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The Ontogeny of the Urogenital System of the Spotted Hyena (*Crocuta crocuta Erxleben*)[1]

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ABSTRACT

Studies were conducted to elucidate the importance of androgen-mediated induction of the extreme masculinization of the external genitalia in female spotted hyenas. Phallic size and shape; androgen receptor (AR) and α-actin expression; and sex-specific differences in phallic retractor musculature, erectile tissue, tunica albuginea, and urethra/urogenital sinus were examined in male and female fetuses from Day 30 of gestation to term. Similar outcomes were assessed in fetuses from dams treated with an AR blocker and a 5α-reductase inhibitor (antiandrogen treatment). Clitoral and penile development were already advanced at Day 30 of gestation and grossly indistinguishable between male and female fetuses throughout pregnancy. Sex-specific differences in internal phallic organization were evident at Gestational Day 45, coincident with AR expression and testicular differentiation. Antiandrogen treatment inhibited prostatic development in males and effectively feminized internal penile anatomy. We conclude that gross masculinization of phallic size and shape of male and female fetuses is androgen-independent, but that sexual dimorphism of internal phallic structure is dependent on fetal testicular androgens acting via AR in the relevant cells/tissues. Androgens secreted by the maternal ovaries and metabolized by the placenta do not appear to be involved in gross masculinization or in most of the sex differences in internal phallic structure.

androgen receptor, clitoris, developmental biology, female urogenital tract, male urogenital tract, penis, spotted hyena, testosterone

INTRODUCTION

Female spotted hyenas display extreme masculinization of the external genitalia and have a hypertrophied penile clitoris traversed by a central urogenital canal opening at the tip of the glans and a pseudoscrotum situated in the position where an external vaginal orifice should be located [1]. Female spotted hyenas urinate, mate, and give birth to infants that weigh 1.33 ± 0.27 kg through this peniform clitoris [2]. They also display erections similar to those of males during meeting ceremonies, with dominant hyenas inspecting the erect penis/clitoris of subordinate members of the clan. Despite masculinization of the external genitalia of female spotted hyenas, internal accessory sexual structures are similar to those of other carnivores [3–5].

Our general understanding of masculine sex differentiation in eutherian mammals is based on a theory that was advanced more than a half-century ago [6]. Accordingly, development of external genitalia from common ambisexual structures is determined by the presence (males) or absence (females) of androgens [7]. Testosterone produced by the fetal testes is metabolized in situ to dihydrotestosterone (DHT), which elicits penile development. This involves fusion of urethral folds to form a penile urethra that traverses the penis to open distally at the tip of the glans. The genital swellings are stimulated by androgens to fuse in the midline caudal to the penis to form the scrotum. In the absence of androgens (in females), growth of the genital tubercle is modest, resulting in formation of the clitoris. Also, in females, the urogenital folds and the genital swellings remain separate to form the labia minora and labia majora, respectively (in humans). Although there are species differences in the arrangement of the female external genitalia, the feminine pattern develops in the absence of androgens, whereas the male pattern develops in response to androgens. In addition, testosterone produced by the fetal testes is responsible for development of the prostate from the urogenital sinus and the epididymis, ductus deferens, and seminal vesicles from the Wolffian ducts. Fetal testicular androgens apparently reach the embryonic Wolffian duct by diffusion [8, 9]. Anti-Mullerian hormone produced by the Sertoli cells of the testes and probably acting via local diffusion elicits regression of the Mullerian ducts, which in females, are the progenitors of the oviducts, uterus, cervix, and upper vagina.

Researchers have postulated an androgenic pathway that could account for the masculinized external genitalia of the female spotted hyena. The fetal ovary was suggested to be the likely source of such androgens [10], but subsequent work demonstrated that the fetal ovary was undifferentiated (ambisexual stage) at the time the genital tubercle exhibited advanced morphogenesis [11]. Subsequent studies suggested placental conversion of androstenedione to testosterone, and transfer to developing fetuses [12, 13], which elicited masculinization of the genital tubercle before functional differentiation of the gonads in hyena fetuses [11].

The unusual pattern of sex differentiation of the spotted

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hyena became even more puzzling when treatment of pregnant female hyenas with various antiandrogens failed to produce typical female mammalian external genitalia (i.e., a small clitoris and a vaginal opening in the perineum) in offspring of treated subjects [14, 15]. Our studies in the spotted hyena consistently showed feminizing effects of antiandrogen treatment in utero, as long as feminization was defined as movement of the male phenotype toward the female phenotype. For example, the penis of the male is longer and thinner than the clitoris of the female, has an angular rather than a rounded glans, and has a much smaller, less elastic urogenital meatus. The penis of antiandrogen-treated males exhibited a female-like configuration; the penis was too short and thick to permit intromission with a receptive female [16]. Likewise, the clitoris of antiandrogen-treated females, although still prominent, was shorter and broader than that of untreated females, and the urogenital meatus was more elastic [16].

The preceding studies call attention to several distinct problems in understanding the coexistence of unremarkable internal feminine genitalia along with masculinized external genitalia of the female spotted hyena: 1) Are androgens responsible for masculine development of external genitalia in female spotted hyenas? 2) What mechanisms account for the sexually dimorphic internal structure of the clitoris and penis in regard to the location of erectile tissue and the fibrous tunica albuginea, the position of retractor muscles, and the configuration of the urethra/urogenital sinus [5, 17]?

Given that genital morphology of female and male spotted hyenas is established at birth, tracking the temporal course of development and differentiation during fetal life is the first step toward understanding the mechanisms that produce the male and female phenotypes in spotted hyenas. The availability of embryonic spotted hyena tissues from mixed-sex twin litters have provided the opportunity to evaluate the course of sexual differentiation within the clitoris and penis, the distribution of AR, and the development of the gonads.

In this paper the following questions were explored: 1) With regard to formation of the external genitalia, if differentiation/development of the basic masculine phenotype is independent of ovarian androgens, or testicular androgens (or both) in female and male embryos, then we would anticipate that a genital tubercle undergoing urethral development would occur before appearance of differentiated fetal gonads. 2) In addition, if development of masculine external genitalia is driven by androgens, then AR should be found in the relevant tissues. 3) If sex differences in the external appearance and internal structure of the phallus were the result of testicular androgens in the male fetus, we would expect (a) emergence of sex differences in clitoral/penile structure coincident with the time of histological differentiation of the testes. We might also expect (b) the distribution of AR in sexually dimorphic structures (i.e., retractor muscles, erectile tissue, tunica, and urethra/urogenital sinus) to exhibit differential distribution in female and male fetal tissues. 4) In terms of development of the internal accessory sexual organs, we know that in adult female spotted hyenas the Wolffian ducts are absent. Thus, regression of the female Wolffian ducts must have occurred despite the presence of placental androgens throughout the bulk of fetal life [11]. This suggests that circulating androgens are not sufficient to stabilize Wolffian duct development in the female spotted hyena.

MATERIALS AND METHODS

The hyenas used in this study were bred at the field station of the University of California at Berkeley. The data presented in this paper are based on 30 hyena fetuses. In the optimal case, estimates of gestational age were derived from observation of mating. In the absence of observed mating, timing of gestation was inferred from crown-rump length, femur length, or both, obtained via ultrasound [18], or by using the midpoint of a period during which animals were paired [11]. Pregnant female hyenas were immobilized with an i.m. injection of ketamine (4–6 mg/kg) and xylazine (1 mg/kg) administered by blow darts. Sterile surgical fetectomy was performed after the pregnant hyenas were anesthetized with isoflurane by procedures approved by the Animal Care and Use Committee of the University of California at Berkeley.

Antiandrogen-Treated Fetuses

Two sets of mixed-sex twins treated with antiandrogens during gestation were stillborn in our colony. Mating was not observed in one case, and treatment was initiated on the basis of elevated progesterone in the maternal circulation (progesterone >40 ng/ml). The mother received oral flutamide (50 mg/kg) and finasteride (2 mg/kg) for 76 days before parturition. Flutamide blocks androgen action by competing for binding to the AR. Finasteride blocks metabolic conversion of testosterone to the more potent dihydrotestosterone. Based on the small size of these fetuses, that the teeth had not penetrated the gums, and that the eyes were not yet open, we concluded that the birth was premature. A best estimate suggests that drug treatment was initiated at approximately 30 days of gestation. The carcasses were retrieved shortly after birth. The urogenital tracts were dissected several hours later, and tissues were preserved in 10% formaldehyde. The mother of the second set of stillborn twins was treated with flutamide (25 mg/kg) and finasteride (1 mg/kg) from Day 12 postmating. The stillborn carcasses were discovered after a delay of approximately 12 h. These carcasses were preserved in 10% formalin.

Morphological Methods and Determination of Sex

After the fetuses were removed they were photographed intact and then decapitated. Legs and tails were amputated to achieve unobstructed lateral and frontal views of the external genitalia, which were photographed. The entire urogenital tract was dissected from the carcass. The isolated penis and clitoris was in turn cut into two to five segments along the proximal-distal axis. Each segment was then fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 5 μm. Sections were mounted on glass slides, deparaffinized, and stained with hematoxylin and eosin or immunostained.

Fetal sex was determined by visual observation of dissected gonads and confirmed by histology. This was not possible in the youngest group of fetuses aged 30–34 days of gestation, which is before sex differentiation of the gonads. For this group sex was determined by polymerase chain reaction (PCR) analysis of a Y chromosomal gene, *SRY,* as reported previously [11] using primers originally designed from the pig *SRY* gene. Alternatively, a portion of the undifferentiated gonad was transplanted under the renal capsule of a castrated male athymic mouse and grown for 1 mo during which gonadal differentiation occurred. Histology of the grafts confirmed testicular or ovarian differentiation.

Immunohistochemistry

Methods for immunohistochemistry have been described [19]. Normal goat serum was applied to the sections for 30 min to bind nonspecific sites. The sections were then incubated with the primary antibodies overnight at 4°C. A rabbit polyclonal anti-AR antibody (Affinity BioReagents, Golden, CO) was used at a dilution of 1/200. This is an antibody that detects AR in prostatic epithelium and stroma of several mammalian species (rat, mouse, human), including spotted hyena prostate. Such nuclear staining was not observed in cells of nonandrogen target organs of the spotted hyena. Given these observations, we therefore assume that this antibody specifically recognizes the AR in the spotted hyena. A mouse anti-smooth muscle α-actin monoclonal antibody (Sigma, St. Louis, MO) was used at a dilution of 1/1000. After incubation with the secondary antibody, sections were incubated with avidin-biotin complex for 30 min at room temperature. Sections were stained for about 1–5 min using 3,3′-diaminobenzidine (DAB) in PBS and 0.03% H_2O_2 and counterstained with hematoxylin.

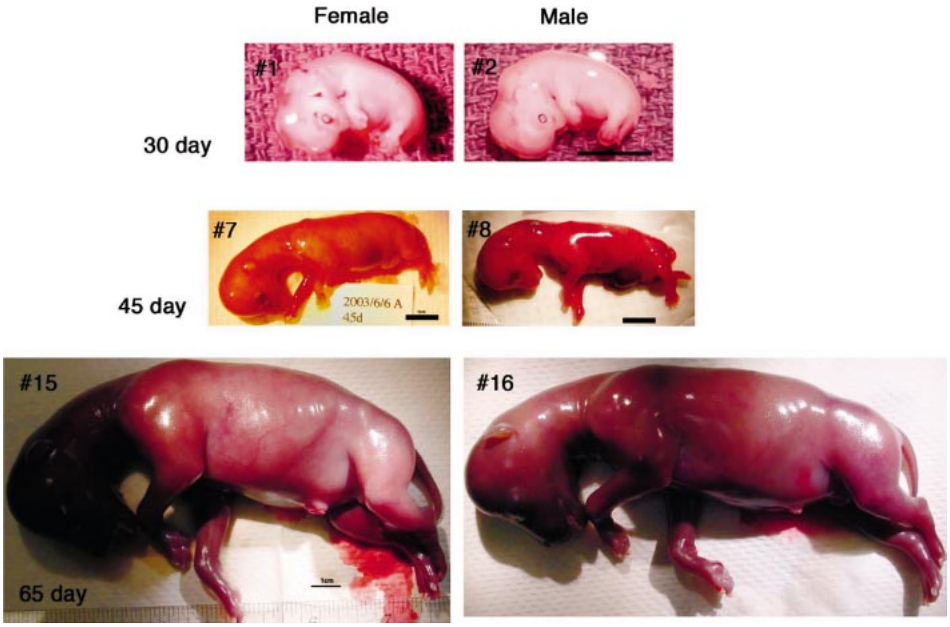

FIG. 1. Montage of male and female spotted hyena fetuses whose age ranges from 30 to 65 days. Females are on the left, males are on the right. Numbers on individual specimens correspond to those in Table 1. The period of 30–65 days of gestation is one of rapid growth and intense morphogenesis, and the critical time for the emergence of sex differences. Bars = 1 cm.

TABLE 1. Sex, age, weight and treatment status of spotted hyenas in this study.

Fetus no.	Sex[a]	Treatment[b]	Age	Age determination[c]	Mass (g)
1	M	Control	34	A	
2	F	Control	34	A	
3	M	Control	30	A	
4	F	Control	30	A	
5	M	Control	31	A	
6[d]	F	Control	33	B	
7	F	Control	45	A	11.70
8	M	Control	45	A	12.60
9	F	Control	45	A	12.30
10[d]	F	Control	48	B	12.00
11[d]	F	Control	48	B	12.00
12[d]	M	Control	50	B	16.00
13[e]	M	Control	58	B	46.00
14	M	Control	58	B	53.00
15	F	Control	65	A	134.26
16	M	Control	65	A	137.19
17[e]	M	Control	78	B	453.00
19	M	Control	90	C	1240.00
20	F	Control	95	A	1124.50
21	M	Control	95	A	1122.60
22	F	Flu/Fin Rx	105	D	880.00
23	M	Flu/Fin Rx	105	D	
24	F	Control	≥100	C	
25	M	Control	≥100	C	
26[e]	F	Control	≥100	C	1100.00
27[e]	M	Control	≥100	C	1120.00
28	F	Flu/Fin Rx	PP1	A	1220.00 (trunk)
29	M	Flu/Fin Rx	PP1	A	
30	M	Control	PP2	A	1600.00

[a] F, female; M, male.
[b] Flu/Fin Rx, flutamide plus finasteride treatment.
[c] Age determination: A, observed mating; B, midpoint of time paired with male; C, initial high progesterone (>40 ng/ml) combined with early and multiple crown-rump measures using ultrasound imaging and size at surgery; D, initial high progesteron with negative ultrasound imaging and size at surgery; D, initial high progesterone with negative ultrasound imaging, followed by multiple crown-rump measurements using ultrasound, and condition at birth (eyes closed, teeth not erupted).
[d] Hormonal data previously reported [11].
[e] Hormonal and anatomical data presented earlier [11].

RESULTS

Ontogeny of Spotted Hyena Fetuses

Figure 1 is a montage of selected male and female hyena fetuses used in this study, highlighting the period of sex differentiation. These images are derived from a series of pregnancies timed by various measures as discussed above (Table 1). This montage represents the first view of the physical features of the developing spotted hyena.

Ontogeny of the External Genitalia: Gross Anatomy In Situ

Our first objective was to compare the external genitalia of male and female hyena fetuses to determine when gross masculinization of the external genitalia occurs in female fetuses. Surprisingly, the overall gross external size and shape of the penis and clitoris (and their precursors, the genital tubercle) were similar at all stages examined (Fig. 2). This is particularly evident in the six youngest specimens (three male and three female) whose age ranged from 30 to 34 days of gestation. Overall gross size and shape of the external genitalia were also similar in four male and four female spotted hyena fetuses in the 45- to 58-day-old group. Likewise, at the gross level the external genitalia of specimens of the oldest group (10 male and 3 female fetuses aged 64 days of gestation to 2 days postpartum) were well advanced and roughly comparable in development in both male and female specimens.

Ontogeny of the External Genitalia: Histology and Immunohistochemistry

The specimens that we examined could be divided into three stages of development: 30–34 days, 45–58 days, and 64 days to birth.

Category 1: The Sexually Indifferent Stage. In the youngest group (30–34 days) the gonads were undifferentiated (Fig. 3A). Therefore, sex was determined by PCR analysis of the *SRY* gene and confirmed by grafting (Fig. 3B). Examination of the isolated genital tubercles by transmitted light with a dissecting microscope revealed a midline translucent structure, which in histological section was the ure-

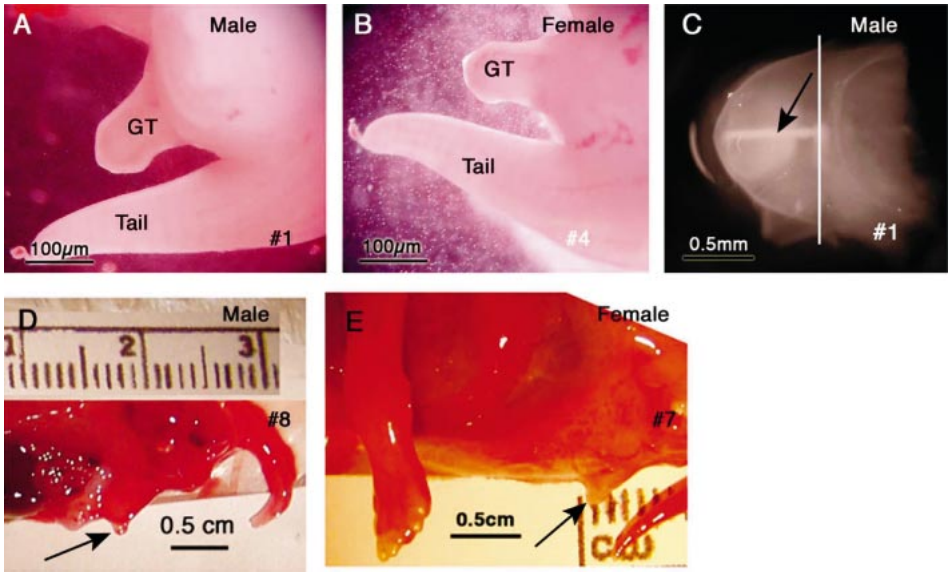

FIG. 2. Gross anatomy of the genital tubercles of spotted hyena fetuses. Brightfield images (**A** and **B**) of male and female specimens at 30 days of gestation (GT, genital tubercle). **C**) Pseudodarkfield image of a genital tubercle dissected from a 30-day-old male spotted hyena fetus photographed with oblique lighting. Note the whitish midline structure (arrow), which represents the solid urethral plate (see Fig. 4). White line in **C** denotes the position of the section in Figure 4. Brightfield images (**D** and **E**) of male and female specimens at 45 days of gestation (arrows indicate GT. Numbers on individual specimens correspond to those in Table 1.

thral plate, a solid cord of epithelial cells extending from the tip of the genital tubercle proximally to near the junction of the genital tubercle with the body wall (Figs. 2C and 4). The urethral plate terminated proximally by joining the canalized endodermal urogenital sinus. This arrangement is indicative of a mechanism of urethral development similar to that observed in mice and humans [7]. The solid urethral plate and associated dense homogenous mesenchyme was surrounded by epidermis (Fig. 4) as reported previously [11]. Androgen receptor immunostaining was not above background (not illustrated) in 30–34 day hyena fetuses. To detect the retractor muscle precursors, we employed immunohistochemical staining with an antibody to smooth muscle α-actin, which is highly reactive to vascular smooth muscle, to skeletal muscle precursors, and to mature skeletal muscle (Figs. 5–10). In the group aged 30–34 days, premuscle mesenchymal condensates representing the retractor muscles were not evident in sections stained with hematoxylin-eosin (Fig. 4) or immunostained for smooth muscle α-actin (not illustrated). As reported earlier [11], the gonads were morphologically undifferentiated (Fig. 3A). Wolffian ducts were present within the urogenital ridge, but the Mullerian ducts had not extended all the way to the cloaca. Thus, from the perspective of sex differentiation hyena embryos at 30–34 days of gestation are undifferentiated.

Category 2: The Emergence of Sexual Dimorphism. At 45–58 days of gestation internal morphology of the external genitalia is sexually dimorphic even though overall gross size and shape of male and female genital tubercles is similar (Fig. 2, D and E). In histological sections through the developing clitoris at midshaft, a distinct corporal body was represented dorsally as a dense mesenchymal condensation (Fig. 5A). The clitoral corporal body was flanked by bilateral arteries, which stained for smooth muscle α-actin (Fig. 5, A and B). A canalized urogenital sinus was situated ventral to the clitoral corporal body. At the 5 and 7 o'clock positions (relative to the clitoral corporal body), α-actin-positive mesenchymal condensations were observed, rep-

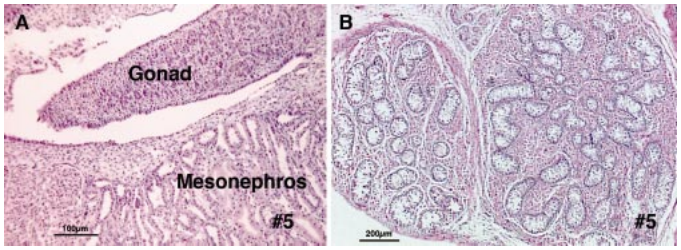

FIG. 3. **A**) Histological section of a gonad from a 31-day-old male fetus (#5, Table 1) before grafting, which was sexed by PCR as male. Note mesonephros and the undifferentiated gonad. **B**) A graft of the gonad contralateral to that shown in Figure 3A grown for 5 wk under the renal capsule of a castrated male nude mouse host. Testicular development has occurred during the graft period, thus confirming the PCR results. The mesonephros persisted (not illustrated).

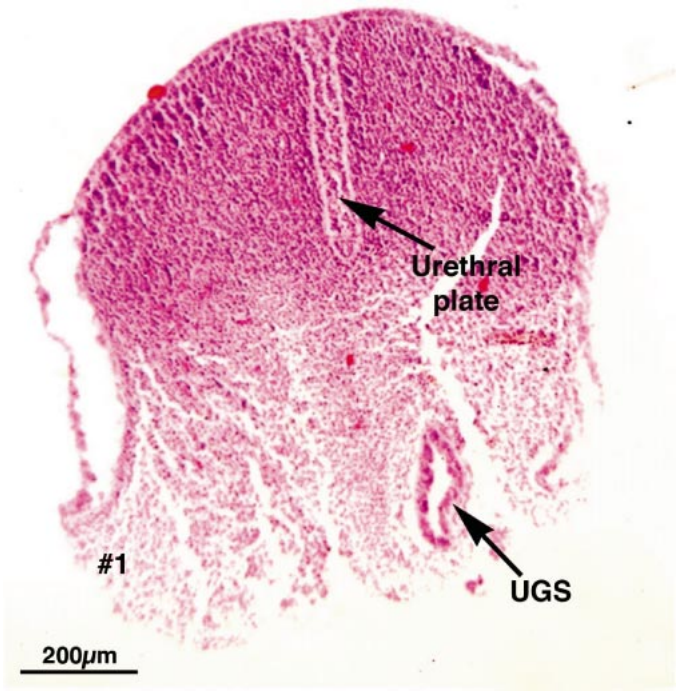

FIG. 4. Histological section of a sexually indifferent genital tubercle of a 34-day-old male fetus. This male specimen (#1, Table 1) was sectioned at the level indicated in Figure 2C. Accordingly, note the solid urethral plate and the dense undifferentiated mesenchyme that constitutes most of the mass of the genital tubercle. Premuscle masses are not detectable at this stage. Note a portion of the endodermal urogenital sinus (UGS), which in other sections, is in continuity with the solid urethral plate.

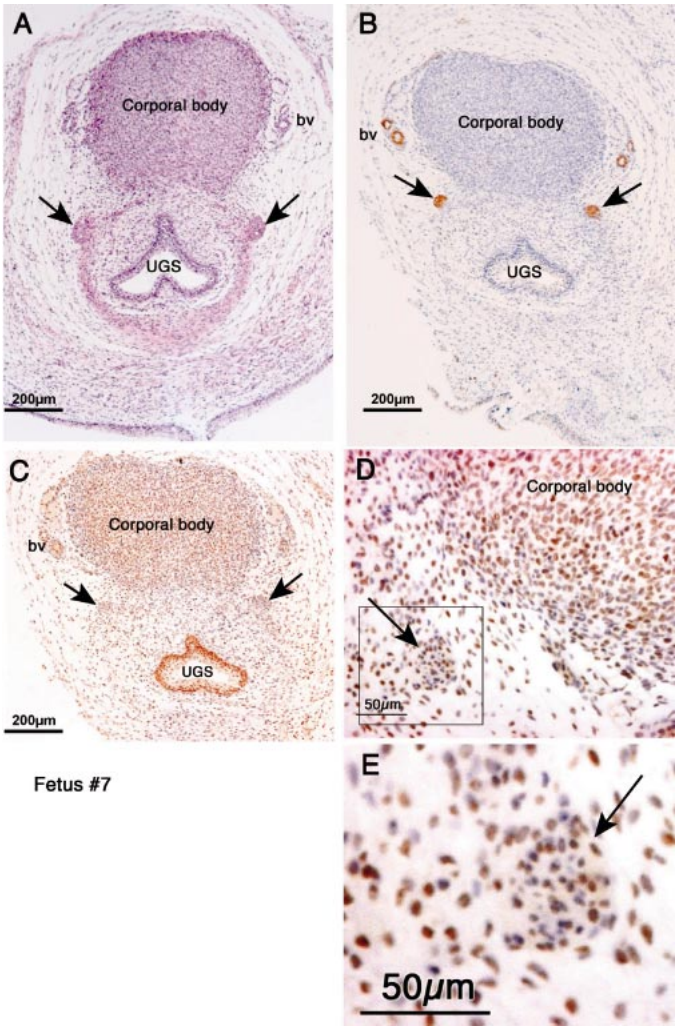

FIG. 5. Midshaft sections of the genital tubercle of a 45-day-old female spotted hyena fetus (specimen 7). Staining: (**A**) hematoxylin and eosin, (**B**) smooth muscle α-actin, (**C**–**E**) AR. Note blood vessels (bv) and premuscle masses of the retractor muscles (arrows), which are stained with the α-actin antibody (**A** and **B**). Androgen receptors (**C**–**E**) are detectable in the urogenital sinus (UGS), in the corporal body and in the premuscle masses of the retractor muscles (arrows).

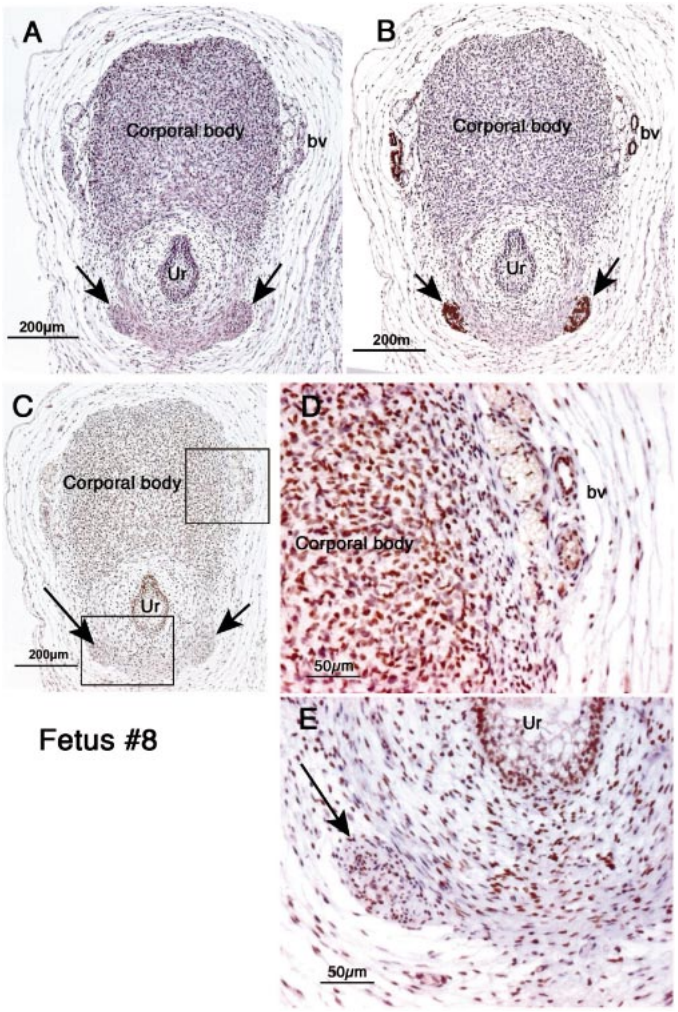

FIG. 6. Midshaft sections of the genital tubercle of a 45-day-old male spotted hyena fetus (specimen 8). Staining: (**A**) hematoxylin and eosin, (**B**) smooth muscle α-actin, (**C**–**E**) AR. Note blood vessels (bv) and premuscle masses of the retractor muscles (arrows), which are stained with the α-actin antibody (**A** and **B**). Androgen receptors (**C**–**E**) are detectable in the urogenital sinus (UGS), in the corporal body and in the premuscle masses of the retractor muscles (arrows).

resenting the retractor muscles (Fig. 5, A and B). In the developing clitoris of specimens aged 45–58 days (Fig. 5, C–E) nuclear AR staining was observed in epithelial cells of the urogenital sinus, in the corporal body, and in mesenchymal cells. Most important, AR-positive mesenchyme cells were observed in and around the clitoral retractor muscle condensates (Figs. 5, C–E).

Comparison of the developing penis with the developing clitoris in the group aged 45–58 days revealed sexual dimorphism of internal structures. A distinct corporal body was present dorsally as a dense condensation of mesenchymal cells in the developing penis (Fig. 6A). A canalized urogenital sinus/urethra was situated ventral to the penile corporal body (Fig. 6A). The position of the retractor muscle mesenchymal condensates was ventral to the urethra in males, and dorsal to the urogenital sinus in females (compare Figs. 5A and 6A). Penile retractor muscle mesenchymal condensates were positive for both smooth muscle α-actin (Fig. 6B) and AR (Fig. 6, C–E). AR staining was also observed in cells of the corporal body, mesenchymal cells of the penis, and epithelial cells of the urogenital sinus (Fig. 6, C–E). Androgen receptor staining intensity was comparable in male and female fetuses.

In the group aged 45–58 days the gonads had differentiated into histologically recognizable testes and ovaries (not illustrated) as reported earlier [11]. Wolffian and Mullerian ducts were both present (Fig. 7, A and B).

Category 3: Maturation of Sexually Dimorphic Morphology. In these older specimens (64 days to birth) the undifferentiated penile and clitoral retractor muscle mesenchymal condensates had differentiated into definitive skeletal muscle, preserving the sexual dimorphic positioning seen in the earlier group; that is, retractor muscles were dorsal to the urogenital sinus in the clitoris and ventral to the urethra in the penis (Figs. 8 and 9). In the developing clitoris of older specimens (64 days to birth) the corporal bodies were circumscribed by a tunica albuginea (Fig. 8A) that was not as thick or prominent as that in the developing penis (Fig. 9A). In addition, the tunica albuginea did not extend ventrally to surround the female urogenital sinus, which was large and pleated (Fig. 8, A–C). Well-differen-

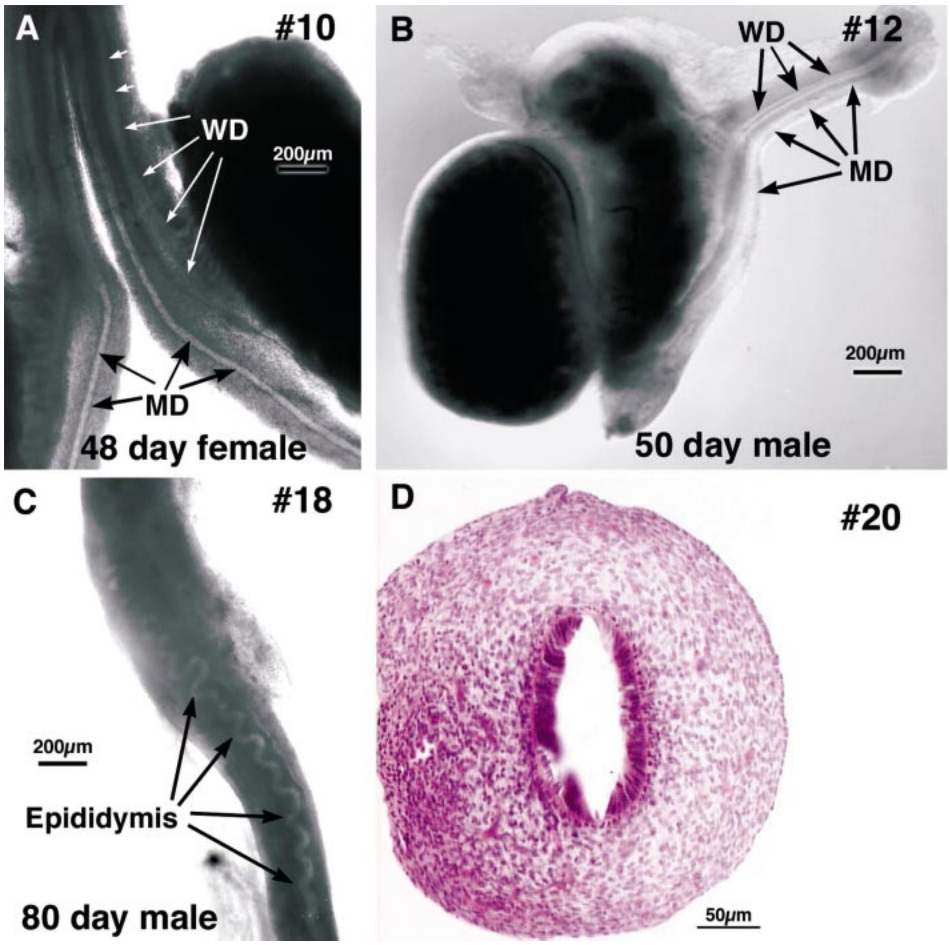

FIG. 7. Whole mount images of spotted hyena genital tracts. **A**) Forty-eight-day-old female specimen (#10, Table 1). Note presence of Mullerian ducts (MD) and Wolffian ducts (WD). **B**) Fifty-day-old male specimen (#12, Table 1). Note presence of MD and WD. **C**) Upper urogenital ridge (testis removed) of an 80-day-old male specimen (#18, Table 1). Note that the WD is beginning to coil to form the epididymis, and that the MD has completely regressed. **D**) Section through the uterus of a 95-day-old female specimen (#20, Table 1). Note the rudimentary uterus and that the WD has completely regressed.

tiated clitoral retractor muscles were small relative to those of the male (Fig. 8, A–C). Arteries and retractor muscles were smooth muscle α-actin-positive (Fig. 8, B and D). Immunostaining of the clitoris revealed faint nuclear AR staining in mesenchymal cells surrounding the urogenital sinus and in the corporal body (Fig. 8, C, E, and F). Mesenchymal cells in and around the retractor muscles were weakly stained for AR (Fig. 8E). It was difficult to discern whether muscle cell nuclei were also AR-positive.

In the developing penis of older specimens (64 days to birth) the corporal bodies were circumscribed by a particularly thick connective tissue capsule (tunica albuginea), which also extended ventrally to surround the urethra. The urethra was flattened dorsal-ventrally and had a slit-like lumen (Fig. 9A). Well-differentiated penile retractor muscles were ventral to the urethra and larger than those of the clitoris (Fig. 9, A and B). Arteries and retractor muscles were smooth muscle α-actin-positive (Fig. 9B). Immunostaining revealed nuclear AR in mesenchymal cells surrounding the urogenital sinus and in the corporal body (Fig. 9, C–F). AR-positive mesenchymal/fibroblastic cells were also observed in and around the retractor muscles (Fig. 9, C–E). It was difficult to discern whether the muscle cell nuclei were also AR-positive.

Sex differentiation of the Wolffian and Mullerian ducts was advanced. For example, in the upper urogenital ridge of an 80-day male fetus, the Mullerian duct had disappeared, and the Wolffian duct had begun to show coiling that is distinctive of the developing epididymis (Fig. 7C). Likewise, in near-term females the Wolffian duct had regressed, whereas the Mullerian ducts were retained and had formed the uterine horns (Fig. 7D).

Effects of Antiandrogen Treatment on Urogenital Tract Development

The rationale for this experiment is that exposure to antiandrogens throughout development should inhibit morphogenetic processes that are dependent on androgens. Accordingly, continuous administration of flutamide and finasteride from Days 12 or 20 of gestation until birth completely inhibited prostatic development in male fetuses (Fig. 10). Figure 10B is a lateral view of the bladder and urethra of a 95-day-old untreated male fetus (#21); the prostatic rudiment is seen as a dorsal elevation on the urethra. Figure 10C is a histological section of this elevation demonstrating the glandular nature of this structure. Figure 10A is a lateral view of a fixed urogenital tract of a male fetus (#29) treated with flutamide and finasteride, which have completely inhibited prostatic development based on the absence of the dorsal elevation and the absence of glandular tissue (not illustrated). The absence of the dorsal elevation in the antiandrogen-treated male specimen corresponds to the absence of this elevation in an untreated 95-day-old female specimen (#20; Fig. 10D). These findings demonstrate that the levels of flutamide and finasteride used in this study were sufficient to inhibit androgen-dependent prostatic development. Examination of the external genitalia revealed little difference in the overall gross size of the antiandrogen-treated male in comparison with untreated males and

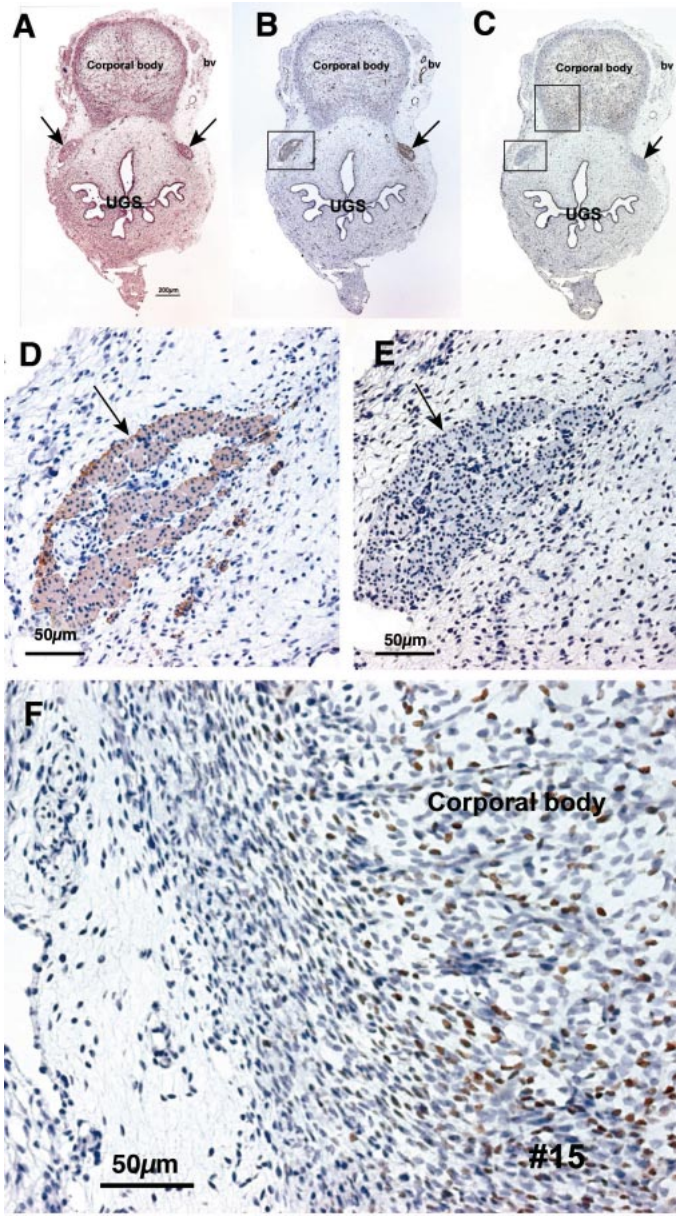

FIG. 8. Midshaft sections of the clitoris of a 65-day-old spotted hyena fetus (specimen 15). Staining: (**A**) hematoxylin and eosin, (**B** and **D**) smooth muscle actin, (**C**, **E**, and **F**) AR. Note blood vessels (bv) and the retractor muscles (arrows), which are stained with the α-actin antibody (**A**, **B**, and **D**). The retractor muscles (arrows) are dorsal to the highly pleated urogenital sinus (UGS). Androgen receptors are weak to undetectable in the UGS and retractor muscles (**C** and **E**, arrow), but clearly present in a subset of cells in the corporal body (**F**).

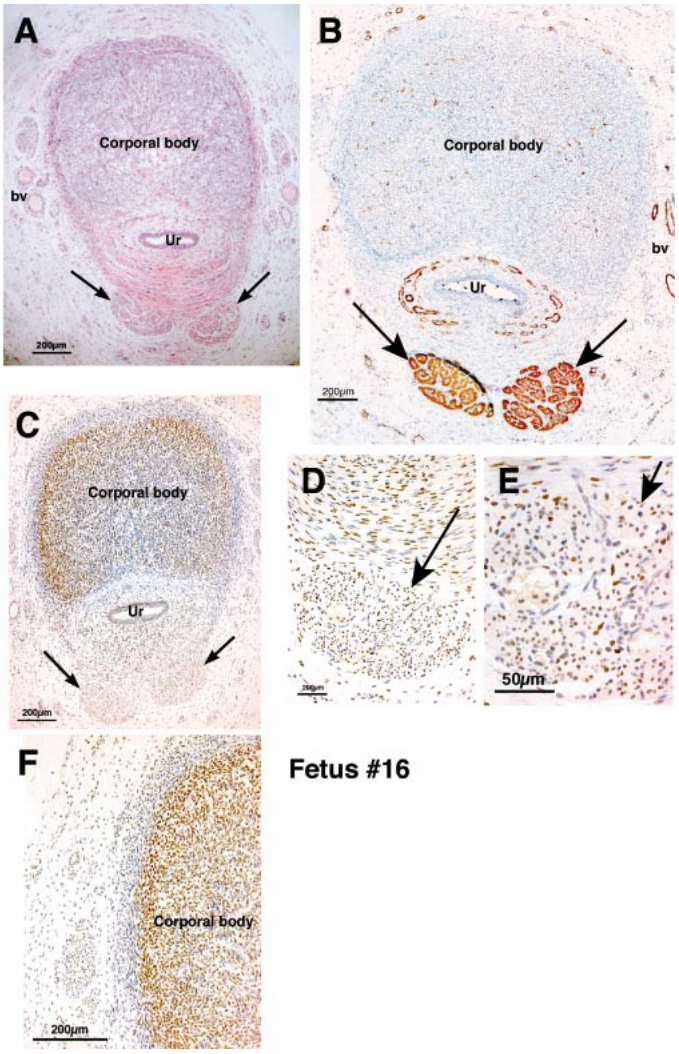

FIG. 9. Midshaft sections of the penis of a 65-day-old spotted hyena fetus (specimen 16). Staining: (**A**) hematoxylin and eosin, (**B**) smooth muscle actin, (**C**–**F**) AR. Note blood vessels (bv) and the retractor muscles (arrows), which are stained with the α-actin antibody (**A** and **B**). The retractor muscles (**A**–**D**, arrows) are ventral to the urethra (Ur). Androgen receptors are strongly expressed in retractor muscles (**C**–**E**, arrows) and in cells of the corporal body (**F**).

females (not illustrated). However, antiandrogen treatment feminized the internal structures of the penis. Normally, the retractor muscles are ventral to the urethra in males and dorsal to the urogenital sinus in females (Fig. 11, A and B). The urethral lumen is small in untreated males, whereas the lumen of urogenital sinus is large and pleated in females. A corpus spongiosum surrounds the urethra in males, but is absent in females (Fig. 11, A and B). The flutamide and finasteride-treated male resembles the female in that the retractor muscles were dorsal to the urogenital sinus, the lumen of urogenital sinus is large and pleated, and the corpus spongiosum is absent (Fig. 11C).

DISCUSSION

The domestic pig provides an interesting point of reference for studies of urogenital development in the spotted hyena. Relatively precocial piglets are born after 115 days; a bit longer than the 110-day gestation of the spotted hyena. Like hyenas, they are born with their eyes open, are fully ambulatory, teeth have erupted through the gums, and neonatal piglet siblings fight, as do spotted hyenas [20]. In the pig, gross masculinization of the genital tubercle occurs at 35–40 days of gestation, following histological differentiation of the testes [21], and initiation of testicular androgen secretion between 30 and 35 days of gestation [22]. Although the timing may vary, in all mammalian species studied to date, masculinization of the genital tubercle is dependent on morphological and functional differentiation of the testes (androgen secretion and local conversion to DHT) [8, 23, 24]. This is not the case in the spotted hyena. We had previously reported the gross appearance of a genital tubercle, with an enclosed urethral plate extending to the

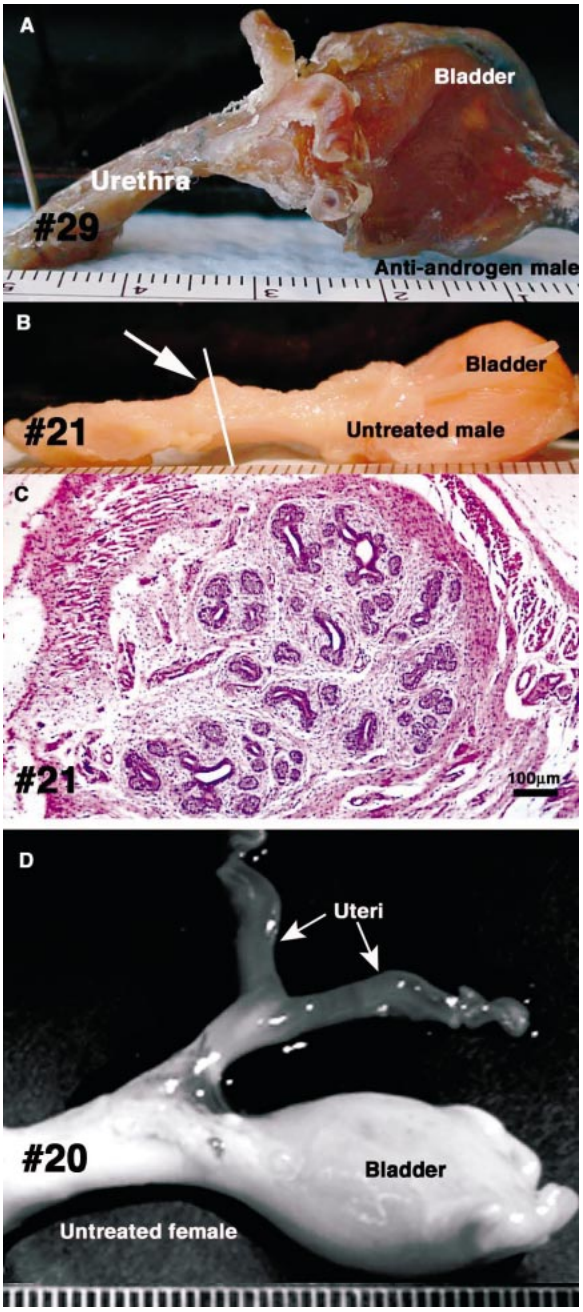

FIG. 10. Comparison of urogenital tracts from late gestation normal male (**B** and **C**), normal female (**D**), and antiandrogen-treated male (**A**) spotted hyenas. Specimen numbers are the same as those in Table 1. **A**) A fixed whole mount of the upper urogenital tract of a stillborn antiandrogen-treated male spotted hyena. Note bladder and urethra. The prostate is absent. **B**) A fresh whole mount of the upper urogenital tract of an untreated male spotted hyena harvested at 95 days of gestation. Note bladder and the elevation on the dorsal aspect of the urethra, which is the prostatic rudiment (**B**, white arrow). The white line indicates the level of the section in **C**. **C**) Section of the prostatic rudiment seen in **B**. **D**) The upper urogenital tract of a normal 95-day-old female spotted hyena fetus. Note the uteri, bladder, and the joining of the reproductive and urinary tracts. As expected, a prostatic rudiment is not present.

tip of the organ in a 33-day female fetus (see [11]; Fig. 2). The gonad of that specimen was undifferentiated, and thus this 33-day-old female specimen was sexually undifferentiated. In the present paper we report similar advanced development of the genital tubercle of 30- to 34-day male fetuses, which also had undifferentiated gonads. Thus, the typical mammalian sequence of testicular differentiation, followed by genital masculinization, is reversed in the spotted hyena because male and female genital tubercles are comparable in size and fully formed with an enclosed urethral plate even though the gonads are histologically undifferentiated.

In our previous studies we hypothesized that placental androgens might have accounted for the masculinization of the external genitalia in both male and female spotted hyenas. To test this hypothesis, pregnant female spotted hyenas were treated with a mixture of flutamide and finasteride. By simultaneously blocking AR activity with flutamide, and conversion of testosterone to DHT with finasteride, we hoped to maximize the possibility of producing female spotted hyenas with a clitoris of modest size, no longer traversed by a central urogenital canal. That did not occur. Both male and female hyenas displayed a prominent phallus (gross masculinization; or phallic size was not affected) following antiandrogen treatment. Either our treatments were inadequate, or gross masculinization of the genitalia of the female spotted hyena occurred through a hitherto unknown, nonandrogenic mechanism. Data presented in this paper and discussed in the next section suggest that our antiandrogen treatment was effective.

Androgen-Induced Sexual Differentiation of the Penis and Clitoris

Although there are no sex differences in either the internal or external morphology of male and female genital tubercles at 30–34 days of gestation, major differences in internal phallic structure were identifiable at 45–50 days of gestation when the gonads are morphologically differentiated. These include the location of the progenitors of the retractor muscles (i.e., ventral to the urethra in males and dorsal to the urogenital sinus in females). Also, at this early stage, a corpus spongiosum surrounds the urethra in the male, but is absent in the female. As gestation proceeds, the lumen of the urogenital sinus becomes large and pleated in females, but remains small in males, while the tip of the corporal body becomes tapered in males and blunt in females. A thick, fibrous connective tissue tunica completely envelops the corporal body and urethra of the male and prevents expansion of the urethra. However, in the female, the tunica envelops only the corporal body, thus permitting expansion of the urogenital sinus during mating and parturition. These functionally significant sex differences, noted previously in adult spotted hyenas [17], are present at birth [5].

The appearance of sexually dimorphic features distinguishing the internal morphology of the penis and clitoris coincided with the histological differentiation of the testes and the presumptive production of testosterone. Androgen receptors were undetectable in the genital tubercle at 30 days of gestation, but were expressed in male and female genital tubercles at 45 days and thereafter. Thus, the basis of androgenic response was in place during the period when the retractor muscles, erectile tissue, and shape of the urogenital sinus/urethra were becoming sexually dimorphic. The most compelling evidence for androgenic activity as the basis for sexual dimorphism within the phallus (internal phallic masculinization) in the developing male genital tubercle was the finding that antiandrogen treatment feminized the penis by 1) repositioning of the retractor muscles dorsal to the urogenital sinus, 2) enlargement and pleating of the urogenital sinus, 3) inhibition of development of the

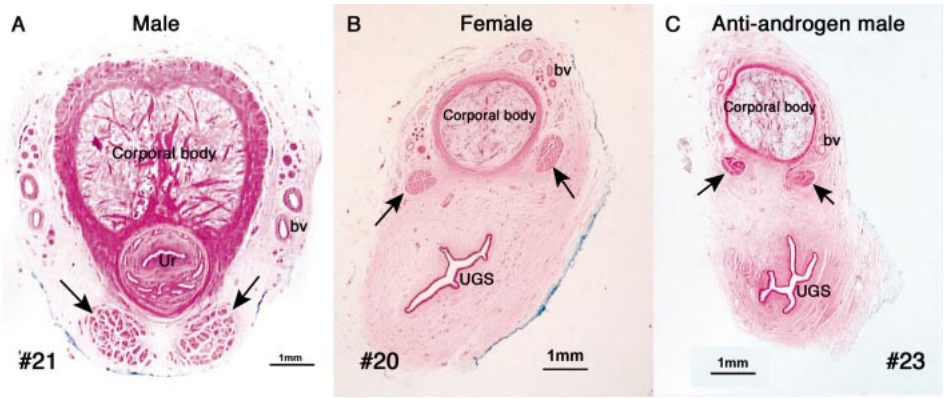

FIG. 11. Midshaft sections though the phallus of (**A**) normal male, (**B**) normal female, and (**C**) and antiandrogen-treated male spotted hyena. Specimens 20 and 21 (**A** and **B**), 95 days old. **C**) Specimen 23, 105 days old. Note that the position of the retractor muscles and the size and shape of the urogenital sinus (UGS) of the antiandrogen-treated male resemble that of the normal female. Thus, the normal patterning of the male phallus (**A**) is dramatically feminized by antiandrogen-treatment (**C**).

corpus spongiosum and associated tunica around the urethra, and 4) inhibition of prostatic development in antiandrogen-treated males.

This suite of morphological changes fit with an array of prior observations. In neonatal hyenas, antiandrogen treatment attenuated/eliminated male-biased sex differences (internal phallic masculinization) in the width of the bulbocavernosus muscle and the number of motor neurons in the Onuf nucleus in the spinal cord [15]. When followed from infancy through adult life, it became apparent that in utero flutamide and finasteride treatment produced male spotted hyenas with a short, thick penis having a large, elastic urogenital meatus, very similar to that of a normal female spotted hyena. The glans of these antiandrogen males assumed a round, feminine appearance, rather than the tapered shape typical of male hyenas [14, 16].

In the spotted hyena, both male and female fetuses receive testosterone from the placenta via the umbilical vein during all stages of gestation that we have examined [11–13]. However, such placental androgens are, evidently, inadequate to support full internal phallic masculinization to stabilize the Wolffian ducts, and to induce prostatic development in female fetuses. Given the timing of events described in this paper, it seems likely that secretion of testosterone by the fetal testes (but not the fetal ovary or placenta) provides the impetus for internal phallic masculinization. In our earlier studies, we emphasized the similarity in circulating testosterone concentrations in late-term male and female fetuses [12], although recognizing that testicular secretions could result in higher plasma testosterone levels in males [11]. Gonads, adrenal glands, and placental tissues obtained from subjects in the present study are currently being analyzed for steroidogenic enzymatic activity. If the preceding line of speculation is correct, we would anticipate an earlier and more robust presence of enzymes involved in the production of testosterone (e.g., 3β-hydroxysteroid dehydrogenase) in the fetal testes than fetal ovaries.

Mechanisms of Androgenic Patterning of the Retractor Muscles

Our results demonstrate that androgens pattern the retractor muscles, and presumably do so by interacting with AR present in cells of the retractor muscle precursors. In this regard, mesenchymal and possibly also muscle cells, were AR-positive, which raises the question of which cells are actually responsible for sexually dimorphic patterning of the retractor muscles. Patterning of limb muscles in avian embryos may provide an answer to this question. Cells of limb musculature arise in the embryo from two sources. The somites contribute cells destined to differentiate into multinucleated skeletal muscle cells. Cells from the lateral plate mesoderm form connective tissue that binds muscle cells into functional groups and that also give rise to the tendons that connect muscle to bone. Ablation studies on each of these two precursor populations and so-called chick-quail marking studies have demonstrated that the cells of the lateral plate form the connective tissue framework of individual muscles and determine the overall size, shape, and pattern of individual muscles [25, 26]. Limb muscles are not sexually dimorphic, as is the case for the penile and clitoral retractor muscles in the spotted hyena. Careful examination of the developing retractor muscles reveals AR within the mesenchymal muscle primordia. Although some of the AR-positive cells may be the muscle cells themselves, clearly, many of the AR-positive cells are mesenchymal/fibroblastic cells located between and around developing skeletal muscle cells. Our observations in conjunction with the embryological studies on avian embryos suggest that sexually dimorphic muscle patterning within the external genitalia is elicited by androgen action on the nonmuscle mesenchymal cells in and around the developing muscle cells, which direct the dorsal-ventral sexually dimorphic patterning of the retractor muscles. Of particular note is that premuscle mesenchymal condensates are absent at 30 to 34 days when the gonads are undifferentiated, and that at 45 days of gestation sexually dimorphic patterning is already evident, even though muscle rudiments are simply represented as undifferentiated mesenchymal condensates. The antiandrogen studies demonstrate that androgens are responsible for muscle patterning, which may be mediated via AR within mesenchymal cells of the genital tubercle.

If Not Androgens, What?

The linkage between androgens and masculinization of mammalian external genitalia is so firmly established that alternative hypotheses have limited precedent. Two alternative hypothetical mechanisms are available: those involving nonandrogenic hormonal mediators, and those based on nonhormonal mechanisms. It is conceivable that growth factors (e.g., insulin-like growth factor 1; IGF1) could act on genital tissues without requiring steroid provocation. Very limited support for such a hypothesis can be found in the coincidence of skeletal growth and IGF1 concentrations in the spotted hyena as a function of age and sex. Female spotted hyenas diverge from males in terms of body size at a period well before puberty (there is no pubertal growth spurt) at a time when IGF1 concentrations peak and are sexually dimorphic (Martin Spencer, personal communication). More directly relevant to the issue of genital differ-

entiation are preliminary studies of interference with estrogen production during fetal life. Place and Glickman [27] report a case in which a male hyena developed hypospadias when treated for the last 30 days of gestation with letrazole, an aromatase inhibitor that inhibits estrogen synthesis. A similar but more pronounced hypospadias was observed in a female hyena treated with letrazole during Days 50–100 of gestation [27]. Obviously, we need to examine the role of estrogens during the earlier stages of gestation, when the genital tubercle is grossly masculinized, to determine whether estrogens are required for formation of a penile clitoris.

Finally, it is possible that gross masculinization of the external genitalia of the female spotted hyena is genetically programmed independent of hormones. In the wallaby, sexually dimorphic characteristics of the external genitalia, such as the formation of a pouch or a scrotum from their common precursor, are the direct result of an XX genotype, as contrasted with an XY genotype, without hormonal intervention [28]. Direct genetic determination of development of the external genital was originally proposed as a metatherian-eutherian dichotomy. However, the present study suggests that such gonadal independence may not be restricted to marsupials. Arnold and his collaborators have summarized a set of cases in which such direct genetic effects can account for sex differences in brain and behavior [29, 30].

Examination of the ontogeny of the genital tubercle, AR, genital tract differentiation, and the effects of antiandrogens on genital tract development has led to the following conclusions:

1. A substantial penis and a robust penile clitoris, with a central urogenital canal that emerges at the tip of the glans, survives antiandrogen treatment in utero. Such gross masculinization of the external genitalia is, therefore, androgen-independent.
2. Significant sexual dimorphism develops in the fine details of internal phallic masculinization, including retractor muscle position, the status of the tunica albuginea, size and shape of the urogenital sinus, and development of the prostate. These are androgen-dependent events. Data presented herein suggest that it is the maturation of the fetal testes, and the actions of testicular secretions on AR in target tissues at critical stages of fetal life that account for the essentially masculine internal phallic phenotype, prostatic development, and maintenance of the Wolffian duct system.
3. Although in gross terms a remarkably masculine clitoris develops in antiandrogen-treated female spotted hyenas, nonetheless, such treatment has effects on clitoral morphology, producing a suite of changes involving further feminization. The clitoris is normally shorter and thicker than the penis and has a larger and more elastic opening at the tip of the glans. All such changes are exaggerated in antiandrogen-treated females (i.e., the clitoris is shorter yet substantially thicker, and has a much larger and more elastic urogenital meatus than that found in normal female spotted hyenas [14, 16]).

That antiandrogens affect female urogenital morphology raises the question: What is the source of the androgenic activity that is blocked by antiandrogen treatment of female fetuses in utero? There is ample evidence for placental production of testosterone and its transfer to female and male fetuses via the umbilical vein [12, 13]. Despite the failure of placental testosterone to support the development of a male phenotype with respect to the internal structure of the clitoris, to maintain the Wolffian duct system or to induce prostatic development, such placental androgens may well modulate the development of clitoral morphology.

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