Differentiation 111 (2020) 98–114

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Review article

Androgen-independent events in penile development in humans and animals

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ARTICLE INFO

Keywords:
Penis
Clitoris
Genital tubercle
Androgen-independent
Androgen-dependent
Androgen receptor

ABSTRACT

The common view on penile development is that it is androgen-dependent, based first and foremost on the fact that the genital tubercle forms a penis in males and a clitoris in females. However, critical examination of the complex processes involved in human penile development reveals that many individual steps in development of the genital tubercle are common to both males and females, and thus can be interpreted as androgen-independent. For certain developmental events this conclusion is bolstered by observations in androgen-insensitive patients and androgen receptor mutant mice. Events in genital tubercle development that are common to human males and females include: formation of (a) the genital tubercle, (b) the urethral plate, (c) the urethral groove, (d) the glans, (e) the prepuce and (f) the corporal body. For humans 6 of 13 individual developmental steps in penile development were interpreted as androgen-independent. For mice 5 of 11 individual developmental steps were found to be androgen-independent, which were verified through analysis of androgen-insensitive mutants. Observations from development of external genitalia of other species (moles and spotted hyena) provide further examples of androgen-independent events in penile development. These observations support the counter-intuitive idea that penile development involves both androgen-independent and androgen-dependent processes.

1. Introduction

Human male external genitalia consist of the penis and scrotum, while human female external genitalia consist of the clitoris and vaginal introitus, which are surrounded by the labia minora and in turn by the labia majora (Clemente, 1985). Masculine differentiation of external genitalia is known to be androgen-dependent, whereas development of female external genitalia occurs in the absence of androgens or the absence of androgen action. The essential role of androgens in masculine development of the external genitalia is based upon several observations: (a) Females, having minimal levels of androgens, undergo development of female external genitalia. (b) Human and animal females exposed to exogenous or endogenous androgens during development exhibit varying degrees of masculinization of external genitalia, presumably dependent upon dosage and timing of androgen exposure (Grumbach and Ducharme, 1960; Jost, 1953; Tarttelin, 1986). Female patients with congenital adrenal hyperplasia, an autosomal recessive disorder characterized by impaired cortisol synthesis, produce androgens in utero and undergo varying degrees of masculinization of the external genitalia, which in the most severe cases can result in development of normal penile morphology (Speiser et al., 2010). (c) Human and animal males with genetic defects in and/or absence of the androgen receptor (AR) exhibit female-like external genitalia (Rodriguez et al., 2012; Wilson et al., 1995). (d) Another important aspect of androgen action involves 5α-reductase, an enzyme within the developing external genitalia that converts testosterone into dihydrotesterone (DHT), a more potent androgen. DHT is required for normal masculinization of external genitalia, and patients with genetic defects in 5α-reductase type 2 exhibit ambiguous external genitalia that resemble the female phenotype (Imperato-McGinley, 1984; Imperato-McGinley et al., 1974).

While the role of androgens in masculinization of the external genitalia is indisputable, penile development is a complicated multi-step process. The under-appreciated fact is that certain individual steps in penile penke development are androgen-independent both in humans and animals. The goal of this report is to emphasize these under-appreciated androgen-independent steps in masculinization of the external genitalia. To understand this fact requires detailed knowledge of the process of development of the male and female external genitalia in both humans and in animal models. Comparison of external genitalia

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https://doi.org/10.1016/j.diff.2019.07.005
Received 2 June 2019; Received in revised form 11 July 2019; Accepted 12 July 2019
Available online 06 September 2019

development in animals and humans leads inevitably to the conclusion that mice are not humans, and thus the relevance of animal studies to development of human external genitalia must be viewed with some skepticism (Cunha et al., 2019d).

For the purpose of this report we will restrict our consideration to development of the penis, clitoris and prepuce, and not to the scrotum or labia. In this review, developmental events occurring in males but not females are considered to be androgen-dependent, while developmental events that occur in both normal males and females are considered to be androgen-independent. This is a reasonably safe assumption as androgen levels in embryonic/fetal females of most species are extremely low and clearly not sufficient to elicit masculine development of either external or internal genitalia. The caveat is that information on actual androgen levels are unknown for many aspects of development of external genitalia in both animals and humans. This report represents a trove of data from several earlier studies as well as novel observations that highlight those developmental events in embryonic/fetal external genitalia that are androgen-dependent or androgen-independent.

2. Materials and methods

Most of the data reported in this paper are derived from previous studies augmented in some cases with new data. For the purpose of this paper we refer the reader to our previous studies whose data are the basis of this report.

3. Results

3.1. Development of human external genitalia

The human penis and clitoris develop from the genital tubercle, which is an embryonic projection within the perineum, described in varying degrees of detail in many species including human (Arey, 1965; Baskin et al., 2018; Cunha et al., 2014; Dos Santos et al., 2018; Gray and Skandalakis, 1972; Hynes and Fraher, 2004b; Kluth et al., 2011). For virtually all animal species, the genital tubercle gives rise to the penis and many species to the clitoris. In mice, clitoral development is only indirectly related to the genital tubercle as reported in a companion paper (Cunha et al., 2019a).

The first event in development of the human penis and clitoris is formation of the genital tubercle whose development (Perriton et al., 2002; Petiot et al., 2005; Seifert et al., 2008) begins before synthesis of testosterone by the fetal testes (Fig. 1), as is the case for several animal species. The genital tubercle initially develops identically in males and females of both humans and laboratory animals. For these reasons, development of the genital tubercle is considered an androgen-independent event. Whether androgen receptors are even present at the earliest stages of genital tubercle development remains to be determined. In humans, the genital tubercle can be first recognized as an elevation in the perineum at 5–6 weeks of development (Arey, 1965; Gray and Skandalakis, 1972; Grumbach and Ducharme, 1960; Sajjad, 2010), while production of androgens by the fetal testes begins at 8–10 weeks of gestation (Fig. 1) when fetal testicular androgen production is only slightly above background (Siiteri and Wilson, 1974). Testosterone production by the human fetal testes increases substantially thereafter. Our studies on development of human external genitalia have focused upon the ages of 6.5–16 weeks. Prior to 10 weeks the genital tubercle of human males and females is identical in size and morphology (Fig. 2). In mice development of the genital tubercle begins on gestation day (GD) 11.75 (Perriton et al., 2002), about 2 days before production of testosterone by the fetal testes, which begins on day GD 13 of gestation (Price and Ortiz, 1965).

By 6.5 weeks (perhaps earlier) both human male and female genital tubercles contain a solid urethral plate (Figs. 3B and 4). The urethral plate in human males extends distally almost to the tip of the genital tubercle (Figs. 3 and 4). Formation of the urethral plate is clearly androgen-independent as it occurs in both males and females (Baskin et al., 2018; Li et al., 2015; Overland et al., 2016), and its development begins prior to the onset of fetal testicular testosterone production at 8–10 weeks of gestation (Siiteri and Wilson, 1974; Tapanainen et al., 1981). Initial appearance of the urethral plate is poorly understood in humans, but animal studies describe its development as an extension of endodermal cloacal epithelium (Hynes and Fraher, 2004a, b; Kluth et al., 2011; Seifert et al., 2008). At about 9–10 weeks, the human urethral plate begins to canalize in both sexes to eventually form a wide diamond-shaped urethral/vestibular groove on the ventral surface of both human male and female genital tubercles (Figs. 4–6). The female urethral plate is more properly called the vestibular plate as it is involved in development of the vaginal vestibule (Overland et al., 2016). Since canalization of the urethral/vestibular plates occurs in both sexes over an identical time frame, this process is considered to be androgen-independent even though testosterone production is elevated during canalization of the male urethral plate.

In males, the lateral edges of the urethral groove (urethral folds) subsequently fuse in the ventral midline, thus converting the urethral groove into a tubular urethra within the penile shaft (Figs. 4–6). This process occurs when testosterone production by the fetal testes is substantially elevated (Siiteri and Wilson, 1974; Tapanainen et al., 1981). In females, the vestibular folds (homologues of the urethral folds) do not fuse in the midline and instead remain separate as the labia minora defining the vaginal introitus (Fig. 6). Since urethral folds only fuse in males (but not females), urethral fold fusion and thus formation of the penile urethra is an androgen-dependent event in humans. Androgen receptors (AR) are expressed in both the epithelium and mesenchyme of the urethral and vestibular folds (Fig. 7) (Baskin et al., 2019), and androgen production is substantial during urethral fold fusion in males (Siiteri and Wilson, 1974; Tapanainen et al., 1981). The presence of AR in cells of the vestibular folds of females (Baskin et al., 2019) is presumably the basis of penile development in female patients with congenital adrenal hyperplasia (Speiser et al., 2010).

From about 12 weeks onward the angle of the male genital tubercle (developing penis) to the body wall approaches 90°, while in contrast the orientation of developing clitoris remains closer to the body wall (Baskin et al., 2018). The molecular basis for the ensuing size differential between the penis and clitoris is not known, but most certainly is androgen regulated, an idea supported by the expression of androgen receptors during human fetal penile growth (Baskin et al., 2019). As the developing penis elongates, the scrotum forms caudal to the penis. In contrast, the developing clitoris becomes surrounded by the vestibular folds, which become the labia minora. Given the more pronounced angle of the genital tubercle to the body wall in males compared to females (Baskin et al., 2018) as well as the considerable final size differential between the developing penis versus clitoris, we infer that these developmental events are androgen-dependent and mediated via AR expressed throughout the genital tubercle (Fig. 8A). The presence of AR within female genital tubercle (Fig. 8B) accounts for the ability of the female genital tubercle to undergo penile development in response to androgens.

The human clitoris, like the penis, develops from the genital tubercle and has an anatomy homologous to that of the penis, as both organs contain a glans, body (shaft) and corpora cavernosa (Clemente, 1985) (Table 1) (Figs. 8 and 9). One feature shared by the adult human penis and clitoris is the presence of a corporal body (Fig. 8) formed via fusion of the crura, which in both sexes are attached proximally to the inferior pubic rami (Clemente, 1985). A corporal body rudiment (condensed mesenchyme) is present in the developing penis and clitoris during the ambisexual stage (8–9 weeks) (Fig. 9) before morphological evidence of androgen action and before testosterone production by the testes (Siiteri and Wilson, 1974; Tapanainen et al., 1981). This suggests that initial formation of corporal bodies in males and females is androgen-independent, despite the fact that AR are expressed in male and

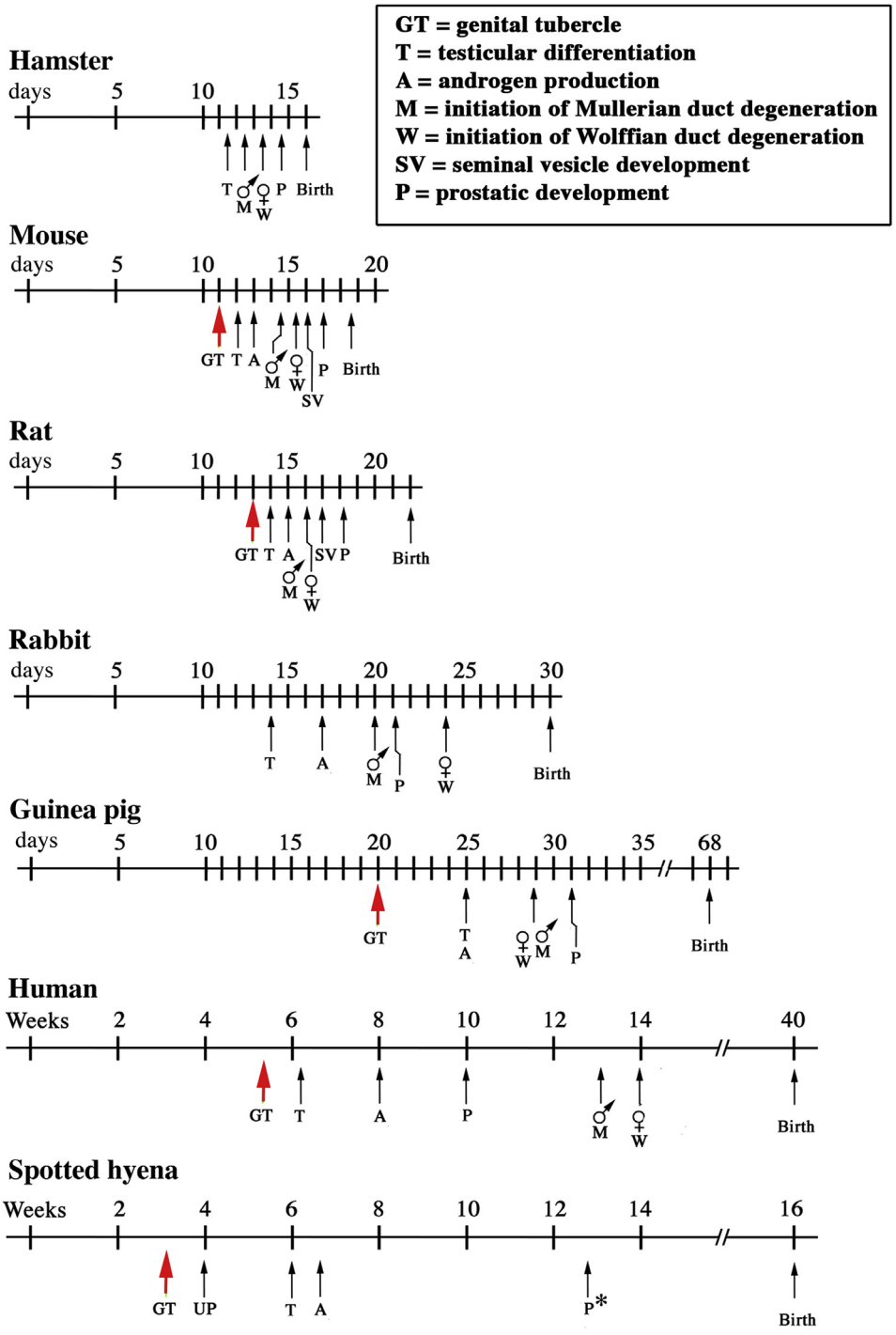

Fig. 1. Comparative sequence of sex differentiation in days for laboratory animals and weeks for human and spotted hyena, modified from (Price and Ortiz, 1965) with addition of data on the spotted hyena and updating data on human and Guinea pig. Abbreviations: GT = genital tubercle (large red arrows), T = testicular differentiation, A = androgen production by the testes, ♀W = initiation of female Wolffian duct degeneration, ♂M = initiation of male Mullerian duct degeneration, SV = appearance of the seminal vesicle, P = appearance of prostatic buds. *In spotted hyena prostatic ducts were observed at ~13 weeks but had developed earlier. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

female corporal bodies during the ambisexual stage (Fig. 8). During subsequent development when testicular androgens are produced in males (but not in females), absolute size of the penis (and the enclosed corporal bodies) becomes far greater than that in the clitoris (Fig. 2). Taken together, in humans development of (a) the genital tubercle, (b) the urethral plate (c) the glans and (d) the corporal bodies occur via androgen-independent mechanisms in both males and females, while subsequent growth of the corporal bodies in males is androgen-dependent. Canalization of the urethral/vestibular plate is also an androgen-independent event occurring in both males and females. Finally, the fact that a glans forms in both the penis and clitoris (Figs. 8 and 9) implies that the formation of the glans is also an androgen-independent event, with subsequent androgen-dependent growth of the glans in males accounting in part for the vast final postnatal penile/clitoral size differential.

The morphogenetic mechanism of human penile urethral development is different in the shaft versus the glans. As indicated above, within the penile shaft the urethra forms via fusion of the urethral folds (Baskin et al., 2018; Li et al., 2015; Shen et al., 2016) (Figs. 4–6). In contrast, within the human glans the urethra forms by direct canalization of the urethral plate without urethral fold fusion (Fig. 10), a process unique to males and therefore presumably androgen-dependent (Liu et al., 2018a).

In Fig. 10C and G note the epithelial connection between the urethra and the epidermis which is removed via a remodeling process in Fig. 10D and H to establish mesenchymal confluence across the ventral

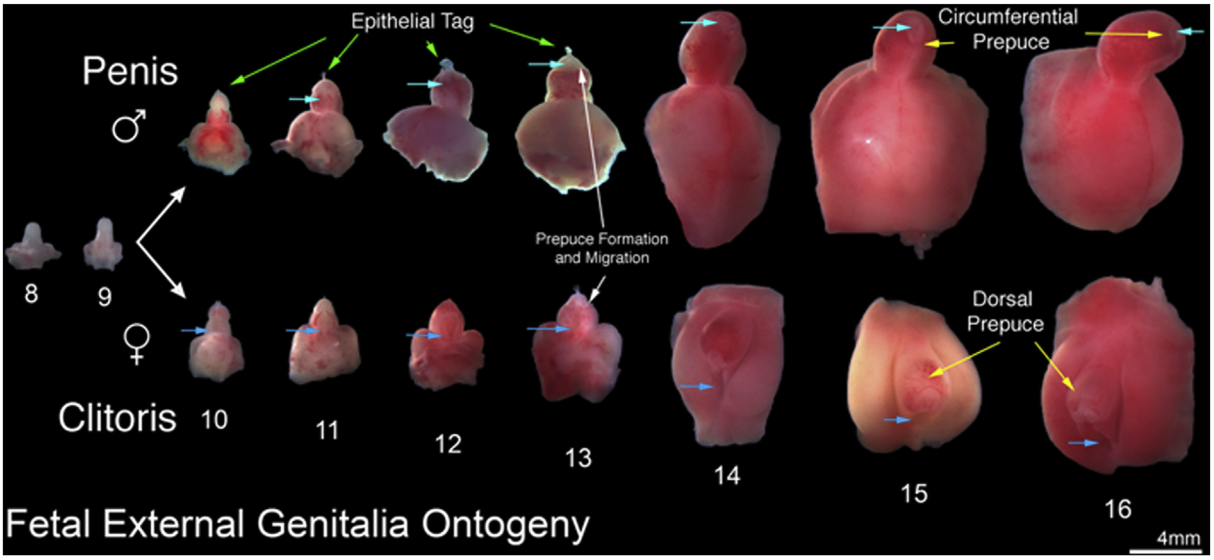

Fig. 2. Representative ventral views of external genitalia of human males (top row) and females (bottom row) from 8 to 16 weeks of gestation. Note the morphologic differences between male and female specimens after the indifferent stage (8–9 weeks of gestation). The penile urethra forms within the shaft due to urethral fold fusion. Urethral (vestibular) fold fusion does not occur in females (light blue arrows depict the location of the urethral meatus in both the male and female specimens). Note the divergent evolution of the male and female prepuce (yellow arrows). Complete circumferential formation of the prepuce occurs by 14–16 weeks of gestation in males. In contrast, in females the prepuce of the clitoris only forms dorsal to the glans clitoris (Clemente, 1985). The epithelial tag is seen in both male (green arrows) and female (clearly visible without arrows) specimens from 10 to 13 weeks of gestation, disappearing after this time point. Reproduced with permission (Shen et al., 2018a). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

midline and thus establish a "stand alone" urethra. In humans this process only occurs in males. The cells undergoing mesenchymal confluence express AR (Liu et al., 2018b). Thus, we interpret this process to be androgen-dependent. Comparable remodeling and mesenchymal confluence also occur in mice, but in a pattern exactly opposite to that in humans (compare Figs. 10 and 13). In mice direct canalization to form a tubular urethra occurs in the proximal aspect of the developing penis (Liu et al., 2018a; Seifert et al., 2008), whereas in humans direct canalization to form a tubular urethra occurs within the glans (Liu et al., 2018b). Likewise, an epithelial fusion mechanism (urethral fold fusion) occurs within the penile shaft in humans (Li et al., 2015) and distally near the meatus in mice (Liu et al., 2018a). In both cases an epithelial seam must be removed to establish midline mesenchymal confluence. Within proximal regions of the developing mouse penis in zones of direct canalization of the urethral plate, the mesenchymal cells involved in midline mesenchymal confluence are AR positive (Liu et al., 2018a), consistent with the idea that this event in mice is also androgen-mediated. Seifert et al. assert that mesenchymal confluence (called septation by these authors) does not occur within the female mouse genital tubercle (Seifert et al., 2008). This point is under-represented in the literature and requires further investigation. Thus, midline mesenchymal confluence appears to be androgen-dependent in both mice and humans.

In summary, penile development in humans is androgen-dependent in a global sense, and clitoral development occurs in the absence of androgens (or impaired androgen action). However, many individual steps in development of the human penis are androgen-independent such as formation of (a) the genital tubercle, (b) the urethral plate, (c) canalization of the urethral plate, (d) initial formation of corporal bodies, (e) formation of the glans rudiment and (f) formation of a

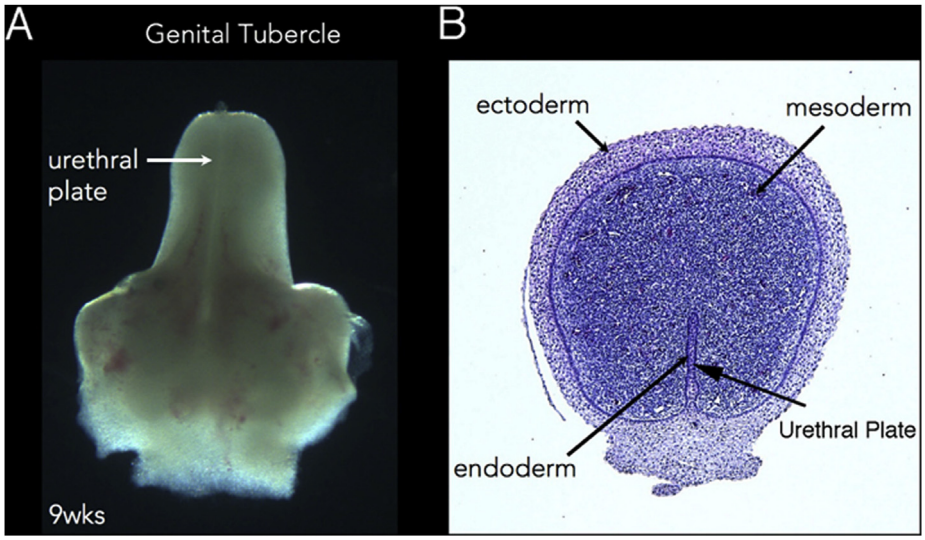

Fig. 3. Human male genital tubercle/future human penis at 9 weeks of gestation. Note the urethral plate in the gross specimen (A). In the corresponding histologic transverse section (B), the three embryonic layers involved in external genitalia development are labeled. Note the urethral plate attached to ventral skin.

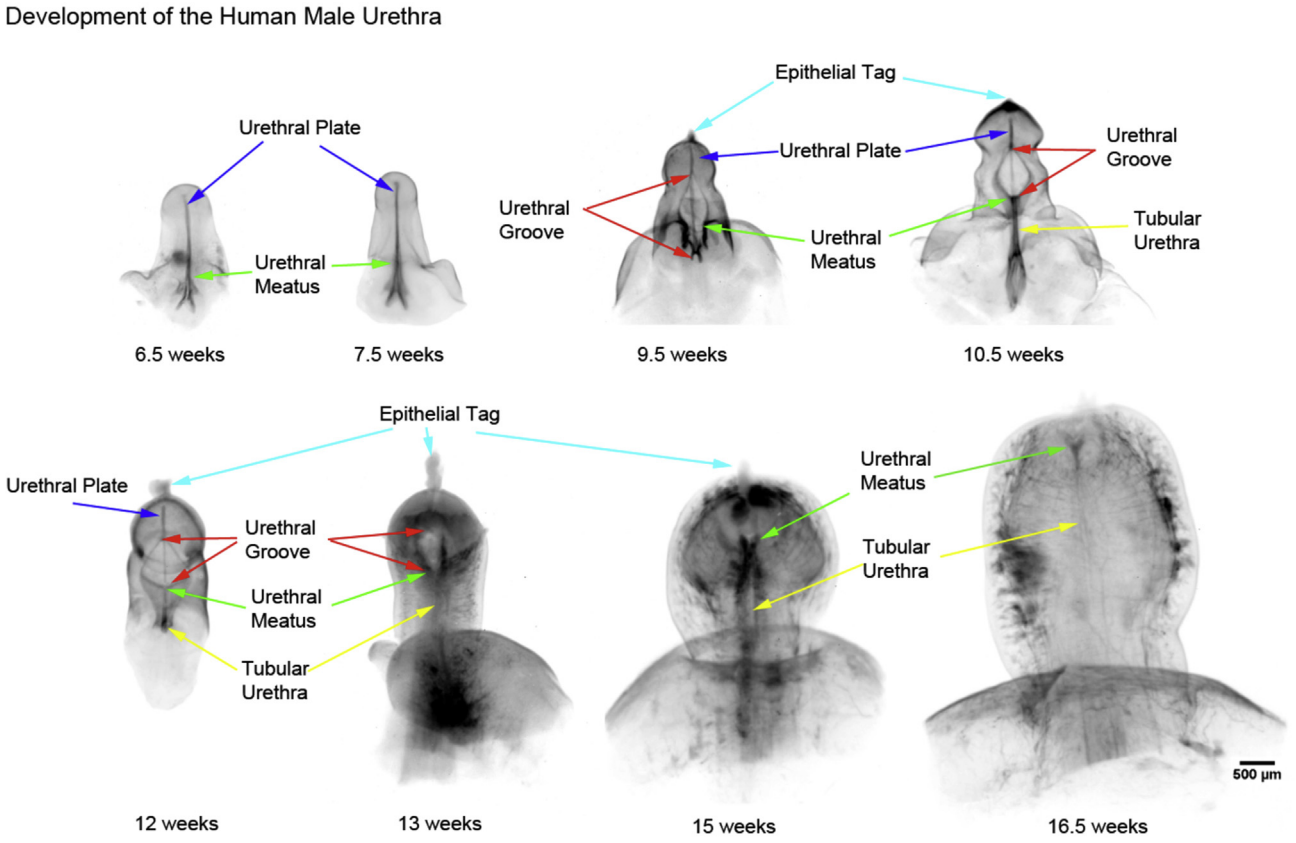

Fig. 4. Optical projection tomography of human penile development from 6.5 to 16.5 weeks of gestation. Note progression of the urethral meatus (green arrows) from the scrotal folds at 6.5 weeks to a terminal position on the glans at 16.5 weeks. The open urethral groove (red arrows) is best seen from 9.5 to 13 weeks with clear progression of proximal to distal fusion of the edges of urethral groove (urethral folds) to form the tubular urethra (yellow arrows). At 13 weeks the urethral plate remains uncanalized within the glans penis with the tubular urethra completely formed within the shaft of the penis. Adapted with permission (Li et al., 2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

circumferential prepuce (Cunha et al., 2019b).

3.2. Development of mouse external genitalia

Development of external genitalia in mice differs substantially from that in humans, even though the initial developmental steps are similar. Vast mouse/human anatomical differences in adult external genitalia are indicative of vast differences in developmental processes, as the adult form represents the culmination of developmental processes. However, on a fundamental level both mouse and human penile development are androgen-dependent globally, an idea demonstrated by the fact that inactivating AR mutations in humans and mice elicit development of female-like external genitalia (Rodriguez et al., 2012; Weiss et al., 2012; Wilson, 1992). Moreover, exposure of female fetuses to androgens elicits masculinization of the external genitalia (Grumbach and Ducharme, 1960). Nonetheless, androgen-independent developmental events can be recognized in mouse penile development. For example, as in humans, formation of the mouse genital tubercle is initiated prior to androgen production by the fetal testes and is initially identical in size and morphology in males and females (Fig. 1 and 11), thus demonstrating that development of the mouse genital tubercle is androgen-independent. Indeed, initial development of male and female external genitalia is remarkably similar during embryonic periods from both gross (Fig. 11) and histologic perspectives. Formation of the urethral plate is clearly an androgen-independent developmental event as it occurs in male (Hynes and Fraher, 2004a; Seifert et al., 2008) and female mouse genital tubercles (Fig. 12).

The first androgen-dependent step in mouse penile development is determination of penile identity, an event that occurs prenatally (Rodriguez et al., 2012) when male and female genital tubercles are grossly similar in size. This conclusion is based upon two observations: (a) Androgen-insensitive X^{Tfm}/Y male mice develop external genitalia characterized as female (Rodriguez et al., 2012). (b) Castration of male mice at birth impairs certain aspects of penile development, even though the resultant external genitalia exhibit unmistakable penile features (Rodriguez et al., 2012). Thus, penile developmental identity is established prenatally via androgen action. The exact timing of this event in mice is not known.

As in humans, the morphogenetic mechanisms of mouse penile urethral development differ in proximal versus distal regions. In proximal regions, the mouse penile urethra forms via direct canalization of the urethral plate (Liu et al., 2018a; Seifert et al., 2008), whereas distally the penile urethra forms via epithelial fusion events (Fig. 13) (Liu et al., 2018a), a process occurring in exactly opposite regions to that in humans (Fig. 10) as described above.

Anatomically, the perineum of adult mice contains a prominent perineal appendage, which is of similar size in males and females, even though it is slightly larger in adult male versus female mice (Fig. 14C and D). It is essential to recognize that in both male and female mice the perineal appendage is neither penis or clitoris, but instead is prepuce (Sinclair et al., 2016c). One of the anatomical features distinguishing human and mouse external genitalia is that mice have two prepuces (external and internal prepuce) and humans have one (Fig. 15) (Blaschko et al., 2013). Since formation of male external genitalia are globally androgen-dependent in mice, what does this similarity in size of male and female perineal appendages mean, and when does the modest size difference appear? In mice, the greater size of the genital tubercle of newborn male versus female mice (and rats) is a well known

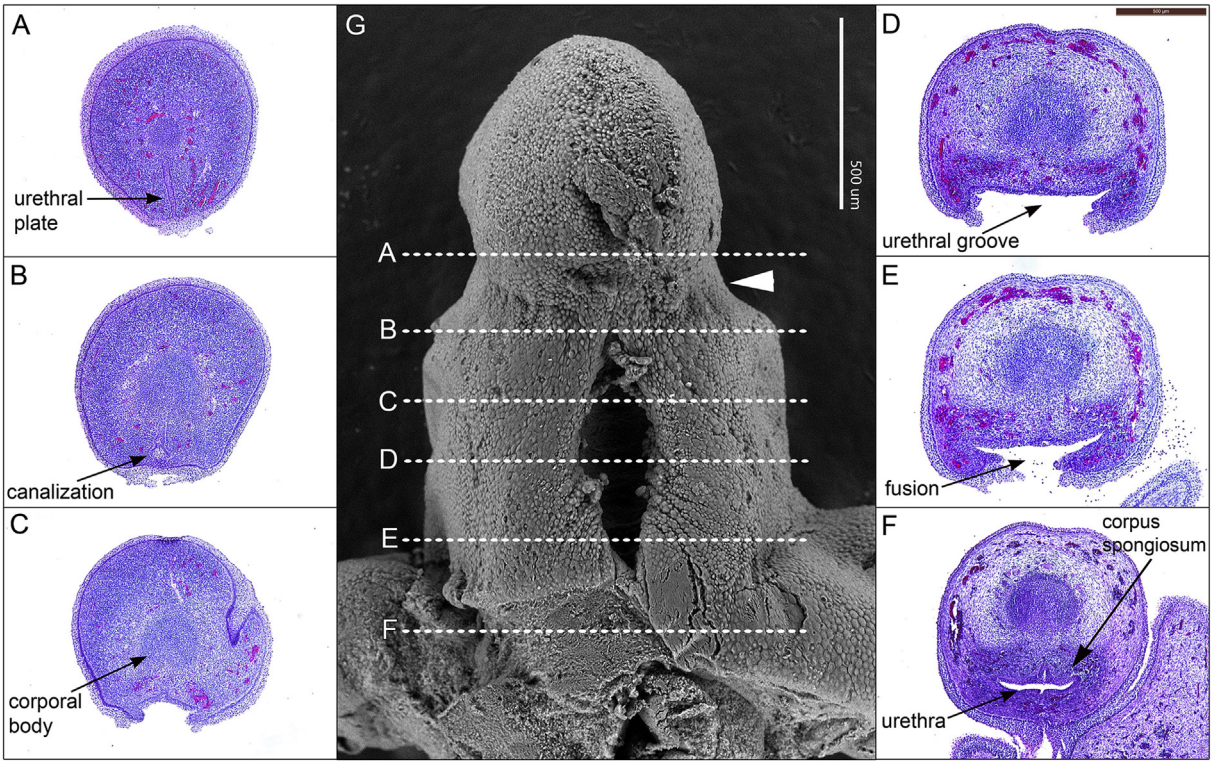

Fig. 5. Scanning electron micrograph (G) and transverse sections (A–F) of a 9-week human fetal penis stained with hematoxylin and eosin demonstrating (A) the solid urethral plate, (B) the beginning of canalization of the urethral plate, (C) urethral plate canalization to form an open urethral groove in the distal penile shaft, (D) mid-shaft showing a widely open urethral groove, (E) beginning of the process of fusion of the urethral folds, and (F) fully formed urethra at the levels indicated in (G). White arrowhead in (G) indicates the transition from penile shaft to glans. Modified with permission (Shen et al., 2016).

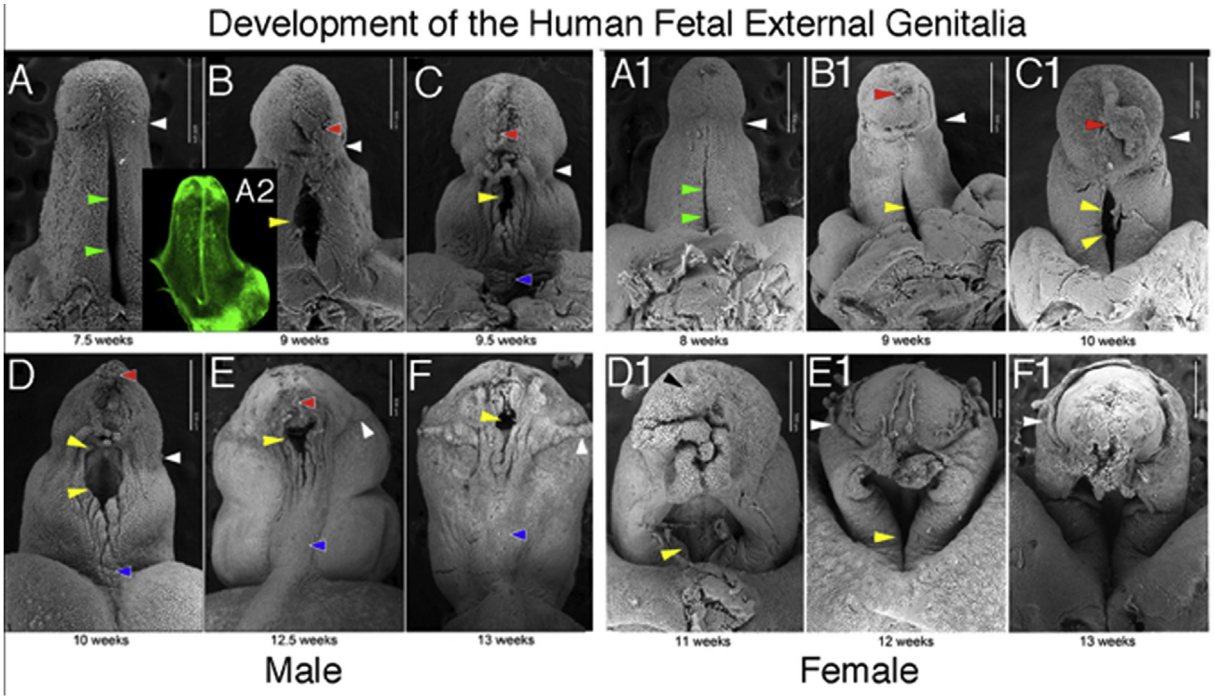

Fig. 6. Scanning electron microscopy of the ontogeny of the developing human fetal penis (A–F) from 7.5 to 13 weeks and developing human clitoris (A1-F1) from 8 to 13 weeks of gestation. White arrowheads indicate the junction of the penile and clitoral shaft with the glans (ages 7.5–11 weeks A-D & A1-D1). At 12 weeks of gestation in both males and females note advance of the prepuce over the glans (white arrowheads, fix) (E, E1, F & F1). Red arrowheads denote the epithelial tag. The blue arrowheads indicate the median penile raphe. Yellow arrowheads indicate the open urethral groove in males and vestibular groove in females. The green arrows in A & A1 denote the urethral plate in males and vestibular plate in females which is not canalized based on histologic analysis (see Fig. 3) and light sheet fluorescence microscopy with E-cadherin (Fig. 4). Modified with permission (Shen et al., 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

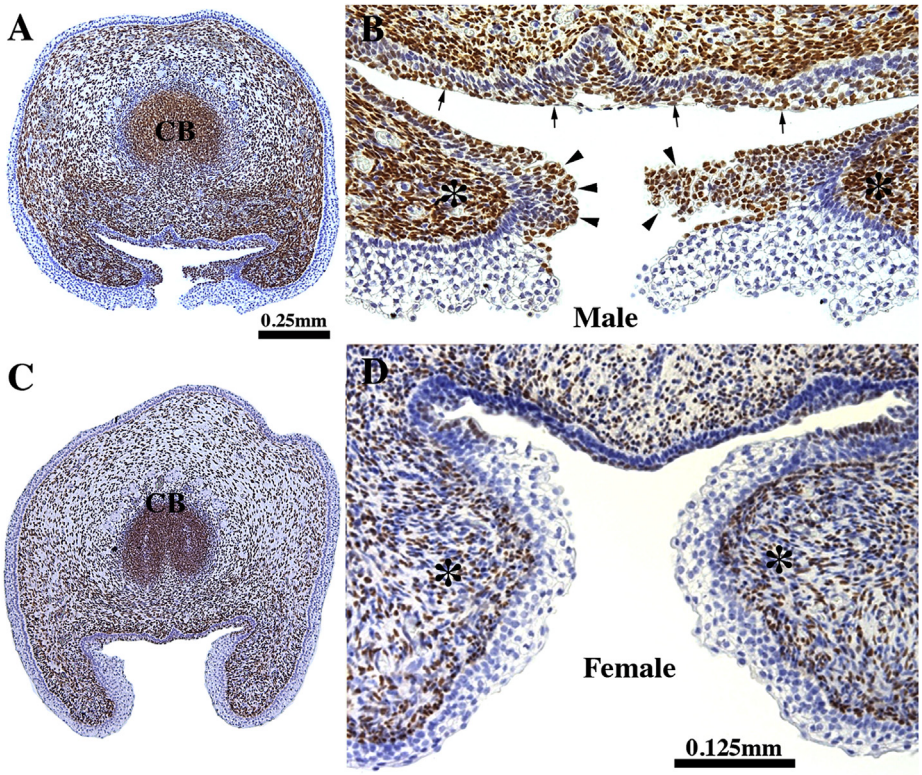

Fig. 7. Androgen receptor immunohistochemistry of 11-week human fetal male (A-B) and female (C-D) genital tubercle at the level of the closing zipper (male). Androgen receptors are detected in mesenchymal cells of the vestibular and urethral folds (black asterisks). In the male, AR are also detected in epithelium of the urethral groove (small arrows) and in the urethral folds (arrowheads). Note the increased amount of AR expression in the male (A-B) in both the epithelium (black arrowheads) and mesenchyme (black asterisks) compared to the female (C-D) in a comparable region. Scale bar in (B) also applies to (D).

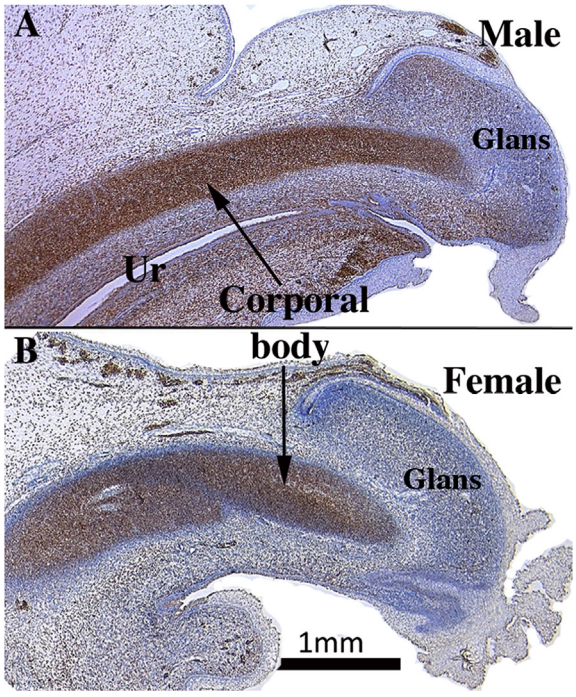

Fig. 8. Androgen receptor immunohistochemistry of 12-week male and female external genitalia. Ur = urethra.

feature allowing sex identification at birth (Murdaugh et al., 2018), and this feature has been attributed to androgen action (Hotchkiss et al., 2007; Hotchkiss and Vandenbergh, 2005). However, perineal appendage size is only marginally greater in males than females at birth and later in adulthood. The clear implication is that most of the growth of the perineal appendage in mice is androgen-independent, and that androgen action in males only accounts for only a modest size differential.

Table 1
Developmental and adult homologies between the human penis and clitoris.

Feature	Penis	Clitoris
Develop from the genital tubercle	Yes	Yes
Contain a urethral/vestibular groove	Yes	Yes
Urethral/vestibular groove canalization	Yes	Yes
Fusion of the urethral/vestibular folds	Yes	No
Phallic elongation	Yes	No
Phallic angle ~90 Degrees from body wall	Yes	No
Corporal bodies	Yes	Yes
Glans	Yes	Yes
Prepuce (albeit dorsal only in females	Yes	Yes
Contains a urethra	Yes	No
Large organ in adulthood	Yes	No

In adult male mice the perineal appendage containing the penis and the scrotum constitute the external genitalia. As mentioned above, the perineal appendage in adult mice is not the penis, but instead is the external prepuce, a hollow tubular structure, which houses the penis within the preputial space (Figs. 15–17) (Sinclair et al., 2016c). This arrangement, namely a perineal appendage (prepuce) housing the penis, is a feature common to small mammals (such as rodents) built low to the ground and is thought to be protective of the penis. Identification of the mouse perineal appendage as prepuce is based upon the following criteria: (a) The perineal appendage lacks erectile bodies, (b) does not contain a urethra, (c) is covered externally with a hair-bearing epidermis, and (d) defines a large space housing the penis lined with a stratified squamous glabrous (non-hair-bearing) epithelium (Figs. 15–17). The epithelium lining the inner surface of the male prepuce (perineal appendage) reflects onto the penile surface deep within the preputial space and thus is continuous with penile surface epithelium (Fig. 17). The ducts of mouse preputial glands open onto the inner surface of the external prepuce near the preputial meatus (not illustrated) as described previously (Sinclair et al., 2016c). Thus, the modest size differential of the adult perineal appendage in male and female mice, as well as the anatomic and histological constellation that

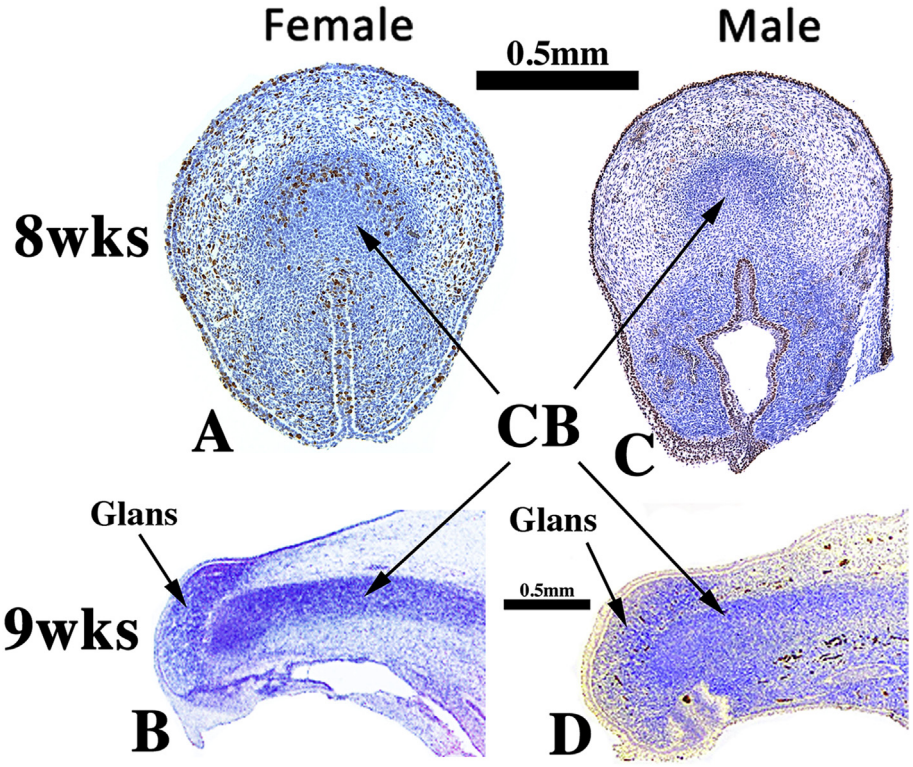

Fig. 9. Mid-sagittal (B & D) and transverse (A & C) sections of human female (A & B) and male genital tubercle (C & D) at 9 weeks (ambisexual stage). Note the condensed mesenchyme of the corporal body (CB) in both sexes at 8–9 weeks (ambisexual stage). Scale bar is 0.5 mm.

defines perineal appendage is androgen-independent.

3.3. Mole external genitalia

Female external genitalia of comparable size to that of males are seen in other animal species. In various species of moles, adult size of male versus female external genitalia (perineal appendages) is similar (Fig. 14A and B). Indeed, adult external genitalia of several mole species defy the conventional visual distinctions between males and females so obvious in humans. In moles, Wood-Jones in 1914 and Matthews in 1935 (Matthews, 1935; Wood-Jones, 1914) were so impressed by the similarity in size and shape of perineal appendages in male and female moles that these structures were incorrectly described as penis and clitoris, respectively. Indeed, Matthews coined the terminology, "peniform clitoris" (Matthews, 1935). This incorrect historic nomenclature (see below) was perpetuated in the modern mole literature until recently when Sinclair et al. critically examined the external genitalia of the broad-footed mole and demonstrated that the perineal appendages of male and female broad-footed moles (Fig. 14A and B) are prepuce and not penis or clitoris (Sinclair et al., 2016c). In male moles the perineal appendage exhibits all of the anatomic features of prepuce as described above for the mouse and houses the penis (Sinclair et al., 2016c). The epithelium lining the inner surface of the prepuce of both sexes of moles is non-hair-bearing and defines the preputial space. The penis of broad-footed moles lies deep within the preputial space and thus is an "internal organ" in the resting state which contains the penile urethra, os penis, and erectile bodies. In female moles the perineal appendage is also prepuce as defined by histologic criteria identical to that of the male. The internally positioned clitoris of broad-footed moles is defined by a U-shaped clitoral epithelial lamina similar to that of the mouse clitoris (Cunha et al., 2019a; Martin-Alguacil et al., 2008b; Sinclair et al., 2016c). These findings of mole external genitalia were confirmed in a subsequent paper on the comparative anatomy of 4 species of moles (Sinclair et al., 2016b). As in mice, adult size of the

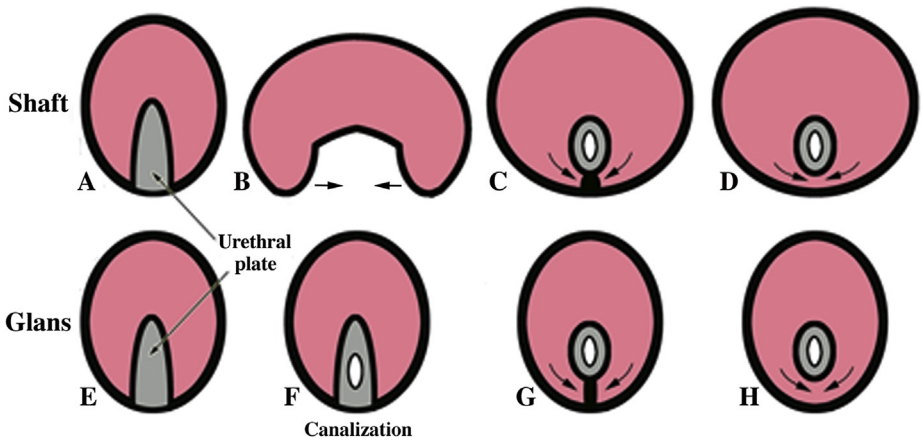

Fig. 10. Different morphogenetic mechanisms of urethral development in the human penile shaft (A–D) versus the glans (E–H). Diagrammatic transverse sections are arranged distal (A & E) to proximal (D & H). Curved arrows in (C-D & G-H) represent the process of midline mesenchymal confluence. Arrows in (B) represent midline fusion of the urethral folds.

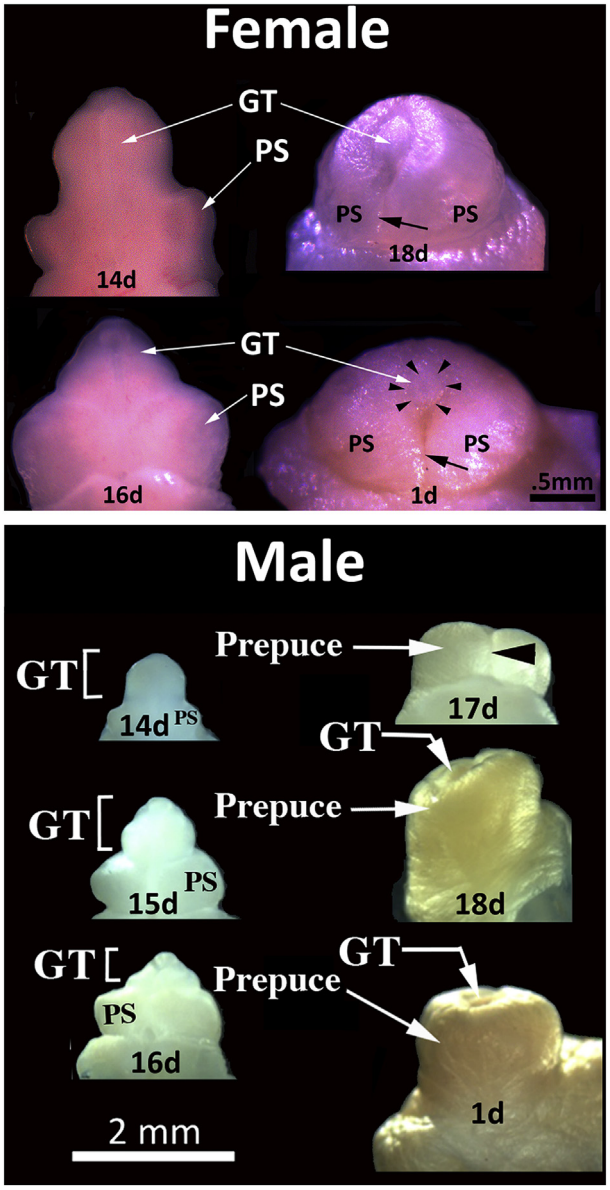

Fig. 11. Wholemount images of female and male external genitalia from embryonic mice at 14–18 (14d-18d) days of gestation and at birth (1d). Note the laterally situated preputial swellings (PS) at 14 days, which grow towards the midline where they fuse in the ensuing days. The preputial swellings also grow distally to cover the genital tubercle (GT). The tip of the genital tubercle is outlined by arrowheads in the 1d female specimen. Evidence of mid-ventral fusion of the genital swelling is manifest in females as the preputial-urethral groove (black arrows at 18d and 1d in the female specimens) and mid-ventral raphe (large arrowhead) in the 17d male specimen. Development of external genitalia is remarkably similar in males and females.

perineal appendage is slightly greater in male than female moles. Thus, the clear implication is that most of the growth of the perineal appendage (prepuce) in several species of moles is androgen-independent, and that the action of androgens in males appears to account for only a modest size differential. Finally, female ring-tailed lemurs (*Lemur catta*) exhibit a profound example of "masculinization" of female external genitalia as female ring-tailed lemurs have a pendulous peniform clitoris (Cunha et al., 2014; Drea and Weil, 2008).

3.4. Development of spotted hyena external genitalia

The spotted hyena presents the most extreme example of "masculinization" of female external genitalia and provides several examples of androgen-independent developmental events in male and female external genitalia. The female spotted hyena is the only extant mammal that mates and gives birth through a pendulous penis-like clitoris (Fig. 18). The hyena penis and clitoris are similar in size in the flaccid state, but profound differences in phallic morphology are seen in the fully erect condition both in pups and adults (Fig. 19). The penis is slightly larger and more flexible than the clitoris, as required of the male for successful intromission during mating (Drea et al., 1999). Following erection, the tip of the penis becomes chisel-shaped, whereas the tip of the clitoris is blunt (Fig. 19). Differences in morphology of the glans penis and glans clitoris are also apparent in the flaccid condition. We believe that this dramatic difference in glans shape, particularly evident upon erection (Fig. 19), is accomplished through a hemo-dynamic mechanism involving bilateral distal glanular erectile bodies (GEB) whose shape and position in the adult glans are sexually dimorphic and dependent upon prenatal androgens (Cunha et al., 2014).

Given the similarity in length of the penis and clitoris of spotted hyenas, the hormonal regulation of phallic growth was explored and surprisingly was found to be independent of gonadal hormones. This conclusion is derived from examination of penile and clitoral growth in spotted hyenas following prepubertal gonadectomy (Fig. 20) (Glickman et al, 1992, 1998). While prepubertal castration results in a marked failure in penile growth in all mammals studied to date (Beach et al., 1983; Beach and Levinson, 1950), in spotted hyenas prepubertal gonadectomy had no effect on growth in length of the penis and only modest reduction in length of the clitoris when examined at ≥2 years of age when adult phallic size is normally achieved (Fig. 20). The obvious interpretation for male spotted hyenas is that penile length is androgen-independent.

Despite the remarkable similarity in size of the penis and clitoris in spotted hyenas, certain aspects of internal and external architecture within these organs exhibit considerable sexual dimorphism. Thus, development of external genitalia in spotted hyenas simultaneously involve both hormone-dependent as well as hormone-independent mechanisms (Cunha et al., 2014). Androgens are clearly responsible for many sex differences in external and internal phallic morphology in male and female spotted hyenas (Table 2), but not for formation of the genital tubercle and urethral plate in male and female hyenas (Cunha et al., 2005; Drea et al., 1998). Thus, initial formation of the genital tubercle complete with a urethral plate is androgen-independent in both male and female spotted hyenas and occurs prior to androgen production by the testes or ovaries (Browne et al., 2006; Cunha et al., 2014).

However, in spotted hyenas certain aspects of urethral development are profoundly different in males and females with these differences being attributed to androgen action based upon treatment of pregnant hyenas with an androgen-blockade cocktail of flutamide (an anti-androgen) and finasteride (a 5α-reductase inhibitor) (Cunha et al, 2005, 2014). In untreated adult spotted hyenas the penile urethra is narrow, inelastic, and surrounded by both a corpus spongiosum and a tunica albuginea, an arrangement that places severe limits on expansion of the penile urethral lumen. In contrast, in females the clitoral meatus is larger and more elastic, and the female UGS (urethra) is pleated and not constrained by either a surrounding corpus spongiosum or tunica albuginea. This permits expansion of the female UGS for receipt of the penis during mating and passage of a 1.5–2 kg fetus at the time of parturition. The penile glans is chisel-shaped upon erection, whereas the clitoral glans is blunt, an important feature in regard to intromission during mating. These anatomical differences are due to the shape and position of the glanular erectile bodies (GEB), which are determined by the presence (males) or absence (females) of androgen action (Cunha et al, 2005, 2014) (Table 2). Thus, penile development in spotted hyenas involves both androgen-dependent and androgen-independent events as is the case for many other species described above.

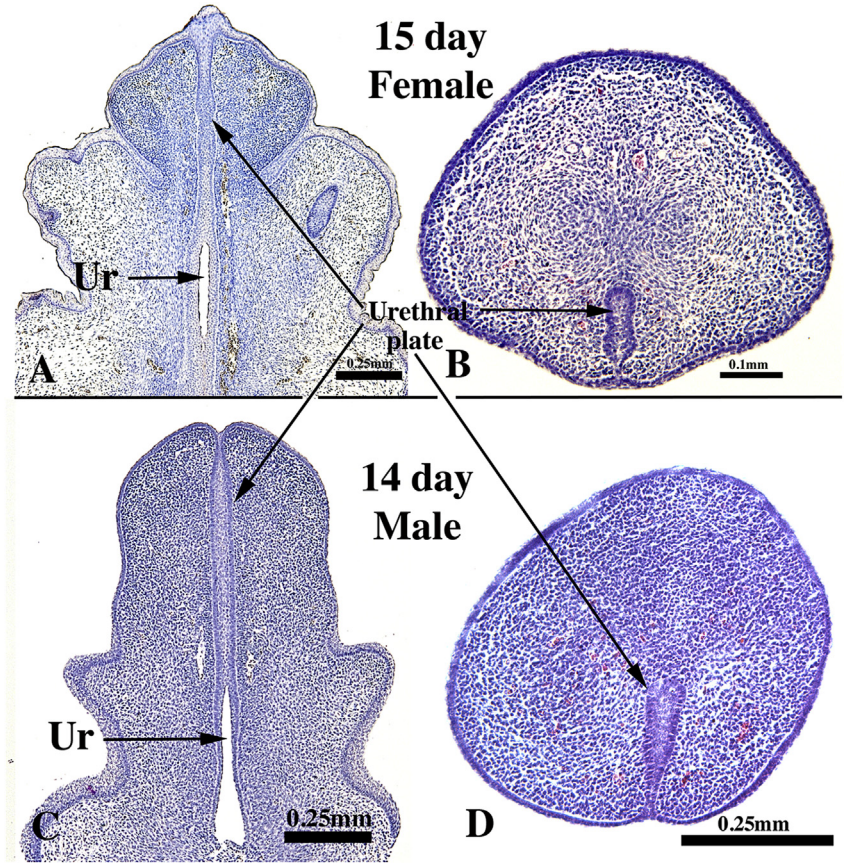

Fig. 12. Coronal (A & C) and transverse (B & D) sections of 15-day female (A & B) and 14-day male (C & D) mouse genital tubercles. Note solid urethral plates and canalized urethra (Ur) in both male and female specimens.

3.5. Abnormal clitoral development in estrogen receptor mutant and aromatase over-expressing mice

The formation of the mouse clitoris and the role of the genital tubercle in mouse clitoral development has been only superficially explored in the past (Schlomer et al., 2013) and is the subject of a companion paper (Cunha et al., 2019a). The mouse clitoris is an internal organ defined by an inverted U-shaped epithelial lamina described previously (Cunha et al., 2019a; Martin-Alguacil et al., 2008a) and subsequently confirmed by numerous studies (Mahawong et al., 2014b; Sinclair et al., 2016c; Weiss et al., 2012; Yang et al., 2010) (Fig. 21A). The anatomical differences in between the adult mouse clitoris and penis are profound (Fig. 21A and B, Table 3) and represent the culmination of radically different developmental processes. Of particular note is that fact that the adult mouse penis contains several erectile bodies (Mahawong et al., 2014a; Rodriguez et al., 2011; Weiss et al., 2012), while the adult mouse clitoris lacks erectile bodies (Christov et al., 1993; Weiss et al., 2012), suggesting that in mice formation of erectile bodies is androgen-dependent, a situation exactly opposite to that in humans as discussed above. Developing penile erectile bodies in male mice express androgen receptors (Blaschko et al., 2013).

The mouse penis has a circular profile in transverse sections (Fig. 21B), while the clitoris of wild-type and X^{Tfm}/Y mice (Tfm = testicular feminization) is an internal organ defined by an inverted U-shaped epithelial lamina (Fig. 21A). Given the similar morphology of the clitoris of wild-type and X^{Tfm}/Y mice (Rodriguez et al., 2012), it is evident that the absence of androgens or the lack of androgen action determines and specifies clitoral fate. However, for

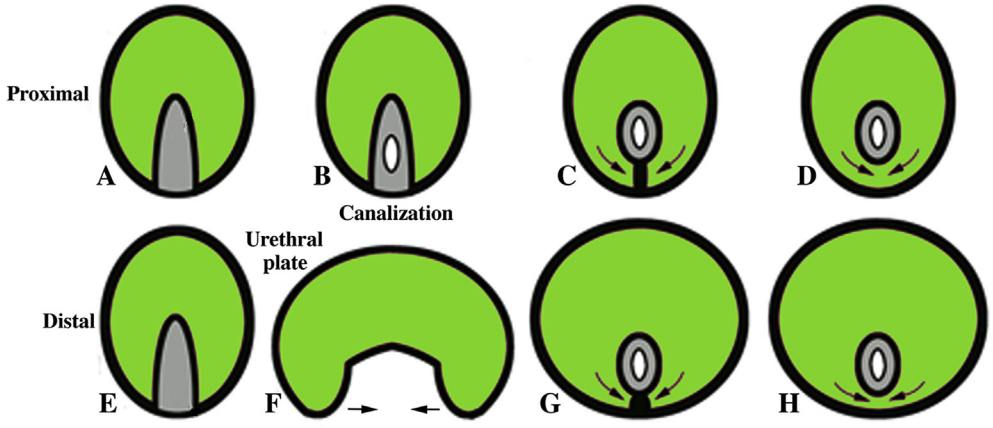

Fig. 13. Different morphogenetic mechanisms of mouse penile urethral development in proximal (A–D) versus the distal (E–H) regions of the genital tubercle. Diagrammatic transverse sections are arranged distal (A & E) to proximal (D & H). Curved arrows in (C-D & G-H) represent the process of midline mesenchymal confluence. Arrows in (F) represents epithelial fusion events. Compare with Fig. 10.

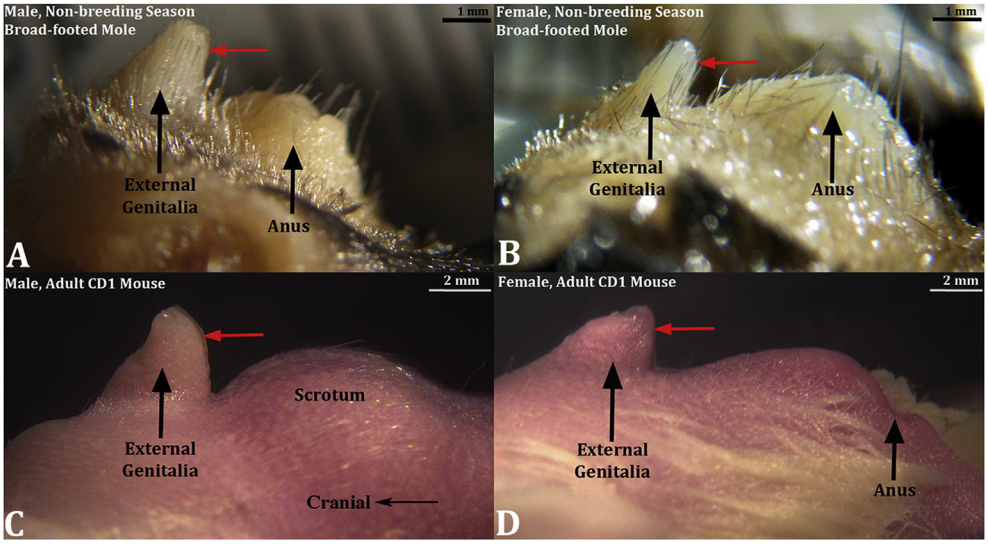

Fig. 14. Side views of the perineum of adult male and female broad-footed moles and mice. Note that male external genitalia are only slightly larger than that of the female.

several reasons a role of estrogen has been proposed for developing external genitalia, an idea formally espoused by the Cohn laboratory in studies on the mouse (Zheng et al., 2015). In light of the potential role for estrogen in development of the external genitalia, we can add some puzzling, but intriguing, observations on female external genitalia of adult estrogen receptor alpha mutant mice and surprisingly mutant female mice with elevated aromatase. In this regard, we have examined the following estrogen receptor mutant mice: αERKO, which lack functional ERα (McDevitt et al., 2007) and NOER (nuclear-only ERα) mice which lack membrane ERα (Pedram et al., 2016). Additionally, we have examined AROM + mice, which over-express aromatase, and thus have elevated levels of serum estrogen (Li et al, 2001, 2003). Adult clitoral morphology was profoundly affected identically in both types of ERα mutant mice (αERKO, N = 6 and NOER, N = 3) and even more profoundly in AROM + mice (N = 4), which over-express the aromatase enzyme that converts androgens to estrogens (Fig. 21). For the purpose of our study a "masculinization index" was created in which we identified in adult mice 10 anatomical features that were unique to penis and not shared by clitoris. Thus, the score for adult penis is 10/10 and the score for adult clitoris is 0/10 (Table 4).

The masculinization score was 6/10 for clitori of female αERKO and NOER mice (Table 4), demonstrating profound masculinization as a result of these inactivating ERα mutations (Fig. 21C and D). Most surprisingly, the "clitoris" of the AROM + female was exceptionally masculinized having 9 of the 10 penile features (Table 4). Indeed, the only feature that differed from that of wild-type penis was ventral tethering due to the presence of a frenulum-like connection to the inner aspect of the epithelium lining the prepuce (Fig. 21 E). Curiously, adult

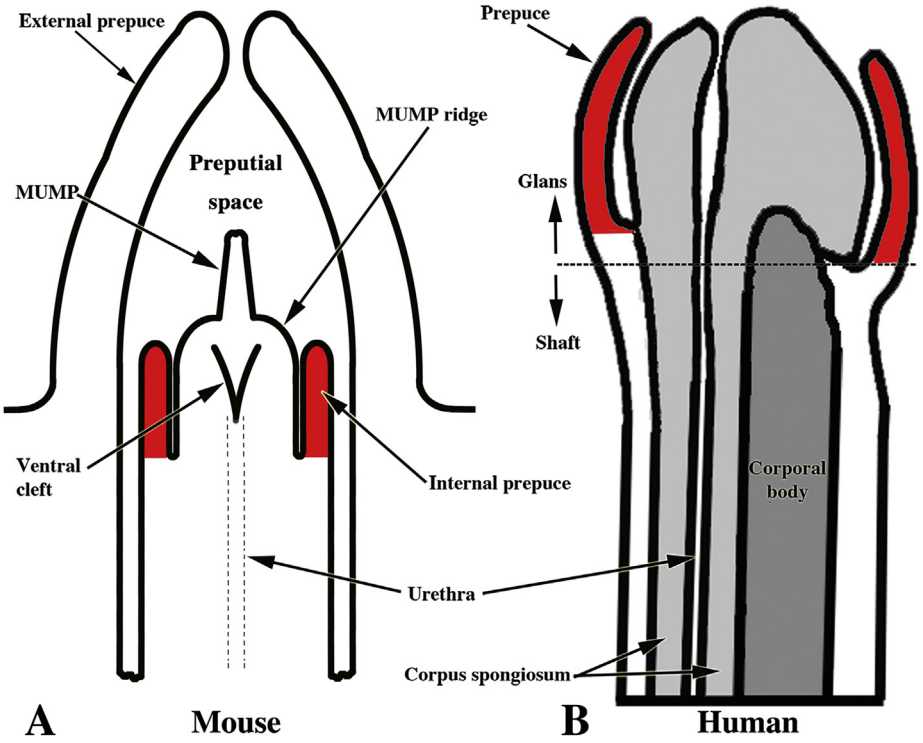

Fig. 15. Sagittal sections of human clitoris at 13 weeks of gestation immunostained for AR. Note the prominent AR expression in the corporal body (A) and in the peri-urethral mesenchyme extending up to the bladder neck, in mesenchyme of the glans clitoris, in mesenchyme associated with the preputial lamina and in mesenchyme ventral to the corporal body (black arrowheads) in the region of the vestibular groove (B).

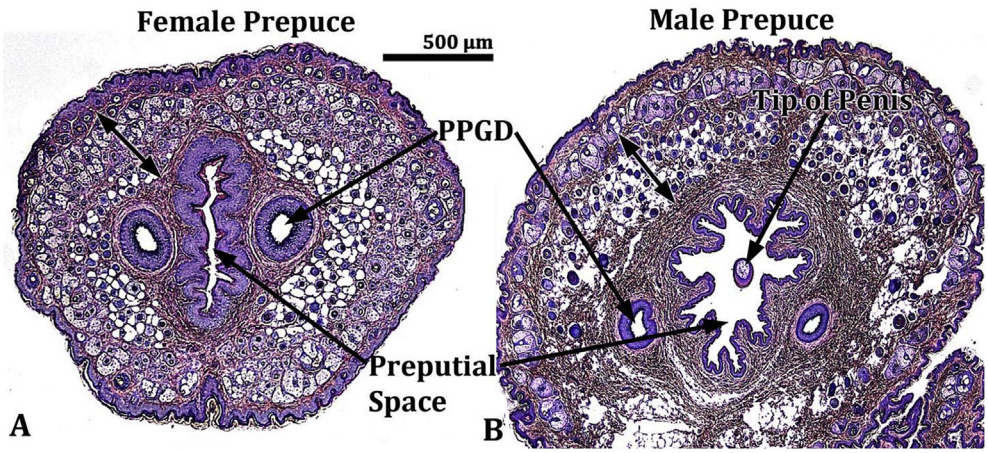

Fig. 16. Transverse sections of the external genitalia of adult male and female mice. The male and female perineal appendages illustrated grossly (Fig. 14C and D) and are illustrated in transverse sections (A–B) and are at the locations denoted by the red arrows in Fig. 14C and D. The sections are indicative of prepuce by virtue of having the following features: (a) covered externally by a hair-bearing epidermis, (b) lined by a non-hair-bearing inner epithelium defining the preputial space, which in the case of males (B) houses the penis (see also Fig. 17). Double-headed arrows denote hair follicles. Preputial gland ducts are labeled PPGD. Note the absence of erectile bodies and a urethra, features indicative of penis but not prepuce. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

penile morphology in all of these "estrogen mutant" male mice differed little from that of wild-type male mice (not illustrated).

4. Discussion

While penile development is globally dependent upon androgens, several steps in this complicated morphogenetic process are clearly androgen-independent. In this regard, formation of the genital tubercle containing a urethral plate is an androgen-independent event that is likely common to all mammals. Initial development of the genital tubercle and the urethral plate is one of the earliest events in urogenital development that precedes production of testosterone by the fetal testes and precedes early morphogenetic effects of androgens such as masculine development of the Wolffian ducts (formation of the epididymis, vas deferens, seminal vesicle) and masculine development of the urogenital sinus (formation of the prostate and bulbourethral glands) (Fig. 1). In humans the genital tubercle appears as an elevation in the perineum at 5–6 weeks of development (Arey, 1965; Gray and Skandalakis, 1972; Grumbach and Ducharme, 1960; Sajjad, 2010), while testosterone production by the fetal testes begins at 8 weeks when testosterone levels are just above background (Siiteri and Wilson, 1974). The first actual manifestation of androgen action (prostate bud formation) occurs at 10 weeks in humans when androgen levels are elevated (Cunha et al., 2018) (Fig. 1). In the mouse, development of the genital tubercle and urethral plate begins on E11.75 (Perriton et al., 2002), testicular differentiation occurs on E12, and production of testosterone by the fetal testes begins on E13 (Price and Ortiz, 1965) (Fig. 1). In rats the genital tubercle appears on E13, testicular differentiation occurs on E14, and androgen production begins on E15 (Kluth et al., 2011; Price and Ortiz, 1965). In spotted hyenas the genital tubercle has been observed as early as gestation day 25 (Cunha and Glickman, unpublished), and the genital tubercle, complete with urethral plate, has been reported at gestation day 30, which is prior to testicular differentiation and production of androgens by the testes (Browne et al., 2006; Cunha et al., 2005). Thus, genital tubercle and urethral plate development precedes androgen production by the fetal testes in several mammalian species.

Once the genital tubercle is fully formed, the first androgen-dependent event is specification of penile developmental fate, which as described above occurs prenatally based upon studies of X^{Tfm}/Y male mice and neonatal castration (Rodriguez et al., 2012). This interpretation is completely consistent with the idea that androgens are required for masculinization of the external genitalia. Our recent mouse studies shed light on this process. A more precise determination of the time course of prenatal establishment of androgen-dependent penile identity awaits further investigation. Welsh and colleagues have explored the programming window for reproductive tract masculinization in rats. They found that androgen-driven masculinization of all components of the reproductive tract is mediated during a common programming window prior to E19.5 (Welsh et al., 2008). In another paper, they determined that the critical programming window for regulating WD development, prostate formation and anogenital distance masculinization was between E15.5 and E17.5 days of gestation (Welsh et al., 2007). Welsh and colleagues "believe the programming window in humans is likely to be 8–14 weeks of gestation" (Welsh et al., 2008). Studies in mice specify two critical windows of androgenic sensitivity

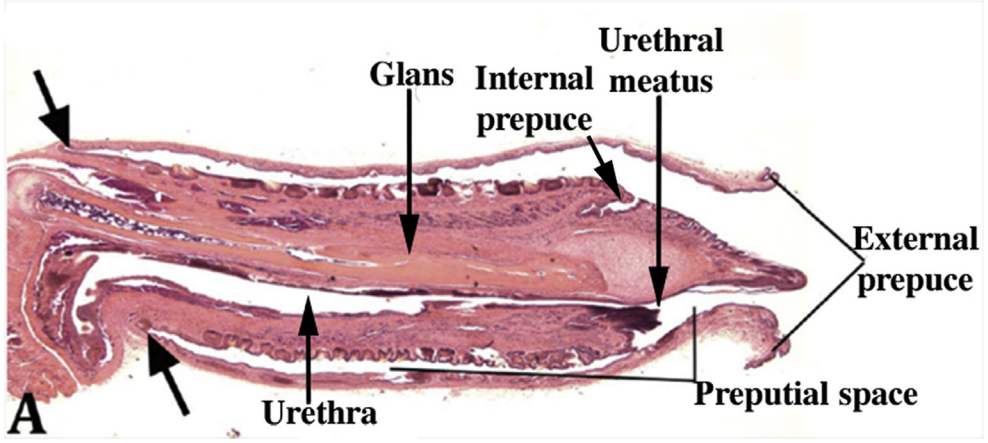

Fig. 17. Mid-sagittal section of the adult mouse penis and associated preputial space. Much of the external prepuce has been removed, but the fact that the mouse penis is housed within the preputial space is clearly evident. Note the continuity of the inner preputial epithelium with penile surface epithelium denoted by the large black arrows.

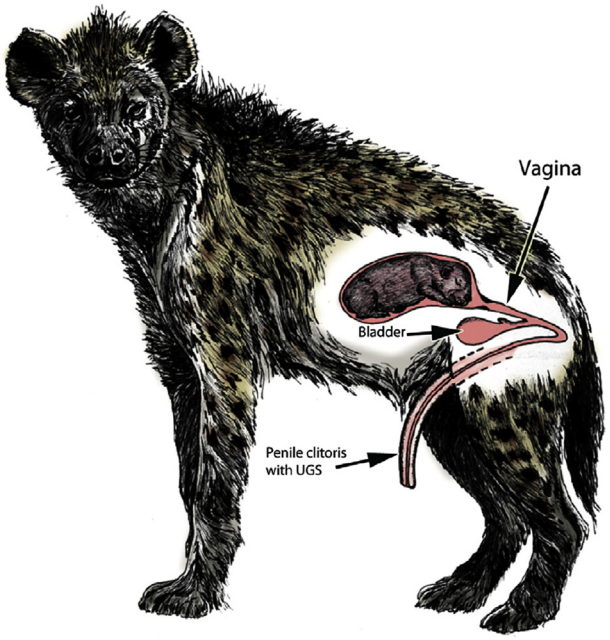

Fig. 18. Drawing of a pregnant female spotted hyena with a fetus in a uterine horn. A segment of the reproductive tract caudal to the uterus exhibits vagina histology. The urethra joins the caudal end of the vaginal segment, and the common urogenital sinus (UGS) thus formed extends through the pelvic outlet and makes a ~180-degree turn to traverse to the exterior through the penis-like clitoris. Note the absence of external vaginal orifice. Adapted with permission (Drea et al., 1998)).

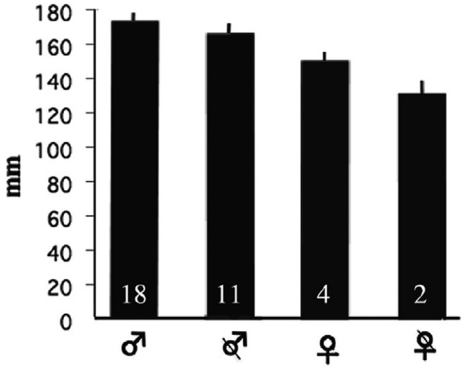

Fig. 20. Phallic length of intact adult (≥30 months) males and females versus adult males and females gonadectomized prepubertally. Analysis of variance revealed that there were highly significant differences among the four groups ($F = 21.74$, $df = 3$, $p < .0001$). Post-hoc tests indicated that there was a small but significant sex difference between intact males and intact females in phallic length (Fisher PLSD, $p < .0001$), and between intact females and ovariectomized females in clitoral length (Fisher PLSD, $p < .01$). Prepubertal castration had no significant effect on penile length (Fisher PLSD, $p = .36$). Numbers at the base of each bar indicates numbers of specimens per group. ♂ = Male ♀ = Female.

during normal penile development, a prenatal window and a neonatal window (Zheng et al., 2015). Feminization of external genitalia in male humans having defects in the androgen receptor gene (Wilson et al., 1981b) further emphasizes the idea that establishment of penile identity in the genital tubercle is a prenatal androgen-dependent event; we presume that this is true for all mammals.

For many species (humans, mice, rats) there is a vast difference in size of the penis versus the clitoris, which is consistent with the idea that penile growth is androgen-dependent, an idea verified by prenatal treatment of rats with testosterone (Welsh et al., 2010). The size differential in the human fetal penis versus clitoris also can be attributed to androgen action during fetal periods. However, human penile growth appears to be androgen-independent from ~1 year postnatal to puberty. For example, flaccid penile length in humans is ~3 cm in normal males at birth increasing to ~4 cm by 6 months to one year of age. This neonatal penile growth is presumably due to the postnatal rise in testosterone at 3 months of age (so-called mini-puberty) (Boas et al., 2006; Wang et al., 2018; Wylie and Eardley, 2007). Human penile size increases by ~1 cm from one year of age until puberty, presumably an androgen-independent event since serum testosterone is undetectable during that time period (Wang et al., 2018). This is in contrast to an overall increase in body length (~2 inches per year) and weight (~6.5 pounds per year) in boys. In contrast, at puberty marked penile growth is initiated in humans, culminating in mean adult penile length of 9 cm, a process coinciding with an increase in testicular volume and a substantial rise in serum in testosterone (Wang et al., 2018). Thus, phallic growth in human is androgen-dependent (a) during fetal development, (b) just after birth at the time of mini puberty and (c) during puberty. In contrast, human penile growth is androgen-independent from ~1 year of age until puberty (Wang et al., 2018). The spotted hyena provides perhaps the best example of androgen-independent penile growth. Penile length is virtually identical at 2 years of age (sexual maturity) in intact versus prepubertally castrated spotted hyenas, consistent with the idea that postnatal penile growth is androgen-independent in this species (Glickman et al, 1992, 1998). Thus, penile growth is both androgen-dependent and androgen-independent over the entire course from fetal to adult stages, an idea that likely generally applies to most if

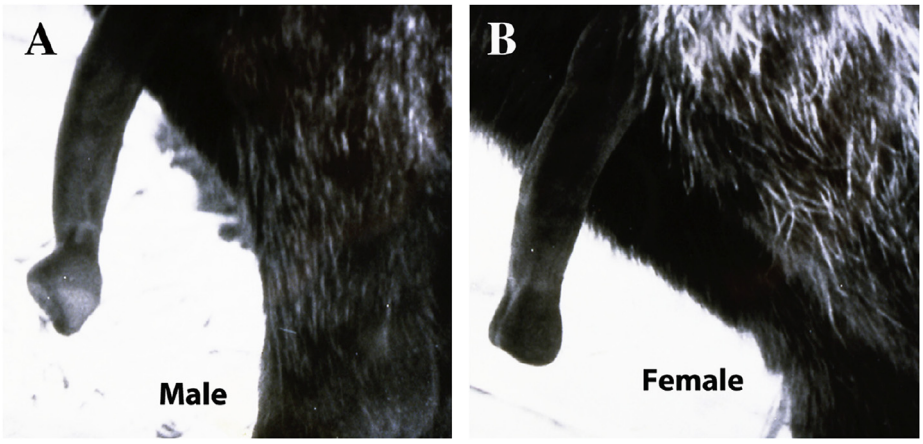

Fig. 19. The erect adult hyena penis (A) and clitoris (B). Note the distinctive and sexually dimorphic shapes of the glans. (A) and (B) adapted from (Place and Glickman, 2004) with permission).

Table 2
Sexually dimorphic androgen-regulated features in external genitalia of spotted hyenas.

Feature	Penis	Clitoris	Male features sex-reversed by anti-androgens	Female features induced by androgen
Position of urethral meatus	Dorsal	Ventral	Yes	ND
Size and elasticity of urethral meatus	Small and Non-Elastic	Large and Highly Elastic	Yes	ND
Glans shape/GEB shape	Chisel-shaped	Blunt	Yes	ND
GEB position re urethra	Surrounds urethra	Dorsal to urethra	Yes	ND
Corpus spongiosum	Present	Absent	Yes	Yes
Urethra/UGS lumen	Slit-like	Pleated/redundant	Yes	Yes
Position of retractor muscles	Ventral	Dorsal	Yes	Yes
Tunica albuginea	Surrounds corporal body and urethra	Surrounds corporal body only	Yes	Yes

ND = not done; GEB = glanular erectile body; UGS = urogenital sinus.

not all mammals.

Development of erectile bodies within the penis and clitoris varies considerably between species. In humans, corporal bodies are present in the adult penis and clitoris, and well-developed corporal bodies were observed in human male and female genital tubercles at 8 and 9 weeks when androgen production is nil or very low (Siiteri and Wilson, 1974). The corporal bodies in these specimens are so distinctive in morphology and location that their identities are unquestionable, leading inevitably to the conclusion that formation of the corporal body in humans is androgen-independent since the androgen production by the fetal testes begins and is barely above background at 8 weeks. Thus, it is unlikely that androgens play a role in initial development of the human penile corporal body. The presence of a corporal body in female genital tubercles at 8 weeks further emphasizes the idea that corporal body development is initially androgen independent in humans.

In mice, the penis contains several erectile bodies (Mahawong et al., 2014a; Rodriguez et al., 2011; Weiss et al., 2012), while the adult mouse clitoris is devoid of defined erectile bodies (Cunha et al., 2019a; Weiss et al., 2012) suggesting that in mice formation of erectile bodies is androgen-dependent. In spotted hyenas, a corporal body develops in both the penis and clitoris (presumably an androgen-independent event), but a corpus spongiosum only develops in males, due to androgen action (Cunha et al., 2014). Retractor muscles are present in phalli of both male and female spotted hyenas indicating that development of these muscles is androgen-independent. However, positioning of these muscles in sexually dimorphic. In males the retractor muscles lie ventral to the urethra, whereas in females these muscles lie dorsal to the urogenital sinus (Cunha et al, 2005, 2014; Neaves et al.,

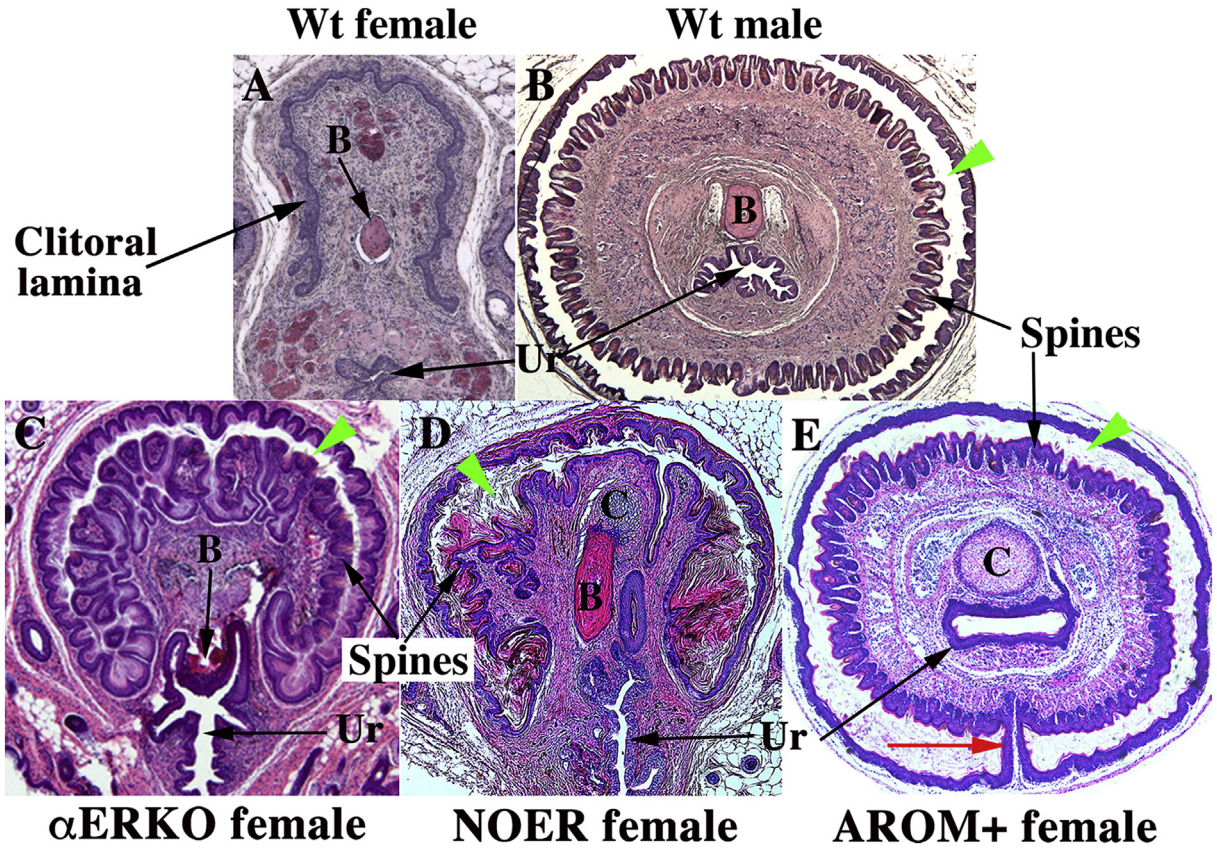

Fig. 21. Morphology of adult penis or clitoris in the following types of mice: (A) wild-type female, (B) wild-type male, (C) αERKO female, (d) NOER female and AROM + female mice. Clitori of αERKO and NOER females are partially masculinized, while clitori of AROM + female mice are almost completely masculinized and exhibit morphology similar to that of wild-type penis with the exception of the ventral tethering (frenulum, red arrow). B = bone, C=Cartilage, Ur = urethra. Note preputial space (green arrowheads) in all specimens except the wild-type clitoris. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Morphological features of penis and clitoris of wild-type mice.

Feature	Wild-type Penis	Wild-type Clitoris
Circular profile[a]	Yes	No
U-shaped epithelial lamina	No	Yes
Epithelial spines	Yes	No
Urethra completely within organ	Yes	No
Proximal hyaline cartilage	Yes	No
Distal cartilage	Yes	No
Large organ	Yes	No
Defined erectile bodies	Yes	No
Long bone	Yes	No
Short bone	No	Yes
Ventral tethering[b]/immobile organ	No	Yes

[a] Circular profile in transverse section.
[b] Clitoral stroma confluent with preputial stroma.

1980). Treatment with an anti-androgenic cocktail of finasteride and flutamide shifts the male anatomical pattern of these muscles to the female pattern, indicating that anatomical positioning of these muscles in androgen-dependent (Cunha et al, 2005, 2014). Finally, the distinctive chisel-shaped male glans and the blunt-shaped female glans in spotted hyenas is determined by the shape and position of the glanular erectile bodies (GEB) which are present in both male and female phalli. Thus, development of the glanular erectile bodies is androgen-independent, while shape and positioning of these erectile bodies are androgen-dependent based upon treatment of male fetuses with an anti-androgenic cocktail of finasteride and flutamide (Cunha et al., 2014). Thus, for spotted hyenas the totality of external genitalia development is particularly complex involving both androgen-dependent and androgen-independent events.

Prepuce morphology is vastly different in human males (which have a single prepuce) versus mice, which have 2 prepuces (Fig. 15) (Blaschko et al., 2013). The morphogenetic process of prepuce development in humans has not been described in sufficient depth and is the subject of a companion paper (Cunha et al., 2019c). Without embarking on the details of preputial development in humans, it is important to note that a prepuce forms in both the human penis and clitoris (Clemente, 1985), which suggests that initial development of the prepuce is androgen-independent, as appears to also be the case in spotted hyenas (Cunha et al., 2014). However, in human males the prepuce completely circumscribes the glans penis, whereas in females the prepuce is only present dorsal to the glans clitoris. This difference is likely attributable to androgen action, which is yet another example of the duality of developmental regulation in the external genitalia.

The external prepuce in mice, rats and moles houses the penis and forms the prominent perineal appendage. The perineal appendage is unquestionably prepuce based upon morphological and histological criteria and is derived from the preputial swellings that completely "over grow" the genital tubercle in both male (Liu et al., 2018a) and female mice (Cunha et al., 2019a), rats and presumably moles. Formation of the perineal appendage (prepuce) occurs identically in male and female mice (Cunha et al., 2019a; Liu et al., 2018a), and thus development of the perineal appendage is an androgen-independent event. For mice, rats and moles the size of the adult perineal appendage (external prepuce) is remarkably similar in males and females, with size being only modestly greater (10–20%) in males versus females (Fig. 14). Since the adult female perineal appendage in mice, rats and moles is ~80% that of males, the additional 20% size differential in males can be attributed to androgen action, while the majority of perineal appendage growth appears to be androgen-independent.

The inference of "androgen independency" based solely on developmental events occurring in males and not females is not the most rigorous assessment of this concept. We are forced to use this convention because for several species discussed in this report, additional corroborating data are lacking. However, developmental events within the embryonic genital tubercle that occur prior to androgen production and prior to evidence of androgen action (masculine differentiation of the Wolffian duct or prostatic bud initiation) are likely to be androgen independent, for example, formation of the genital tubercle and urethral plate. Developmental events that are inhibited by an anti-androgenic cocktail of finasteride and flutamide are androgen-dependent. In spotted hyenas the formation of the corporal body in untreated males, untreated females and anti-androgen-treated males provides one of many examples suggesting that corporal body formation is androgen-independent. Continued development/growth of the male phallus following prepubertal castration provides strong evidence that postnatal growth of the hyena penis is androgen-independent. Anti-androgen studies in spotted hyenas illustrate additional events that are androgen-dependent (Cunha et al., 2014) such as positioning of retractor muscles and shape/positioning of the glanular erectile bodies as discussed above. While evidence for androgen-independent development/growth of the penis could be bolstered by measurement of androgen levels at relevant time frames, such data are lacking for many species. In any case, on balance it is evident that substantial aspects of penile development are androgen-independent.

Those developmental events that we can ascribe to androgen action are consistent with the timing of androgen production by the testes (Fig. 1), by castration studies (Glickman et al, 1992, 1998; Rodriguez et al., 2012), AR mutants (Rodriguez et al., 2012; Wilson et al., 1981a), and the detection of androgen receptors in sites, tissues/cells and at times when presumed androgen-dependent developmental processes are occurring (Baskin et al., 2019). In this regard, many androgen-dependent developmental processes are recognized: (a) human urethral fold fusion, (b) human and mouse phallic growth, (c) genital tubercle angulation in humans, (d) induction of penile identity, (e) preputial growth in moles and mice, (f) erectile body development in mice, and (g) the shapes and positions of internal phallic anatomical features in the developing spotted hyena phallus (Baskin et al., 2018; Blaschko et al., 2013; Cunha et al., 2005; Liu et al., 2018b; Rodriguez et al., 2012;

Table 4
Masculinization index for penis and clitoris of wild-type mice and clitori of various female "estrogen mutant" mice.

Feature	Wild-type Penis[a]	Wild-type Clitoris	αERKO Clitoris	NOER Clitoris	AROM + Clitoris
Circular profile	Yes	No	No	No	Yes
Epithelial spines	Yes	No	Yes	Yes	Yes
Urethra completely within organ	Yes	No	No	No	Yes
Proximal hyaline cartilage	Yes	No	No	No	Yes
Distal cartilage	Yes	No	Yes	Yes	Yes
Large organ	Yes	No	Yes	Yes	Yes
Defined erectile bodies	Yes	No	No	No	Yes
Long bone	Yes	No	Yes	Yes	Yes
Ventral tethering/immobile organ	No	Yes	Yes	Yes	Yes
Resides in preputial space	Yes	No	Yes	Yes	Yes
Score	10/10	0/10	6/10	6/10	9/10

[a] Features indicative of the wild-type penile phenotype are indicated in red.

Development event	Human	Mouse
Genital tubercle development	Independent	Independent
Urethral plate formation	Independent	Independent
Urethral plate canalization	Dependent (in glans)	Independent
Urethral/vestibular groove formation	Independent	Not relevant
Urethral fold fusion	Dependent	Not relevant
Glans rudiment	Independent	Not relevant
Phallic growth	Dependent	Dependent
Preputial growth	Dependent	Independent & dependent
Prepuce development	Independent	Independent
Erectile body formation	Independent	Dependent
Erectile body growth	Dependent	Dependent
Penile identity	Dependent	Dependent
Midline mesenchymal confluence	Dependent	Dependent
Incidence of androgen independent	6 7	5 6

Fig. 22. Summary of androgen-dependent (red) and androgen-independent (green) developmental events during morphogenesis of the penis of humans and mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Shen et al, 2016, 2018b; Zheng et al., 2015). It is perhaps worth noting that the mere presence of AR does not in itself imply androgen action; the other critical factor is the actual timely presence of androgens. Androgen receptors are present in the developing human female genital tubercle (Baskin et al., 2019), and this apparently allows for penile development in female patients with congenital adrenal hyperplasia (Speiser et al., 2010).

Our studies relating to androgen-dependent versus androgen-independent events in development of external genitalia have focused on 4 species (human, mouse, moles and spotted hyena with additional reference to rat and guinea pig). The most detailed and complete data set is based upon human and mouse. Development of external genitalia in mouse and human is a complex multi-step process for which we have identified 13 individual developmental events in human and 10 developmental events in mice. For both species, while penile development is undeniably androgen-dependent in a global sense, surprisingly approximately half of the individual penile developmental events are androgen-independent in both species (Fig. 22). Further investigation may elucidate additional events in penile development which should be characterized as androgen-dependent or androgen-independent.

Finally, an additional factor is the role of estrogen or estrogen/androgen synergism/antagonism in development of external genitalia. It has been known for decades that developing external genitalia are susceptible to the teratogenic effects of estrogens, an idea bolstered by the presence of estrogen receptors in developing mouse external genitalia (Blaschko et al., 2013; Cunha et al., 2015; Rodriguez et al., 2012; Sinclair et al., 2016a; Zheng et al., 2015). Studies from the Cohn laboratory have formally raised the possibility of androgen/estrogen signaling in normal and abnormal development of the external genitalia (Zheng et al., 2015). Perhaps a balance of androgen/estrogen signaling is an important feature of development of external genitalia. We have analyzed 3 estrogen mutant female mice (αERKO, NOER [nuclear only ERα] and AROM+). The "clitori" of the 2 estrogen receptor mutant mice exhibit a masculinization score of 6/10, indicating substantial masculinization. While these two estrogen mutants are defective for estrogen action, androgen receptor action remains intact. Thus, in these estrogen receptor mutants the balance of estrogen/androgen signaling is perhaps perturbed in favor of androgen action. The result is a considerable degree of masculinization of the clitoris. As a follow up experiment we tested whether a feminizing dose of the anti-androgen, flutamide, could revert clitoral masculinization in these ER mutants. This was not the case. Thus, the profound masculinization of the mouse clitoris in certain mice strains with disrupted estrogen signaling remains a mystery to be resolved by further investigation. The AROM + female mouse, whose external genitalia exhibit almost perfect penile morphology, presents the surprising enigma that physiologic elevation in serum estrogen leads to profound masculinization of the external genitalia.

Funding

Supported by NIH grant K12DK083021.

Acknowledgements

This study was supported by NIH grant R21 HD088006 (to P.S.C.). And K12 DK083021 (to LSB and AWS).

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