below). Representative WDs from at least three animals from at least three litters from the aforementioned groups of rats and mice were subsequently used for the studies detailed below. Histological analysis was performed on WD sections stained with hematoxylin and eosin, using standard protocols, and careful note was taken of any histological abnormalities.

Immunohistochemistry

Immunohistochemistry was performed on WDs recovered from mouse and rat fetuses using previously published standard avidin-peroxidase protocols and citrate antigen retrieval (11). WDs were stained for AR (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), laminin (1:100; Abcam plc, Cambridge, UK), cleaved caspase 3 (1:200; Cell Signaling Technologies, Beverly, MA), phospho-histone H3 (1:1000; Upstate Biotechnology Inc., Lake Placid, NY), and pan-cytokeratin (1:200; Sigma-Aldrich). Cellular sites of expression were determined and slides photographed using a Provis AX70 (Olympus Optical, London, UK) microscope fitted with a Canon DS6031 camera (Canon Europe, Amsterdam, The Netherlands). To ensure reproducibility of results and allow accurate comparison of immunostaining between groups, sections of WDs from control and treated/knockout animals were processed in parallel on at least three occasions; sections of WDs from at least three animals in each group were run on each occasion. Appropriate negative controls were included, whereby the primary antibody was replaced by normal goat serum alone, to ensure that any staining observed was specific; none of the antibodies used showed other than minor nonspecific staining.

Epithelial cell height analysis

WD sections were immunostained for pan-cytokeratin as detailed previously to label clearly all epithelial cells. Sections were viewed using an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK). Image-Pro Plus version 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) was used to measure epithelial cell height. Using a $\times$63 objective, WD epithelial cell height was measured in every fifth epithelial cell per section. Only epithelial cells in which the nucleus could be clearly identified were measured, thus excluding from analysis any epithelial cells from treatment/knockout animals that were severely flattened or degrading.

Statistical analysis

Values have been expressed as means ± SEM. Data were analyzed using Fisher's exact test (incidence of prostates and WDs) or one-way ANOVA (AGD and epithelial cell height), using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA).

Results

Effectiveness of the rat models in manipulating masculinization endpoints (AGD and prostate formation)

Both AGD and prostate formation depend upon androgen action (8, 29, 30), and are commonly used markers of fetal androgen action. At e21.5, AGD in fetal male rats is approximately twice as long as in females, and at this age, elaborate prostatic bud branching can be identified in control male rat fetuses but not in females (Fig. 1). Fetal exposure to exogenous testosterone increased female AGD to a length comparable to control males and induced elaborate prostatic bud branching in female fetuses at e21.5. In contrast, masculinization was prevented at e21.5 in male rat fetuses exposed to DBP in combination with flutamide (DBP plus F), as evidenced by reduction of AGD to a length comparable to that in control females and complete prevention of prostatic bud branching (Fig. 1).

Timing of normal WD development in male and female rat fetuses

At e16.5–e17.5, a patent WD was readily identified in female rats lying medial to the müllerian duct (MD): this WD began to

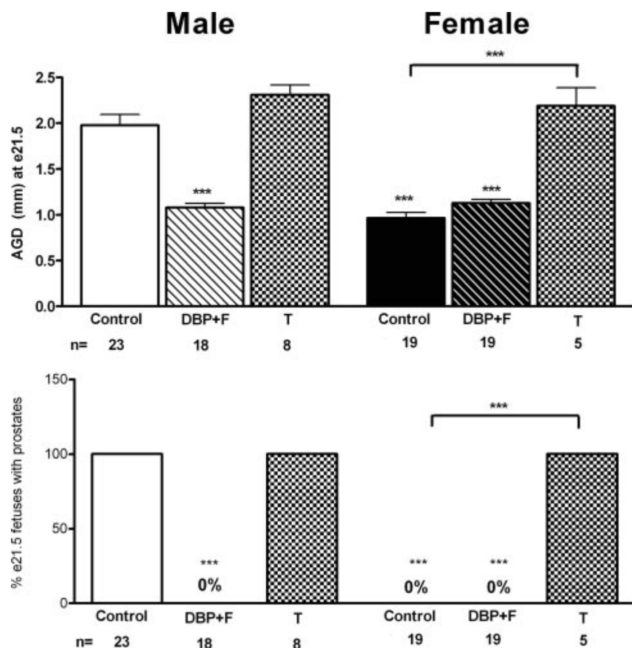

FIG. 1. Effectiveness of the treatments in manipulating masculinization endpoints (AGD and prostate formation). Quantification of AGD and the presence of a prostate in male and female fetuses from control (*solid bars*), DBP plus F-exposed (*striped bars*), and testosterone (T)-exposed (*checkered bars*) litters at e21.5. Note that AGD was significantly smaller in control female fetuses than in males. Exposure to testosterone completely masculinized AGD in females but had no effect on male AGD. Conversely, exposure to DBP plus F reduced male AGD to female levels but had no effect on female AGD. Testosterone exposure induced prostate formation in females, whereas exposure to DBP plus F prevented prostate formation in males. ***, $P < 0.001$, compared with control males. Values are means ± SEM.

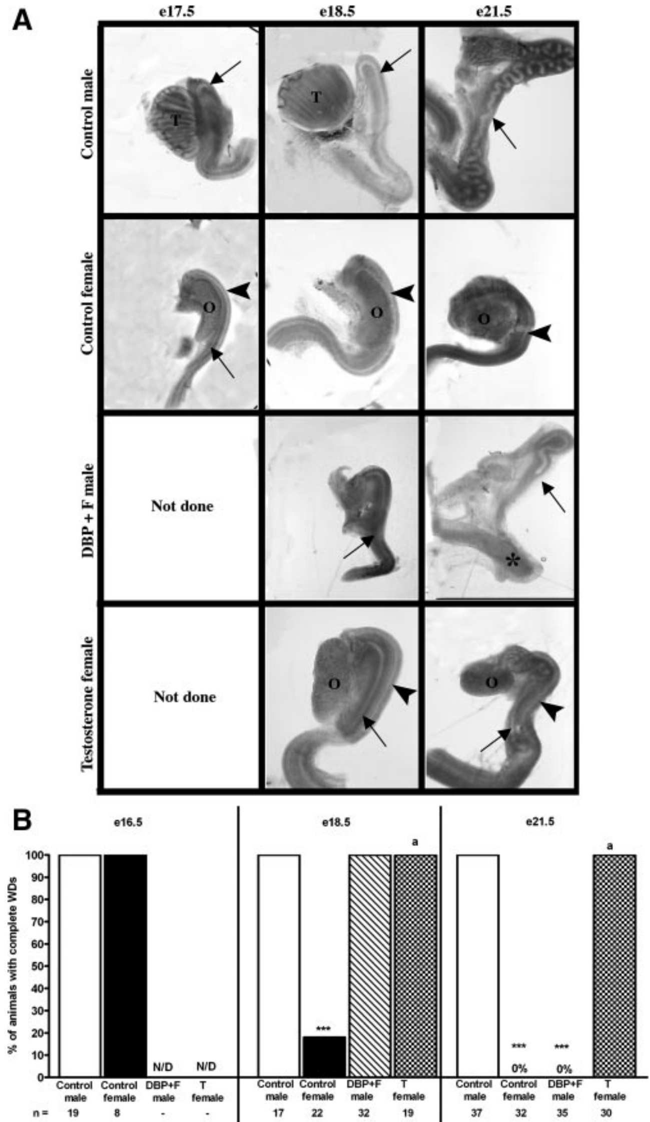

FIG. 2. Timing of WD development in representative male and female rat fetuses at e17.5–e21.5. A, Note that at e17.5, the WD (*arrow*) is obvious in females, lying medial to the MD (*arrowhead*). At e18.5 the WD has almost completely regressed in females and is completely absent at e21.5. Exposure to testosterone prevented WD regression in females, with the WD evident at both e18.5 and e21.5 (*arrow*). At e18.5 the WD is a simple straight duct in males, but at e21.5 the future epididymal segment has differentiated and become highly coiled. The WD is present and looks morphologically normal at e18.5 in males exposed to DBP plus F, but at e21.5 the WD has almost completely degenerated with little patent lumen apparent, leaving a remnant mesenchymal-like structure (*). The presence or absence of WDs in male and female fetuses was quantified at e16.5–e21.5 (B), and is summarized in Table 1. All images are at the same magnification (×25). O, Ovary; T, testis. **, $P < 0.001$, compared with age-matched control males; a, $P < 0.001$ compared with age-matched control females. N/D, Not determined.

regress in a cranio-caudal direction and, by e18.5, was barely identifiable upon gross examination (Fig. 2), with a patent lumen only visible at the caudal end. Conversely in males, the WD remained a simple straight duct throughout this period while the MD regressed between e17.5–e18.5 (Fig. 2). Therefore, a detailed investigation of WD regression and stabilization was undertaken in all subsequent studies at e18.5, when the WD has almost completely regressed in control females. Any experimen-

TABLE 1. Summary of the incidence of WD regression and the histological processes involved

	WD regressed	Apoptosis epithelia	Proliferation		BM interrupted	Epithelial height reduced
			Epithelia	Stroma		
Mouse						
e15.5						
WT male	0% (0/5)	X	√	√	X	X
ARKO male	0% (0/5)	X	√	√	X	X
Female	**100% (6/6)**	**N/A**	**N/A**	v	**N/A**	**N/A**
e16.5						
WT male	0% (0/16)	X	√	√	X	X
ARKO male	30% (3/10)	X	√	√	X	X
Female	**100% (17/17)**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**
Rat						
e18.5						
Control male	0% (0/17)	X	√	√	X	X
DBP plus F male	0% (0/32)	X	√	√	X	X
Control female	**82% (18/22)**	√	**X**	√	√	√
T female	0% (0/19)	X	√	√	X	X
e21.5						
Control male	0% (0/37)	X	√	√	X	X
DBP plus F male	**100% (35/35)**	√	**X**	√	√	√
Control female	**100% (32/32)**	√	**X**	**X**	√	√
T female	0% (0/30)	X	√	√	X	X

Values are the number of animals the WD regressed in, out of the total number of animals examined. *Bold* values are different from age-matched control males. *Non-bold* values are the same as age-matched control males.

tal perturbation of the timing of WD regression should be readily identifiable at this age.

Ability of exogenous testosterone to stabilize and differentiate the WD in female rats

At e18.5 and e21.5, WDs were present in all females exposed *in utero* to testosterone, with 87% of them showing some degree of coiling at e21.5; however, this coiling was less extensive than that observed in WDs of control, age-matched males (Fig. 2 and Table 1). These "stabilized" WDs in females persisted into adulthood but never developed as fully as the equivalent "epididymal" organ in males (data not shown). Exposure to flutamide (100 mg/kg^{-1}) in combination with testosterone (20 mg/kg^{-1}) prevented WD stabilization in females, and, in this treatment group, no WDs were identified in any female examined at e21.5 (data not shown). These data confirm that females can respond to androgens, and that testosterone alone can stabilize and partially differentiate the WD in females; this experimental masculinization can be prevented by flutamide exposure. There was no obvious effect of exogenous testosterone exposure on the gross morphology of WDs in males at either e18.5 or e21.5, *i.e.* epididymal coiling was not initiated any earlier or to any greater extent than in control males (data not shown).

Impact on WD development of ablating androgen action in male rats

Treatment with DBP plus F did not induce any gross WD abnormalities in males at e18.5 (Fig. 2), and a complete and patent WD was observed in all e18.5 fetuses examined (Fig. 2 and Table 1). In contrast, at this age the WD in control females had completely regressed (Fig. 2). However, exposure to DBP plus F resulted in the loss of all WD structures in 25% of males at e20.5 (data not shown) and in all male fetuses by e21.5 (Fig. 2 and Table 1), with a patent lumen only apparent in the residual caput segment of the WD, whereas the rest of the WD appeared as a remnant mesenchymal-like structure (Fig. 2). Thus, although DBP plus F resulted in the absence of the WD, the timing of its regression (e20.5–e21.5) was 2–3 d later than occurred in normal females (e18.5) (Table 1).

WD regression in ARKO mice

In WT male mice, the WD could clearly be identified as a simple straight duct at e15.5, but by e16.5, some coiling could be identified in the future caput epididymis (Fig. 3). By the day of birth (pnd0), the future epididymis was highly coiled. Conversely in WT females, the WD had regressed almost completely by e15.5, with a patent lumen only visible at the caudal end (Fig. 3), lying medial to the MD. In contrast, in ARKO males a complete WD could still be readily identified at e15.5 (Fig. 3 and Table 1). By e16.5, the WD had started to regress in ARKO males, but a patent lumen could still be identified in the caput by gross inspection (Fig. 3). At pnd0 the WD had almost completely regressed, with a patent lumen only apparent in the residual caput segment of the WD, whereas the rest of the WD appeared as a remnant mesenchymal-like structure. Therefore, complete ablation of AR signaling in ARKO mice resulted in an absence of the WD by birth, but its regression (around e16.5) occurred at approximately 2 d later than occurred in normal females (Table 1).

Apoptosis, proliferation, and morphology of the epithelium in WDs from male and female fetuses

Analysis of apoptosis, cell proliferation, and epithelial degeneration was undertaken in WDs recovered from male and female fetuses because these cellular processes have been suggested to

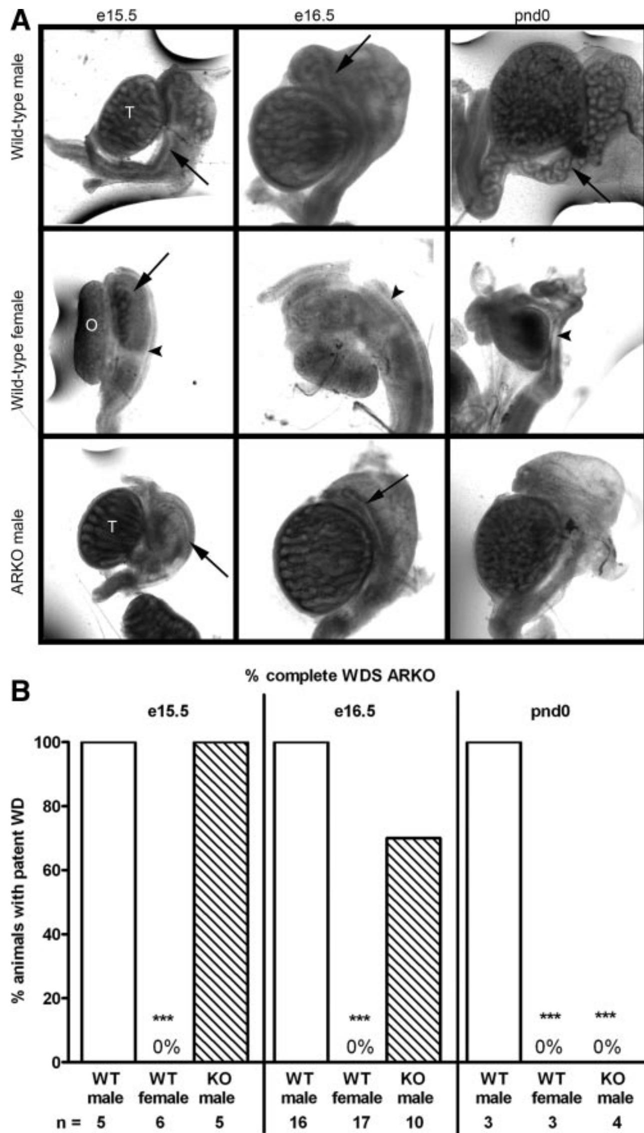

FIG. 3. Timing of WD development/regression in ARKO mice compared with WT males and females at e15.5–pnd0. A, In WT males the WD (*arrow*) was clearly identified at all ages examined (e15.5–pnd0). However, in WT females the WD had almost completely regressed by e15.5, with a patent lumen only visible at the caudal end (*arrow*), lying medial to the MD (*arrowhead*). The WD was completely absent in females at e16.5 and pnd0. Conversely, in ARKO males a complete WD could still be readily identified at e15.5 (*arrow*). By e16.5 the WD had started to regress in ARKO males, but a patent lumen could still be identified at the caput (*arrow*). At pnd0 the WD had almost completely regressed in ARKO males, with a patent lumen only apparent in the residual caput segment of the WD. The presence or absence of WDs in male and female fetuses was quantified at e15.5–pnd0 (B) and is summarized in Table 1. All images are at the same magnification (×25). KO, Knockout; O, ovary; T, testis. ***, $P < 0.001$.

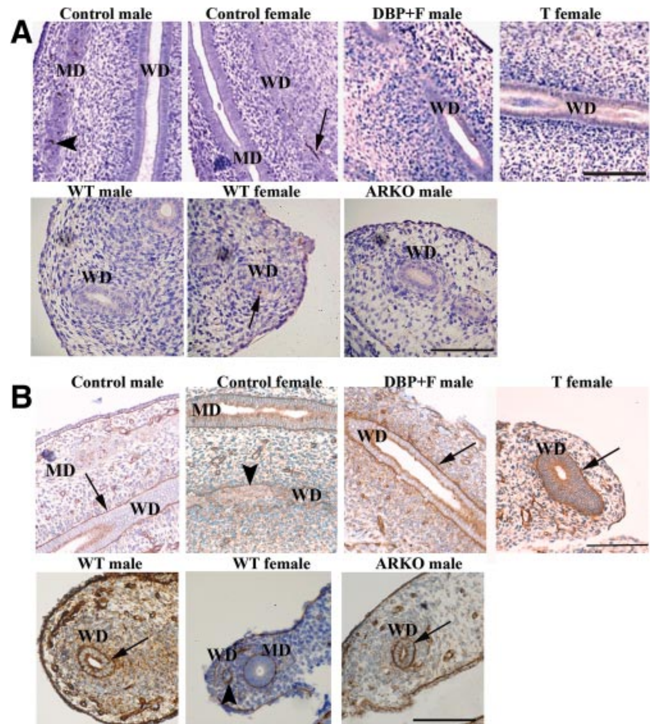

FIG. 4. Effects of androgen action on apoptosis and the basement membrane in male and female WDs from rats (e18.5) and mice (e16.5). A, Representative images of apoptotic cells (immunopositive for cleaved caspase 3, *brown* staining) in WD from male and female fetuses. Apoptosis is evident in the epithelium of WDs from control females (*arrow*) but is rarely seen at e18.5 in WDs from control or DBP plus F-exposed male rats or from testosterone (T)-exposed female rats. Conversely, apoptotic cells are evident in the epithelium of the MD in control males (*arrowhead*). Apoptosis is rarely seen at e16.5 in WDs from WT or ARKO male mice. Occasional apoptotic cells can be seen in the residual epithelium still remaining in WDs from female mice at e16.5 (*arrow*). B, Demarcation of the basement membrane in WDs from male and female fetuses by immunostaining for laminin (*brown*) in the basement membrane. Note that laminin forms a defined "ring" at the basement membrane in control male WDs (*arrow*) and also in WDs from DBP plus F-exposed male rats, from ARKO male mice, and from testosterone-exposed female rats. Conversely, in normal females the WD basement membrane is disrupted and the epithelium flattened, leaving patches of diffuse laminin expression (*arrowhead*). Note that laminin forms a defined "ring" at the basement membrane of the MD in control females. *Scale bar*, 100 μm.

play a role in MD regression in males (5, 31–33) and in causing WD abnormalities in males exposed to antiandrogens during fetal life (11, 14). These results are summarized in Table 1. Note that in control females, little epithelium was present in the WD, therefore, the images shown in this paper were selected to show the phenotype of any persisting epithelium.

Apoptotic cells (positively immunostained for cleaved caspase 3) were noted in the epithelium of the WD from female rats at e17.5 (data not shown) and in the remnant WD epithelium at e18.5 (Fig. 4A); this was in contrast to age-matched control males in which the WDs were immunonegative for cleaved caspase 3. Similarly, apoptotic cells were rarely detected in either the epithelium or stroma of WDs from testosterone-exposed female fetuses at e18.5 (Fig. 4A) or 21.5 (data not shown). Apoptosis was not apparent in WDs from DBP plus F-exposed male fetuses at e18.5 (Fig. 4A) or at 21.5 (data not shown), or in ARKO male mice at e15.5 or 16.5, although it is emphasized that by e21.5 in rats and e16.5 in mice, very little epithelium was present in the WDs of these males. Because epithelial cell apoptosis was evident in the regressing WD from females but not in the WD of DBP plus F-exposed or ARKO males, this highlights a potential difference in the cellular mechanisms of WD degeneration in each of these models.

Immunostaining for phospho-histone H3 showed that mitotic cells were present in the stroma surrounding the regressing WD from e16.5–e18.5 females, but they were rarely detected in the epithelium (data not shown) (summarized in Table 1). This was in contrast to control males in which mitotic cells were evident in both the stromal and epithelial cell compartments of the

WD at all ages examined (e16.5–e21.5, data not shown). Similar patterns were seen in WT mice (data not shown). Mitotic cells were noted in the WD epithelium and stroma at e18.5 and e21.5 (data not shown) in female rats exposed to exogenous testosterone. Phospho-histone H3 positive cells were still evident in both the stromal and epithelial cell compartments of WDs from DBP plus F-exposed male rats at e18.5 and in ARKO male mice at e16.5 (data not shown). However, by e21.5 in DBP plus F-exposed rats and pnd0 in ARKO male mice, mitotic cells were rarely noted in the residual epithelium of WDs but could still be identified in the remnant stromal compartment (data not shown). Therefore, cell proliferation was evident in the stromal compartment of WDs from both males and females in all models examined, but variation was noted in epithelial cell proliferation between males and females (Table 1).

Immunostaining for laminin highlighted that the basement membrane around the WD epithelium was interrupted and incomplete in the regressing WD from control female rats at e16.5 compared with the defined "ring" of laminin evident in the basement membrane of WDs from control male rats at e16.5–e21.5 (data not shown) (Table 1). By e18.5 the WD in control female rats had almost completely regressed, leaving only patches of laminin staining where the epithelium had once been (Fig. 4B). Similar patterns were seen in WT mice. Exposure of rats to exogenous testosterone prevented this interruption to the basement membrane and resulted in a defined "ring" of laminin in the basement membrane in WDs from females at e18.5 (Fig. 4B) and e21.5 (data not shown). In contrast to normal females, exposure of male rats to DBP plus F did not interrupt laminin expression in the basement membrane of WDs at e18.5 (Fig. 4B), but by e21.5, laminin expression was poorly defined and was often absent (data not shown). Similarly, in e16.5 ARKO male mice, laminin expression in the basement membrane was comparable to that in WT littermates (Fig. 4B), but by pnd0 this laminin "ring" was absent (data not shown).

Epithelial abnormalities were noted in the regressing WD of control females at all ages examined (e16.5–e18.5) in comparison to age-matched control male WDs. These included an apparent reduction in epithelial cell height and a narrowing or absence of a patent lumen at e18.5 (Fig. 5A). Exposure to exogenous testosterone prevented these abnormalities because the WD epithelium from exposed female fetuses was histologically comparable to WDs from age-matched control males at e18.5 (Fig. 5A) and e21.5 (data not shown). Exposure to DBP plus F did not result in reduced height of the WD epithelium in male fetuses at e18.5 (Fig. 5A), but by e21.5 the majority of these animals had very little epithelium evident, especially distal to the caput; any epithelium present was flattened with a grossly abnormal lumen (data not shown). Similar patterns were noted in WDs from male ARKO mice, whereby epithelial cell height was not reduced at e16.5, compared with WT littermates, and the lumen was patent (Fig. 5B). However, by pnd0, epithelium was rarely present in WDs from any ARKO male mice (data not shown). Quantitative measurement of epithelial cell height confirmed these histological observations (Fig. 5, A and B). No WD epithelium was present in female mice to measure at e16.5.

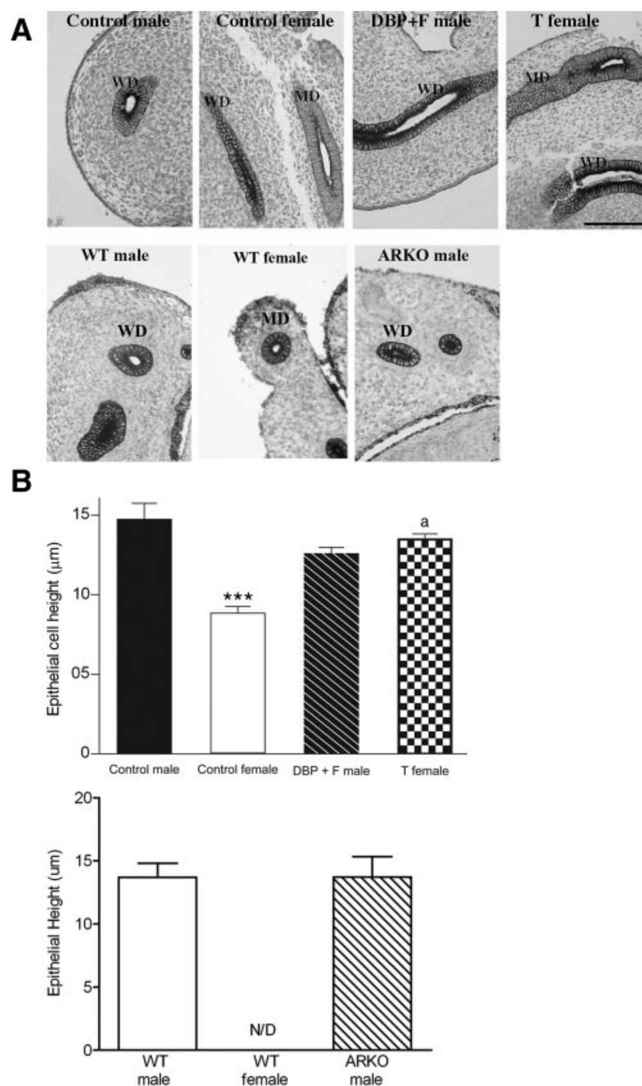

FIG. 5. WD epithelial cell height in male and female rats at e18.5 and in mice at e16.5. A, Representative images in which the epithelium is immunostained for cytokeratin. Note that the epithelium appears flatter in control females, but not in DBP plus F-exposed or ARKO males or in testosterone-treated (T) females, compared with age-matched control males. *Scale bar*, 100 μm. B, Quantification of epithelial cell height, demonstrating a significant reduction in control females compared with males. Note that epithelial cell height is not significantly reduced in DBP plus F-exposed or ARKO males or in testosterone-exposed females, compared with control males. Note also that epithelial cell height is significantly different in WDs from testosterone-treated females compared with control females. ***, $P < 0.001$ compared with control male values; a, $P < 0.001$ compared with control female values. Values are means ± SEM for three to six animals per group from at least two different litters. N/D, Not determined.

Discussion

Various studies have shown that androgens play a critical role in WD development (15, 17, 22, 34–36). The majority of studies used experimental impairment of fetal androgen action with subsequent evaluation of the males postnatally and demonstrated an absence of WD-derived tissues. The interpretation from these studies was that, in the absence of fetal androgen action, the WD regressed in males at the same time as in age-matched (normal) females. However, results from previous studies in rats by our group caused us to question this interpretation because we had shown that, at e18.5, an age when the WD has completely re-

gressed in normal females, WDs recovered from males exposed *in utero* to high concentrations of flutamide were comparable to those from control males (11). However, subsequent differentiation of the WD in these flutamide-exposed males was impaired by e21.5, and the WD subsequently degenerated during puberty. These results suggested that either WD stabilization can proceed in males in the presence of lower levels of androgen action than that required for subsequent differentiation or that WD stabilization is regulated by different mechanisms to those required for differentiation. We addressed this issue in the present studies using rodent models in which fetal androgen action was ablated in males or increased in females, and then examined the impact on WD development, compared with age-matched controls. These studies demonstrate that testosterone alone can masculinize the WD in females. However, genetic or chemical ablation of androgen action in males does not induce WD regression at the same time as in normal females, suggesting that there may be fundamental differences in WD programming in males *vs*. females, with factors other than androgens contributing to WD stabilization, particularly in the absence of androgen action in males.

Although little is known about the timing of, or mechanisms underlying, WD regression in females, the majority of understanding derives from studies in rats (3, 11, 14, 21, 37, 38). In our rat colony, a patent WD is readily identifiable in female fetuses at e16.5, but by e18.5 it has almost completely regressed. These timings agree with previous publications (21, 31, 39). Testosterone measurements confirmed that, at e17.5, testosterone was present in the fetal testis but was barely detectable in the fetal ovary (12). This agrees with data that plasma testosterone concentrations are at least 4-fold lower in female than in male rat fetuses (4). Conversely, AR protein was detected in WDs from females whenever a WD was present (data not shown), indicating that females appear capable of responding to androgens if the ligand is available. Furthermore, AR protein was immunolocalized to the WD epithelium in females at an age when it was rarely detected in the epithelium in males (our unpublished data). This highlights sexually dimorphic expression of AR in the fetal WD, suggesting that there may be fundamental differences in the WD in normal males compared with normal females. This difference in AR expression might play a role in the different timing of WD regression in males and females, and merits further investigation.

Because exposure to high doses of flutamide (100 mg/kg^{-1}) did not induce WD regression in fetal life in male rats (11), we sought a model in which more complete blockade of androgen action could be achieved, to determine the dependence of WD stabilization on androgens alone. Therefore, pregnant rats were exposed to DBP in combination with flutamide (DBP plus F); DBP reduces testicular testosterone production in fetal male rats by 70–90% (15–18, 23, 40, 41), whereas flutamide competes with residual endogenous androgens for binding to the AR and so prevents transcriptional activation of AR in target tissues (11, 22, 42, 43). Combined exposure to DBP plus F should result in a near complete blockade of androgen action and, therefore, provides a model in which to test if WD stabilization in males is solely dependent on androgen action.

At e18.5, WDs from all DBP plus F-exposed males were intact and had a similar morphology to WDs from control males, whereas WD regression was complete in all males at e21.5. These timings contrast with normal female rats in which WD regression was nearly complete by e18.5, 2–3 d earlier than in DBP plus F-exposed males. Because the MD is present in control females but not in control or DBP plus F-exposed males at e18.5, it is possible that this could contribute to these differences in the timing of WD regression. Furthermore, it is possible that DBP may have a direct effect on the WD, but there is no way of testing this because it would be impossible to separate any possible direct effect of DBP on the WD from the impact of DBP-induced reduction in testosterone production. Another obvious explanation for this discrepancy is that DBP plus F still did not result in complete blockade of androgen action within the WD, but there is no obvious way to assess this directly. Therefore, to address this we examined WD regression in ARKO mice, a rodent model accepted to lack completely a functional AR due to a genetic mutation (28, 44). In ARKO male mice (n = 11 litters), as in DBP plus F-exposed male rats, WD regression is temporally delayed by approximately 2 d compared with normal littermate females. We also found similar results in *tfm* male mice, in which androgen action is genetically ablated due to a different inactivating mutation in the AR (n = 5 litters; unpublished data). Therefore, these studies confirmed our findings of delayed WD regression in males in two different rodent species using three different models to ablate androgen action.

It is not obvious why the WD should regress slower in males devoid of androgen action than in normal females, and raises the question of whether unknown factors could actively promote WD regression in females or whether factors other than androgens may help maintain the WD in males, particularly in the absence of androgen action. The precise source of these factors is unknown; they could be produced by the testis or be endogenous to the WD itself. This merits further investigation. However, this "maintenance" mechanism is clearly not sufficient to stabilize the WD long term in males because, in both the rat and mouse models lacking androgen action, the WD ultimately regresses by birth. This mechanism might serve to prolong survival of the WD in males to maximize its opportunity to respond to testosterone. Further investigations are required to identify the factor(s) involved, but possible candidates include inhibins and/or insulin-like factor 3 (Insl3). Body weight is not significantly different between that of ARKO or DBP plus F-exposed males and control females, therefore, we do not believe the differences in the timing of WD regression can be explained by growth rate (unpublished data) (28, 45). Tfm/ARKO mice provide rodent models for complete androgen insensitivity syndrome (CAIS), in which patients are genetically male but have a female phenotype (46–50). Most evidence from CAIS patients is derived from postnatal examination (51), with no definitive published evidence on the status of the fetal WD. Therefore, it is unclear whether in CAIS patients the WD fails to stabilize during early fetal life, as occurs in females, or whether the absence of WD structures in later life results from a "post-stabilization" degeneration of WD-derived tissues, as occurs in this study in rats exposed *in utero* to DBP plus F and in ARKO male mice.

Because these findings in males question whether androgens are the only determining factor in WD stabilization, we investi-

gated the ability of exogenous fetal androgens to stabilize the WD in females. Exposure to exogenous maternal testosterone in rats can masculinize female offspring to varying degrees (25, 39, 52–55). However, some studies were unable to stabilize the WD, even in females in which prostates were readily identifiable (25). In the current study, female reproductive tissues were masculinized by exogenous maternal testosterone. This is a direct effect of androgens, rather than due to testosterone being aromatized to estrogen, because they could be blocked in female fetuses by combined exposure to testosterone plus flutamide. In our study, testosterone exposure not only stabilized the WD in all female rats examined but could even induce some degree of differentiation and compartmentalization, as evidenced by the initiation of "epididymal" coiling at e21.5. Interestingly, this coiling was never as pronounced as in control males. It may be that the dose of testosterone used in this study was not sufficient to fully stimulate coiling, or that the presence of the normal MD alongside the WD in females may physically prevent the "stabilized" WD from fully coiling, or that basic differences exist in the WD in females *vs*. males that prevent the WD in females from undergoing complete differentiation. Examination of testosterone-exposed females after birth showed that the stabilized female WD persisted postnatally and into adulthood, even though exposure to exogenous testosterone ceased at birth (data not shown). This suggests that patterning of the fetal WD is established early in reproductive development (e14.5–e17.5), and, once stabilized, the female WD persists postnatally. This is in contrast to males exposed to flutamide *in utero*, in which WD derivatives were present at birth but were usually absent by adulthood (11). This contrast further highlights fundamental differences between males and females, and merits further investigation.

Histological comparison of naturally regressing WDs in females with those from males deprived of androgen action revealed fundamental differences that support the view that the WD may be subtly different in males and females. First, apoptosis was observed in the WD epithelium from control females but not in WDs from control, ARKO, or DBP plus F-exposed males or in testosterone-exposed females. This is in agreement with previous studies in control female rats (5, 21, 56, 57). Second, cell proliferation was not observed in the WD epithelium of control females but was noted in both the epithelium and mesenchymal compartments of WDs from control and DBP plus F-exposed males, and in testosterone-exposed females at e18.5 and in ARKO males at e16.5. Third, our results suggest that during WD regression in females, epithelial cells lose their attachment to the basement membrane; these changes may be the trigger for apoptosis (58). Exposure to exogenous testosterone prevented these WD cellular abnormalities in females. WDs recovered from e18.5 males exposed to DBP plus F or e16.5 ARKO males showed no obvious histological abnormalities, whereas around the time of birth, similar abnormalities were noted to those observed in the regressing female WD at earlier ages. Together, these data demonstrate differences in the timing of and/or presence of the cellular processes observed in the WD epithelium in normal females compared with males. Furthermore, they highlight that exposure to exogenous testosterone induces "male" like changes in females but that deprivation of androgen action (genetically or chemically) cannot induce the cellular changes in males at the same age as they are seen in control females. These observations suggest that a similar mechanism for WD degeneration is operating in males and females in which androgen action is absent but that in males these changes occur several days later than in females.

In summary, the present study sought to gain new insight into WD development by examining the role for androgens in WD stabilization, rather than in later WD differentiation into its adult derivatives. These studies have shown that testosterone alone can stabilize and partially differentiate the WD in females but that genetically or chemically ablating androgen action in males does not induce WD degeneration at the same time as it occurs in females. This suggests either that factors other than androgens prolong the survival of the WD in males, possibly to maximize the opportunity for WD stabilization by endogenous fetal androgens, or that unknown factors actively promote WD regression in females. Although androgens are critical in WD stabilization and differentiation, these studies offer the first evidence that they may not be the only factor involved in dictating the fate of the WD in males. Our studies suggest there are fundamental differences in the preprogrammed setup of the WD in males compared with females and that the differential response in the fate of the WD may not depend solely on the presence or absence of androgens.

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