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Estrogens and development of the mouse and human external genitalia

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Abstract

The Jost hypothesis states that androgens are necessary for normal development of the male external genitalia. In this review, we explore the complementary hypothesis that estrogens can elicit abnormal development of male external genitalia. Herein, we review available data in both humans and mice on the deleterious effects of estrogen on external genitalia development, especially during the "window of susceptibility" to exogenous estrogens.

The male and female developing external genitalia in both the human and mouse express ESR1 and ESR2, along with the androgen receptor (AR). Human clinical data suggests that exogenous estrogens can adversely affect normal penile and urethral development, resulting in hypospadias. Experimental mouse data also strongly supports the idea that exogenous estrogens cause penile and urethral defects. Despite key differences, estrogen-induced hypospadias in the mouse displays certain morphogenetic homologies to human hypospadias, including disruption of urethral fusion and preputial abnormalities. Timing of estrogenic exposure, or the "window of susceptibility," is an important consideration when examining malformations of the external genitalia in both humans and mice. In addition to a review of normal human and mouse external genital development, this article aims to review the present data on the role of estrogens in normal and abnormal development of the mouse and human internal and external genitalia. Based on the current literature for both species, we conclude that estrogen-dependent processes may play a role in abnormal genital development.

Keywords

External genitalia development; Estrogen; Mouse, and human

1. The Jost hypothesis

The Jost hypothesis states that male sexual differentiation is an active process based on production by the fetal testes of two hormones: testosterone and Mullerian inhibiting substance (MIS) (Jost, 1971, 1972). Testosterone, and/or its more potent metabolite dihydrotestosterone (DHT), promotes masculine development of the mesonephros, the

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mesonephric duct (Wolffian duct), the urogenital sinus, and the external genitalia. MIS (also called anti-Mullerian hormone [AMH]) elicits regression of the Mullerian duct in males. The Jost hypothesis has withstood the test of time and remains a useful model to explain clinical disorders of sex development (Baskin and JMaT, 2020; Wilson et al., 1981) (Fig. 1).

The short arm of the Y chromosome contains genes necessary for testicular development (Wilson et al., 1981; Lamb, 1995). The sex-determining region on the Y chromosome contains the gene for testis determining factor, SRY (Capel, 1996). The SRY gene directs development of the testis from the bipotential gonad by interacting with SOX-9 and at least four other genes: Wilms' tumor suppressor gene (WT-1), Fushi–Tarza factor 1 (FTZ-F1), steroidogenic factor 1 (SF-1), and LIM-1 (Fig. 1). Testicular development (formation of seminiferous cords) is evident at approximately 7 weeks of gestation in humans (Satoh, 1991). Originally, it was thought that ovarian development from the bipotential gonad is a default developmental pathway due to the absence of the SRY cascade. It is now known that the dosage-sensitive sex reversal gene (DAX-1) located on the short arm of the X chromosome is essential for ovarian development. The gene products of both SRY and DAX-1 compete to stimulate expression of the steroidogenic acute regulatory protein (StAR). The StAR protein is the first step in steroidogenesis, facilitating the conversion of cholesterol to pregnenolone, which occurs in the gonads as well as the adrenal glands (Miller, 2017).

Simply put, in normal XY male development, SRY overwhelms DAX-1, stimulating the bipotential gonad to form a testis with subsequent testosterone and MIS production. In normal XX female development, two copies of DAX-1 are present (one on each X chromosome (Niakan and McCabe, 2005)) and SRY is absent, resulting in downregulation of StAR, inhibition of testicular development, and initiation of ovarian development (Baskin and JMaT, 2020) (Fig. 1).

At approximately 8–9 weeks gestation, Sertoli cells of the human fetal seminiferous cords begin to secrete MIS (Behringer et al., 1994). This protein acts by inducing regression of the Müllerian ducts in male fetuses. Because MIS acts locally, Müllerian duct regression occurs ipsilaterally, in close proximity to the fetal testis. MIS also induces further development of the testis through differentiation of the primordial sex cords into seminiferous cords and ultimately mature seminiferous tubules (Satoh, 1991).

In 1850, Franz Leydig identified interstitial cells in the mammalian (Leydig, 1850) that approximately 50 years later were shown to produce androgens (Bouin and Ancel, 1903). Now known as Leydig cells, they appear as two distinct populations: fetal and adult, each with their own fetal and adult androgen production profiles (Zirkin and Papadopoulos, 2018). Testosterone acts directly on the embryonic mesonephros and Wolffian duct (Habert et al., 2001), and is converted to the more potent androgen dihydrotestosterone by 5-alpha-reductase type 2 in the urogenital sinus (UGS) and external genitalia (Wilson et al., 1993). Fetal testicular testosterone induces retention of a subset of mesonephric tubules and stimulates their development into the efferent ducts of the rete testis, as well as inducing the Wolffian duct to form the epididymis, vas deferens, and seminal vesicle. DHT elicits prostatic development from the UGS and masculinizes the external genitalia.

The post-pubertal ovary produces estrogen as well as progesterone. Evidence in humans shows that the fetal ovary produces estradiol in the second trimester (Vaskivuo et al., 2005), although fetal ovarian production of estrogen is miniscule relative to maternal and placental estrogen production (Zondek and Zondek, 1979; Oakey, 1970).

In addition, the human fetal adrenal gland secretes androgens as a substrate for placental production of estrogens (Rainey et al., 2004), contributing to the complex *in utero* hormonal milieu. Maternal estrogens at the end of the second trimester and throughout the third trimester are noteworthy for being particularly high (Noyola-Martinez et al., 2019), and are biologically active, eliciting prostatic squamous metaplasia (Zondek and Zondek, 1979), maturation of vaginal epithelium (Cunha GR et al., 2017), temporary lactation, and formation of transient breast nodules in both newborn males and females (Madlon--Kay, 1986).

1.1. Human external genitalia development

The embryologic rudiments of human external genitalia are the bipotential genital tubercle (GT) and genital swellings, which can be first recognized at 7–8 weeks of gestation (Carnegie collection of human embryos) (Shen et al., 2018). At approximately 9 weeks gestation, the external genitalia begins to differentiate into either the male or female phenotype (Baskin et al., 2018a) (Fig. 2). In the case of the penis, this is the consistent with the onset of testosterone production by the fetal testis (Siiteri and Wilson, 1974). Masculine development of the human external genitalia involves both androgen-dependent and androgen-independent morphological events (Cunha et al., 2020a), while development of the female external genitalia is androgen-independent (Baskin et al., 2018a; Overland et al., 2016). Similar androgen-independent events occur in both the developing human penis and clitoris, including: (a) development of the GT, (b) formation of the urethral plate, (c) formation of the genital folds and genital swellings, and (d) canalization of the urethral plate, which becomes the urethral groove in males (Cunha et al., 2020a). Canalization of the homologous vestibular plate in females results in the vestibular groove (Overland et al., 2016). Additional androgen-independent morphological events include formation of the corporal body and glans in both the developing human penis and clitoris (Baskin et al., 2018a; Liu et al., 2018a).

In contrast, androgen-dependent events distinguishing the developing human penis from the developing clitoris include fusion of the urethral folds to form a tubular urethra within the penile shaft and formation of the glanular urethra by a complex process of direct urethral plate canalization followed by mesenchymal confluence ventral to the newly formed urethra (Liu et al., 2018a). The final step in formation of the penis is circumferential development of the foreskin that ultimately covers the entire glans by 18 weeks gestation (Cunha et al., 2019a). The foreskin of the clitoris forms only on the dorsal aspect of the glans clitoris, but never fuses ventrally to become circumferential, as in the male, resulting in a dorsal hooded prepuce in the clitoris (Liu et al., 2018a; Baskin and Ebbers, 2006). Under the influence of androgens, the penis ultimately elongates and protrudes when erect at a 90○ angle from the body (Baskin et al., 2018a), while the female clitoris lies more adjacent to the body. In summary, the human clitoris is a smaller structure analogous to the human penis, differing

primarily due to the absence of urethral development within the body and glans of the clitoris and absence of circumferential preputial development (Baskin et al., 1999, 2018a; Overland et al., 2016).

1.2. Mouse external genitalia development

A full understanding of development of murine external genitalia requires an appreciation of the morphologic changes that occur during embryonic, neonatal, and adult time periods. As in development of human external genitalia, the mouse penis and clitoris are characterized by androgen-dependent and androgen-independent developmental events. Androgen-independent events include development of the mouse GT, formation of the urethral plate followed by its canalization, development of the preputial lamina (Liu et al., 2018a; Cunha et al., 2020b), and formation and distal growth of the external prepuce (perineal appendage). Androgen-dependent events in penile development include: (a) establishment of mouse penile (versus clitoral) identity (Rodriguez et al., 2011), (b) phallic growth, (c) erectile body formation and growth, and (d) establishment of the midline mesenchymal confluence ventral to the urethra (Rodriguez et al., 2011, 2012; Weiss et al., 2012a). In direct contrast to the human, the mouse penis and clitoris are not analogous structures and exhibit highly divergent development processes, culminating in drastically different adult morphologies (Cunha et al., 2020b). For example, the adult mouse penis has epithelial spines, a urethra completely enclosed within the organ, proximal hyaline cartilage, a distal male urogenital mating protuberance (MUMP), a relatively long os penis, defined erectile bodies, and a circular morphology in transverse section (Rodriguez et al., 2011, 2012; Weiss et al., 2012a). End-on SEM views of the penis demonstrate that the MUMP is fused proximally to a circumferential ring known as the MUMP ridge (Blaschko et al., 2013a; Weiss et al., 2012b; Yang et al., 2010a) (Fig. 12A). A ventral cleft partially bisects this MUMP ridge (Fig. 12A–B) to form the stem of the characteristic Y-shaped urethral meatus (Fig. 12A). In contrast, the adult mouse clitoris is defined by a U-shaped epithelial lamina, a miniscule os clitoris, absence of defined erectile bodies and spines, and ventral tethering, rendering it an immobile organ, relative to the much larger mobile mouse penis (Cunha et al., 2020b; Rodriguez et al., 2011, 2012; Liu et al., 2018b). The proximal portion of the mouse glanular penile urethra develops through an extension and canalization of the urethral plate, whereas the distal portion, especially the urethral meatus, develops via multiple fusion events (Kim et al., 2004; Cunha et al., 2015; Blaschko et al., 2013b). This is an especially important consideration when comparing the body of literature on the mouse to that of the human, where the exact opposite process occurs.

1.3. Mouse indifferent stage

In mice, the GT originates in part from the ventral tail bud mesenchyme (Tschopp et al., 2014). Development of the mouse GT can first be recognized as a perineal elevation around embryonic day (E) 11.75 (Perriton et al., 2002). Shortly thereafter, the urethral plate develops within both the male and female GTs (Hynes and Fraher, 2004a, 2004b; Seifert et al., 2008; Petiot et al., 2005; Yamada et al., 2006). Bilateral preputial swellings can be recognized lateral to the GT in both sexes at E14 (Perriton et al., 2002; Petiot et al., 2005; Cunha, 1975a; Suzuki et al., 2002). The preputial swelling in male mice are not as analogous to the genital swellings in human embryos. Mouse preputial swelling do not form the

scrotum, and female mice do not develop a labia majora or labia minora. In both male and female embryonic mice, as the preputial swelling grow distally, the preputial lamina is laid down (Cunha et al., 2020b; Liu et al., 2018b). This appears to occur as a result of epidermal fusion of the preputial swelling with the epidermis of the GT (Liu et al., 2018b).

1.4. Normal adult mouse penis

The external genitalia of the adult male mouse is defined by the surface elevation in the perineum, which is called the external prepuce (Liu et al., 2018b) (Fig. 3). The penis normally rests within the external preputial space (Rodriguez et al., 2011, 2012). The MUMP is the bifid tip of the adult mouse penis and contains a central core of cartilage, (Rodriguez et al., 2011; Yang et al., 2010b). The MUMP cartilage extends proximally within the penis to dorsally overlap the os penis. The MUMP appears shortly after birth from a central mesenchymal condensation circumscribed by epithelium (Schlomer et al., 2013).

The urethra within the mouse glans develops via two entirely different morphogenetic mechanisms: (a) epithelial fusion events and (b) direct canalization of the solid urethral plate. In the proximal portion of the mouse glans the urethra forms via direct canalization of the urethral plate (Cunha GR et al., 2017; Seifert et al., 2008; Hynes and Fraher, 2004c) (Fig. 4A–D) in contrast, the distal portion of the mouse urethra, and especially near the urethral meatus, forms via an epithelial fusion events (Liu et al., 2018b) (Fig. 4E–H). In both regions (proximal and distal) note that a transitory epithelial seam (Fig. 4C & G) is removed resulting in midline mesenchymal confluence (opposed curved arrows in Fig. 4) to generate a stand-alone urethral tube completely surrounded by glans mesenchyme, as well as eliminating the physical connection between the penis and the inner surface of the preputial mucosa (Liu et al., 2018b; Cunha et al., 2019b). The terms “body” and “glans” of the penis are substantially different in mice versus humans, however, so care must be taken when comparing development between the two species (Rodriguez et al., 2011; Cunha et al., 2015).

Conversely, formation of the human urethra within the glans penis occurs via direct canalization of the urethral plate, whereas proximally within the penile shaft, the urethra forms via canalization of the urethral plate to form an open urethral groove and then subsequent fusion of the urethral folds (Li et al., 2015; Baskin et al., 2018b) (Fig. 5).

Thus, while both mechanisms of urethral formation occur in mice and humans, the location of the developmental mechanisms is exactly opposite in these species (Figs. 4 and 5). Of note, hypospadias in humans occurs most frequently at precisely the junction of these two disparate morphogenetic mechanisms, the glans-shaft interface (Liu et al., 2018a, 2018b; Baskin and Ebbers, 2006; Cunha et al., 2015; Baskin et al., 1998).

1.5. Normal mouse clitoris

As in the adult male mouse external genitalia, the surface elevation in the perineum of the adult female mouse is the hair-bearing female prepuce (perineal appendage) and not the clitoris (Cunha et al., 2020b) (Fig. 6). The adult mouse clitoris is deeply placed within the perineum and is defined by a U-shaped epithelial lamina derived from the embryonic preputial lamina (Cunha et al., 2020b). The adult mouse clitoris contains a small bone (os

clitoris) analogous to that of the os penis, lacks a cartilaginous MUMP (Weiss et al., 2012a), and is tethered to (or confluent with) the ventral stroma and therefore immobile. The mouse female urethra lies mostly outside the clitoral stroma (Fig. 6B) except the distal portion (Fig. 6C and D), where the ventral stroma surrounds the dorsal aspect of the urethra, and differs from the adult male in which the urethra resides completely within penile stroma (Qiao et al., 2012).

The mouse embryonic perineal appendage is called the GT and its initial development is identical in both sexes, rendering the embryonic female GT indistinguishable from that of the male (Cunha et al., 2020b; Liu et al., 2018b). The clitoral stroma arises from the mesenchyme of the GT, a feature shared with the penis. The embryonic female GT, like that of males, is flanked laterally by the preputial swelling, which fuse in the ventral midline and grow distally to completely cover the GT to form the hair-bearing perineal appendage (external prepuce) (Cunha et al., 2020b). At birth, the female perineal appendage is slightly smaller than that of males (Murdaugh et al., 2018) and is colloquially (and perhaps incorrectly) termed the GT. During fusion of the embryonic preputial swelling with the embryonic female GT, a preputial lamina is laid down, which circumscribes and encloses the mesenchyme of the female GT (Cunha et al., 2020b). The process of preputial lamina formation in female mice is identical to that in males (Liu et al., 2018b), and has no counterpart in human clitoral development (Cunha et al., 2020b). For additional detail on mouse versus human clitoral development see Cunha et al., 2020 (Cunha et al., 2020b).

At birth and thereafter, the female mouse GT is completely covered by the preputial swelling, and is more appropriately termed the “perineal appendage” or female prepuce, which has a histologic signature identical to that of the male. In adulthood, the female perineal appendage is not the clitoris. It is a hair-bearing perineal appendage with the female urethral meatus located at its base. The actual mouse clitoris is a deeply placed internal structure defined by a U-shaped clitoral lamina (Cunha et al., 2020b; Weiss et al., 2012b; Martin-Alguacil et al., 2008a) as discussed above (Fig. 6A).

In summary, the adult mouse clitoris is a much smaller organ than the mouse penis (Cunha et al., 2019b, 2020b) and is immobile due to ventral tethering. The urethra is not entirely enclosed within the clitoral lamina, unlike the male urethra. The os clitoris is quite diminutive compared to the os penis and does not contain cartilage. The mouse clitoris unlike the mouse penis does not contain epithelial spines.

1.6. Role of estrogens in development of the external genitalia

The groundbreaking work of Jost demonstrated that androgens dominate as the key instigator of masculine sexual differentiation, a concept supported by a comprehensive literature on sexual development of the human and mouse (and many other species) (Jost, 1972; Baskin et al., 2018a; Overland et al., 2016; Li et al., 2015; Shen et al., 2016). What is less clear is the role of estrogens in development of the external genitalia. Are they involved in normal development? Are they involved only in abnormal development such as hypospadias? Can Jost’s original hypothesis be extended into a model in which sexual differentiation is not only androgen-dependent, but a balance between androgenic and

estrogenic action? Here we summarize the current literature on the role of estrogens in development of the human and mouse external genitalia.

1.7. Human clinical data

There are a number of examples of disorders of sex development in humans that fit the hypothesis that both androgen and estrogens play a role in normal development of the external genitalia. Individuals with complete androgen insensitivity (CAIS) have a XY genotype. The phenotype of their external genitalia, however, is almost indistinguable to that of normal XX females (Quigley et al., 1995), though subtle physical differences have been identified suggesting a possible role of estrogens. XY CAIS patients have a defect in the AR gene, accounting for the observed insensitivity to the effects of androgens. This results in reduced clitoral width, reduced vaginal size and depth, and abnormal genital hair distribution (Wilson et al., 2011). These studies in humans demonstrating the importance of androgen action in masculinization of the external genitalia are supported by studies of androgen-insensitive mice, either the spontaneous “Tfm mutation” or genetic alteration of the AR gene in transgenic mice (Rodriguez et al., 2012; Weiss et al., 2012a; Cunha, 1975b).

Another example of abnormal development of the external genitalia is 5α-reductase type 2 deficiency, defined by an unusually severe form of hypospadias at birth (Wilson et al., 1993) (Fig. 7). The higher-than-normal levels of testosterone at puberty are converted to estrogen via peripheral aromatase, leading to gynecomastia. At the time of normal male penile and urethral development in humans (approximately 8–18 weeks gestation), testosterone without conversion to DHT leads to undervirlization of the external genitalia with severe hypospadias and the presence of a pseudo vagina (Fig. 7).

1.8. Human hypospadias

Human hypospadias is an extremely common congenital malformation occurring in approximately 1:250 newborn males (Baskin et al., 2001; Baskin, 2017). Hypospadias is defined as an ectopic urethral opening on the ventral aspect of the penis, along with a deficiency in the surrounding corpus spongiosum, an arrest in foreskin development resulting in a dorsal hooded foreskin, and in more severe cases, penile curvature (Baskin, 2017) (Fig. 8). While the cause of human hypospadias is very likely to be multifactorial, recent studies have implicated exogenous estrogenic endocrine disruptors in the etiology of this malformation (Baskin et al., 2001; Yiee and Baskin, 2010). This hypothesis is based on the fact that multiple estrogenic endocrine disruptors are associated epidemiologically with human hypospadias (Vilela et al., 2007; Wang and Baskin, 2008) (Kalfa et al., 2011, 2015). For example, diethylstilbestrol (DES) exposure during pregnancy is associated with an increased incidence (~2.5×) of hypospadias in male offspring (Klip et al., 2002). Human epidemiologic studies on the influence of pesticides, vegetarian diets, and oral contraceptives on hypospadias have shown a mild association with hypospadias but have not been definitive (Baskin et al., 2001; Yiee and Baskin, 2010; Swan et al., 2005; Willingham and Baskin, 2007; North and Golding, 2000). A number of estrogen-responsive genes or genes that interact with the estrogen receptor(s) have also been associated with hypospadias, including CYR61, CTGF, ATF3, and GADD45b (Wang et al., 2007). Polymorphisms of the estrogen receptor may also predispose to hypospadias (Beleza-Meireles et al., 2006).

Exposure to exogenous estrogenic compounds may distort the estrogen/androgen signaling balance during the first trimester when human urethral formation occurs (Baskin et al., 2001; Yiee and Baskin, 2010). Exogenous estrogens during development can lead to a reduction in serum testosterone, which is mediated via the pituitary/gonadal axis (Cooke et al., 2017). The developmental effects of exogenous estrogens can result in permanent elevation (Richter et al., 2007) and/or reduction in AR levels in androgen target tissues (Prins, 1992). Finally, estrogens can act by up-regulating the estrogen receptor (Zheng et al., 2015). While the exact role of estrogens in normal development of human male and female external genitalia is unknown, it is notable that the human fetal penis and clitoris express both estrogen receptors, ESR1 and ESR2 (See below) (Baskin et al., 2020) (Cunha et al., 2020c).

1.9. Estrogen receptor expression in the humans penis and clitoris

Estrogen receptor alpha (ESR1 or ERα) and estrogen receptor beta (ESR2 or ERβ) are expressed to varying degrees and locations in the developing human penis and clitoris (Baskin et al., 2020). This is also true of the androgen receptor, whose location in the penis is in close proximity to the urethral plate and urethral groove and in the area of formation of the penile and glanular urethra. The location of the androgen receptor supports the role of androgens as necessary for fusion of the urethral folds during formation of the penile urethra in the shaft and in formation of the glanular urethra (Baskin et al., 2020). Similar expression of the androgen receptor in the developing clitoris provides an explanation of masculinization of the clitoris into a normal penile-like structure in females with congenital adrenal hyperplasia (Speiser et al., 2010).

The localization of ESR1 and ESR2 in epithelial cells of the preputial lamina and in the area of urethral plate canalization, as well as in the remodeling of the glanular urethra plate is consistent with the possible role of estrogens in normal and/or abnormal development. ESR1 expression was consistently less prominent than ESR2 expression in developing human penis and clitoris (Fig. 9), as is also the case in mice (Blaschko et al., 2013b). ESR1 expression was not present in the corporal body of the penis and clitoris from 9 to 16 weeks gestation. ESR1 was strategically localized to the epithelial cells of the preputial lamina and in areas of canalization and remodeling of the glanular urethral plate. These sites of ESR1 expression support the idea that exogenous estrogenic endocrine disruptors may play a role in human hypospadias (Baskin et al., 2001; Yiee and Baskin, 2010). In the 16-week male, ESR1 expression localized to the basal epidermal cells of the future penoscrotal junction. The expression of ESR1 at the penoscrotal junction may also play a role in the etiology of penile webbing (Bonitz and Hanna, 2016).

ESR2, in contrast to ESR1, is consistently more highly expressed in the developing penis and clitoris (Baskin et al., 2020) (Fig. 9). ESR2 expression is first observed during the indifferent stage at 8 weeks of gestation in the urethral plate and epidermis, as well as sparsely in the GT mesenchyme. ESR2 expression was seen in the corporal body and glans at 11 and 12 weeks of gestation in both the penis and clitoris. ESR2 is prominently expressed in the urethral epithelium and epithelium lining the vestibular groove, the preputial lamina, the area of urethral/vestibular plate canalization, and during remodeling in the penile glanular urethra. It is reasonable to hypothesize that ESR2 (in contrast to ESR1)

plays the more prominent role during normal, and perhaps abnormal development of male and female external genitalia based upon its more extensive expression compared to ESR1.

The expression of ESR1 and ESR2 in the developing human penis is consistent with the hypothesis that interference of ESR1 and/or ESR2 can lead to under-virilization of the penis, resulting in hypospadias by impairing fusion of the urethral folds and remodeling of the glanular urethra (Baskin et al., 2020). Furthermore, data derived from analysis of human infant foreskin from hypospadiac patients versus normal newborns undergoing circumcision showed that mRNA expression levels of ESR1 and ESR2 were significantly decreased in foreskin harvested at the time of hypospadias repair compared to age matched control skin from normally-developed patients (Qiao et al., 2012). ESR2 immunostaining was strong in normal foreskin, but weak in hypospadiac foreskin and immunoreactivity was most intense in the stratum basale and stratum spinosum. ESR1 immunostaining was weak in normal and mild hypospadias foreskin, and undetectable in severe hypospadias (Qiao et al., 2012).

1.10. Mouse knockout data

Our knowledge regarding the role of estrogen signaling in normal and abnormal external genitalia development comes mostly from studies in the mouse. Initial studies of αERKO (ESR1-KO) and βERKO (ESR2-KO) mice demonstrate that the male and female internal and external genitalia form normally (Lubahn et al., 1993; Krege et al., 1998), as all internal and external male and female reproductive tract organs form in these estrogen receptor mutant mice. However, an interesting and perplexing phenotype was subsequently observed in ESR1-KO and NOER (Nuclear-Only ESR1) female mice, which exhibited profound masculinization (Fig. 10 C and 10D) of their external genitalia (Rainey et al., 2004). To further describe this phenotype, we established a “masculinization index” based upon seven anatomic features that were present in males but absent in females (Table 1). Accordingly, external genitalia of normal wild-type males scored 7, while normal wild-type females scored 0. Female ESR1-KO and NOER mice were profoundly masculinized and scored 5.5 on average. Even more perplexing is the phenotype of female AROM + mice that over-produce estradiol (Li et al., 2003a). AROM + mice have been engineered to express both mouse and human aromatase, which results in elevated serum estrogen levels (Li et al., 2001, 2003b). These female AROM + mice exhibit almost complete masculinization of their external genitalia (Fig. 9E). Such a severe and unexpected phenotype need further exploration to elucidate the mechanisms that produce this phenomenon.

2. Effects of exogenous estrogens on the external genitalia of mice

2.1. Effects on the urethra: Mild Mouse Hypospadias

Hypospadias in the mouse is quite different from that in human. Hypospadias in male mice may involve two structures: (a) the prepuce and (b) the glanular urethra, either alone, but usually in tandem. Preputial hypospadias in mice is simply an abnormality in the form of the perineal appendage (external prepuce), while estrogen-induced urethral hypospadias is typically an abnormality in the urethral meatus. A mild form of mouse hypospadias is defined by the anatomic relationship between the ventral cleft in the MUMP ridge (see Fig. 11A) and two internal structures: (a) the tip of the os penis and (b) the urethral flaps (Fig.

11B–E). Normally, in transverse sections of the penis of oil-treated adult mice, the urethral flaps and os penis are located proximal to the open ventral cleft in the MUMP ridge, and thus transverse sections containing an open ventral cleft do not contain the urethral flaps or os penis (Fig. 11B–C). In prenatally DES-treated mice, transverse sections containing an open ventral cleft also contain the os penis and/or urethral flaps (Mahawong et al., 2014a) (Fig. 11D–E). Analysis of serial sections of oil-treated and DES-treated mice indicates that the ventral cleft extends further proximally in DES-treated mice versus oil-treated mice. Indeed, the MUMP ridge may also have a greater proximal-distal length in DES-treated mice than in untreated controls (Mahawong et al., 2014a). Curiously and unexpectedly, a similar mild form of mouse hypospadias has been reported in aromatase knockout mice in which levels of serum estradiol are decreased (Cripps et al., 2019; Govers et al., 2019).

2.2. Effects on the urethra: severe mouse penile hypospadias

The salient feature of human hypospadias is mal-positioning of the urethral meatus from the tip of the penis to more proximal positions along the glans and/or penile shaft. In the mouse, estrogen-induced urethral malformations are localized to the glans penis, especially in the urethral meatus (Weiss et al., 2012a). Severe malformations of the glans penis are observed in mice subjected to perinatal treatment with exogenous estrogen. In particular, neonatal (DES) treatment of mice elicits striking malformation of the penile urethral meatus, which is well illustrated by scanning electron microscopy (SEM).

As a result of perinatal treatment with DES, the glans of the mouse penis and the urethral meatus are profoundly malformed (Cunha et al., 2015; Mahawong et al., 2014a, 2014b; Sinclair et al., 2016a) (Fig. 13A–D, as compared with Fig. 12). The MUMP is severely truncated (Sinclair et al., 2016a) (Fig. 13B) with a bulbous distal tip and patterning of the MUMP ridge is altered into a multitude of individual processes across the epithelium separated by deep grooves (see asterisks in Figs. 13A and 13C–D). Penises of AROM + mice also exhibit profound malformations of the distal aspect of the penis, including an abnormal urethral meatus and a truncated bulbous MUMP (Blaschko et al., 2013a) (Fig. 13E–F). In addition, penises of adult neonatally DES-treated (P0–P10) mice (Fig. 13B–D) and AROM + mice develop a ventral tether connecting the surface of the penis with the inner surface of the preputial mucosa (Blaschko et al., 2013a; Mahawong et al., 2014b; Sinclair et al., 2016a) (Fig. 13B–D). It should be noted that such ventral tethering is not seen in mice treated prenatally with DES for embryonic day 12–18 (E12–E18) (Mahawong et al., 2014a), emphasizing the importance of timing of DES exposure.

The normal MUMP ridge has a constellation of processes that extend across the epithelial surface, shallow grooves and a ventral cleft as seen in SEM images (Fig. 12C). Transverse histologic sections of untreated adult mice penises reveal many of the individual processes and shallow grooves and clefts that make up the MUMP ridge (Fig. 14), as well as a shallow cleft between the MUMP and the MUMP ridge. As transverse serial sections are followed proximally, the processes fuse together and become shallow grooves. This constellation of clefts and processes within the MUMP ridge and between the MUMP and the MUMP ridge in untreated adult mice forms the basis of proposing that the Y-shaped urethral meatus forms as a result of bilateral fusion of the surface processes.

We propose that neonatal mouse DES treatment perturbs the multiple fusion events involved in normal formation of the urethral meatus described above. Malformations revealed in wholemount and SEM images (Figs. 13A and 13C–D) of the urethral meatus in mice treated perinatally with DES are also observed in histologic sections and three-dimensional reconstructions (3DRs) (Fig. 15), providing further evidence for DES-induced perturbation of fusion between the MUMP and processes that constitute the MUMP ridge.

In summary, striking disturbances in the penile development of neonatally DES-treated mice include shortening of the MUMP, abnormal size and patterns of MUMP ridge processes, perturbation of fusion between individual MUMP ridge processes, absence of the ventral cleft, and the presence of a frenulum-like ventral tether attached to the inner surface of the external prepuce, resulting in an abnormal urethral meatus and gross penile structure.

2.3. Effect of perinatal DES treatment on mouse penile growth

While perturbation of fusion events may be to blame in DES-induced malformation of the MUMP and urethral meatus, another disturbed mechanism in DES-induced penile malformations involves inhibition of growth of the distal mouse penis, which can be observed at 5 days postnatal (P5) both at the macroscopic (Figs. 16 and 18) and microscopic levels (Fig. 17). DES-induced inhibition of growth was also verified by morphometric analysis of various penile components (Sinclair et al., 2016a, 2016b) (Fig. 18). The developing male external genitalia of postnatally DES-treated mice are markedly reduced in size by P5 (Fig. 16), including a reduction in the diameter of the preputial lamina, as measured from its internal basement membrane (green lines in Fig. 17C–D, 17K–L, and 17O–P). Continuation of postnatal DES treatment through P10 results in permanent growth impairment of the adult glans: in DES-treated males, the glans measured only 3.80 mm in length, as compared to an average oil-treated glans length of 5.44 mm (Fig. 18).

3. Effect of DES treatment timing

3.1. Timing of DES effects on mouse external prepuce

The perineal appendage or external prepuce exhibits minimal abnormalities in oil-versus prenatally (E12–E18) DES-treated mice (Fig. 19A–D), while neonatal DES treatment (P0–P10) elicits profound malformation of the external prepuce (Fig. 19E–H). Deleterious effects of DES are more severe in C57BL/6 than CD-1 mice, which is consistent with the known increased estrogenic sensitivity of C57BL/6 (Spearow et al., 1999).

3.2. Timing of DES effects on mouse erectile bodies

The corpora cavernosa urethrae (CCUr) are bilateral erectile bodies that begin in the urethral flaps and extend proximally to where they lie ventral to the urethra. The CCUr are circumscribed by fibromuscular capsules and are closely associated with the urethra. A CCUr index was devised from transverse sections based upon three criteria: (a) well-defined erectile bodies with a distinct surrounding fibromuscular capsule, (b) red blood cells and/or blood vessel spaces within the structure, and (c) urethral flaps are present and contain CCUr erectile tissue. A score of 0 for each criterion was given if the feature was absent, a score of 0.5 was given if the feature was present but reduced or poorly developed, and a score of 1

was given if the feature was present and morphologically normal. Thus, scoring ranged from 0 to 3. The severity of CCUr malformation as quantified by the index was dependent upon the timing of the DES treatment period (Fig. 20D). A similar inhibitory effect was seen for the MUMP corpora cavernosa (Table 2). Histologically, the CCUr of oil-treated mice (Fig. 20A2) have well-developed fibromuscular capsules, which are indistinct in the DES-treated groups (both DES E12–P10 and DES P0–P10) (Fig. 20B1–B2 & 20C1–C2). Thus, the most severe defects were seen when treatment occurred at least in part during the first ten days after birth.

3.3. Timing of DES effects on the MUMP

DES treatment inhibits the development of the MUMP, specifically (a) growth of the MUMP and (b) differentiation of the MUMP cartilage, and the effect is dependent upon the timing of DES treatment (Sinclair et al., 2016b) (Fig. 21S). Truncation of the MUMP is minimal in mice treated with DES from E12–E18 and P5–P10, with no significant effect in the P10–P20 group (Fig. 21S). Severe truncation of the MUMP was seen in mice treated with DES from E12–P10 and P0–P10 (Fig. 21I–J & 21O–P)., and the underlying mechanism may be DES-induced inhibition of growth as discussed above. However, careful examination of relevant transverse sections (Fig. 17I–K & 17M–O) demonstrates an undifferentiated mesenchyme in place of MUMP cartilage, implying a defect in cartilage differentiation. Importantly, defects seen at P10 or P15 persist into adulthood, indicating that effects seen during development are likely irreversible. DES treatment terminated before P0 or initiated after P10 (E12–E18 and P10–P20) did not impair MUMP cartilage differentiation (Sinclair et al., 2016b) (Table 2). Bone length is also inhibited in mice treated from P0–P10 with DES (Fig. 21T).

3.4. Normal development of the murine preputial lamina and timing of DES effects on tethering

‘At E14, the normal mouse external genitalia consist of the GT, which projects from the perineum, flanked laterally by the preputial swelling (Fig. 22). At E16 and thereafter, the PS grow distally to (a) cover the developing GT (Baskin et al., 2002) and (b) converge and fuse in the ventral midline (Perriton et al., 2002; Petiot et al., 2005). During this process, the preputial lamina is laid down (Fig. 23), presumably as a result of fusion of epidermis of the preputial swelling with the epidermis of the GT (Cunha et al., 2020b; Liu et al., 2018b). Ventral tethering of the penis was observed in mice treated with DES from E12–P10 and P0–P10, but not in mice treated prenatally with DES from E12–E18, P5–P15, or P10–P20 (Fig. 21I–J & 11O–P). DES-induced penile tethering is a perturbation of the development of the preputial lamina, whose initial embryonic development is identical in males and females (Cunha et al., 2020b; Liu et al., 2018b). The salient feature of ventral tethering is retention of a confluent stromal channel linking the stromal wall of the prepuce with the stroma of the penis. The connection between these two stromas is covered externally by epithelium, and thus the surface epithelium of the penis is continuous with the epithelial lining the preputial space when a tether is present (Figs. 10, 13, 15 and 26).

As the preputial swelling approach each other to fuse in the ventral midline (Fig. 24C, black arrows) late in gestation, a ventral groove is formed which is called the preputial urethral groove (PUG) (Fig. 24, red arrowheads). The edges of the preputial urethral groove fuse to

form the urethra (Fig. 24E), while the ventral portion of the preputial urethral groove flattens to contribute to the preputial epidermis (Fig. 24F–I).

In normal mouse development, a ventral gap formed by the separation of the preputial lamina and the urethral epithelium (Fig. 24G–I) and a confluence of the penile and preputial stromas (Fig. 25) are evident at P0, remain open at P15 (data not shown), and disappear completely by P20. From P0 to approximately P25, the preputial lamina remains solid, but from P25–P30, it canalizes to create the preputial space that houses the penis (Fig. 25). The timing and process of canalization of the preputial lamina has previously described (Mahawong et al., 2014a; Cripps et al., 2019).

DES treatment results in the penile-preputial stromal confluence persisting into adulthood, and ventral tethering as the preputial lamina canalizes. In many cases, the tether is very narrow and frenulum-like, but it can be quite wide in some DES-treated specimens. The process of preputial delamination is affected by reduced estrogen levels, as preputial delamination is initiated precociously in aromatase knockout mice at 15 days postpartum, as compared to 25 days postpartum in wild-type mice (Cripps et al., 2019).

Penile tethering has severe consequences for affected males by affecting penile mobility and preventing distal extrusion of the penis during mating, which impairs fertility. In addition, there are two urinary consequences of penile tethering. Affected males exhibit a perpetually wet and urine-stained perineum and preputial stones form within the preputial space (Mahawong et al., 2014b; Warner et al., 1979). These stones are composed of crystallized urine and particularly large stones can erode the penile surface (Warner et al., 1979). Our interpretation of these events is that during urination in normal mice, the “freely mobile” penis is extruded beyond the preputial meatus so that urine can be expelled completely exterior to the body. In mice with penile tethering, urine is expelled into the preputial space and then subsequently dribbles out, while urine retained in the preputial space crystallizes to form stones.

3.5. Strain differences in response to perinatal diethylstilbestrol

While this topic has not been explored adequately, mouse strain has a predictable influence on perinatal effects on penile and preputial development. As noted above, the mild form of mouse hypospadias involves the anatomic relationship between the open ventral cleft in the MUMP ridge and the os penis and urethral flaps (Fig. 11). Whether the os penis and urethral flaps are associated with an open ventral cleft is affected by the mouse strain. The more estrogen-sensitive C57BL/6 strain (Spearow et al., 1999) shows a higher incidence of this defect in comparison to CD-1 mice (Table 3). Likewise, DES-induced malformation of the external prepuce was more severe in C587BL/6 versus CD-1 mice (Fig. 19).

3.6. Estrogen receptor expression in the mouse penis and clitoris

The developing mouse penis and clitoris express the ESR1 and ESR2 (Zheng et al., 2015; Martin-Alguacil et al., 2008b; Yamashita, 2004; Agras et al., 2007), as well as the androgen receptor (Agras et al., 2006). As in humans, ESR2 is the predominant receptor and is widely expressed (Blaschko et al., 2013b). In P10 mice, ESR1 and ESR2 were detected in urethral epithelium and MUMP cartilage, with ESR1 being expressed in virtually all chondrocytes

(Blaschko et al., 2013b) (Figs. 27 and 28), while ESR2 was detected in only a subset of chondrocytes. ESR2, but not ESR1, was detected in the MUMP ridge groove epithelium (internal preputial lamina) and in the epithelium of the external preputial lamina. The expression profiles of both estrogen receptors in the erectile bodies was also varied: the corpus cavernosum glandis was positive for ESR2 only; the MUMP corpora cavernosa was positive for ESR1 and weakly positive for ESR2; and the corpus cavernosum urethrae were positive only for ESR1, (Blaschko et al., 2013b). The fact that ESR1 and ESR2 are broadly expressed in the developing murine external genitalia supports the idea that estrogens may be involved in normal development and are likely the basis for some penile malformations that cannot be explained by abnormalities in androgen action alone.

Based on the results of studies affecting estrogen levels in the developing external genitalia, ESR1 and/or ESR2 should be expressed in a temporo-spatial pattern consistent with DES induction of the many malformations discussed above. Unfortunately, there are many gaps in the ontogeny of ESR1 and ESR2 expression in mouse external genitalia throughout the critical timing window of estrogenic teratogenic sensitivity and across the myriad of structural elements that constitute the developing mouse penis.

4. Discussion

There is an extensive literature on the role of androgens in the normal development of mouse external genitalia (Zheng et al., 2015; Agras et al., 2006). Recent evidence, however, has emerged that estrogens may play a role along with androgens during normal and abnormal external genitalia development (Yang et al., 2010b; Zheng et al., 2015; Govers et al., 2019; Sinclair et al., 2016b). Indeed, penile abnormalities may be due to alteration in the estrogen and/or androgen signaling balance, leading to induction of penile abnormalities characterized as hypospadias in animal models treated with exogenous estrogen (Kim et al., 2004; Blaschko et al., 2013b; Mahawong et al., 2014a, 2014b; Sinclair et al., 2016b, 2016c).

One of the key controversies recognized in the literature is an imprecise definition of mouse hypospadias and a failure to define whether malformations of the external genitalia documented prior to birth, at birth, and in the neonatal period persist as true urethral defects in adult mice (Kim et al., 2004; Cunha et al., 2015; Sinclair et al., 2016c). It is imperative that the consequences of developmental exposure to exogenous estrogens be assessed in adulthood to eliminate the possibility that effects observed during perinatal periods are not merely developmental retardation that can be corrected with future compensatory growth. The study of “mouse hypospadias” requires exacting anatomical terminology and an understanding of the similarities and differences between the mouse and human and normal and abnormal genitalia. In humans, hypospadias is defined by an ectopic urethral meatus located on the ventral aspect of the penis on the glans, coronal margin, penile shaft, scrotum, or perineum with deficient spongiosal support (Baskin, 2017) (Fig. 8). Human hypospadias is also associated, with penile curvature, especially in severe cases.

In contrast, the aspects of male murine external genitalia relevant to “mouse hypospadias” are (a) the penile glans, that portion of the penis protruding outside the body (albeit within the external preputial space) (Rodriguez et al., 2011, 2012), and (b) the external prepuce or

perineal appendage. The so-called body of the mouse penis is situated deep below the body surface (Rodriguez et al., 2011, 2012; Liu et al., 2018b; Sinclair et al., 2016c), and has not been considered to be involved in hypospadias. Therefore, by definition, estrogen-induced malformation of the mouse urethra translates solely to glanular hypospadias in humans (Weiss et al., 2012a; Schlomer et al., 2013; Baskin, 2017).

During normal external genitalia development in both humans and mice, it has become clear that there is a “window of susceptibility” for the action of hormones such as androgens, estrogen, and Mullerian inhibiting substance (MIS). In human males, normal urethral development occurs from 8 to 18 weeks gestation with the formation of the tubular urethra in the penile shaft and glans (Baskin et al., 2018a; Liu et al., 2018a). Without androgens present during this time period or if androgen exposure occurs later in gestation, normal urethral development does not occur (Baskin and JMaT, 2020; Baskin, 2017). This is consistent with the clinical observation that postnatal administration of androgens to patients with hypospadias does not correct hypospadias or elicit normal urethral development (i.e. the penis of infants will grow in response to exogenous androgens, but the ectopic urethra/hypospadiac defect remains) (Baskin and Ebbers, 2006; Baskin, 2017). The same is true of MIS, which in humans acts between 9 and 13 weeks gestation to elicit regression of the Mullerian duct (Taguchi et al., 1984). If the onset of MIS production occurs later that expected in gestation, this will result in retained or persistent Mullerian structures in XY genetic males since the MIS receptor is no longer sensitive to the action of MIS (Salehi et al., 2012).

In humans, based on the timing of urethral development, it is clear that developmental exposure to endocrine disruptors would need to occur in the first trimester to elicit effects. This is the case of maternal ingestion of DES, which was administered to pregnant women from the 1940’s to the 1970’s to allegedly prevent miscarriage (which it did not (Dieckmann et al., 1953)), resulting in a spectrum of teratogenic effects throughout the female reproductive tract (Cunha et al., 2020c), clear cell vaginal carcinoma in their daughters, and an increased incidence of hypospadias in their sons (Klip et al., 2002). For females fetuses, the period of susceptibility to the adverse effects of DES was 7–15 weeks of gestation (Jefferies et al., 1984), a slightly narrower window than that for males, as discussed above.

In rodents, there is also a window of susceptibility in respect to hormonal sensitivity of the developing external genitalia to exogenous estrogens. Prenatally-administered estrogen-induced mouse hypospadias is characterized by subtle alteration in the patterning of the urethral meatus, which is defined by abnormalities of the MUMP and MUMP ridge (Kim et al., 2004; Mahawong et al., 2014a; Sinclair et al., 2016c) (Fig. 11). There is also abnormal positioning of the internal penile elements such as the os penis and urethral flaps relative to the ventral cleft in the MUMP ridge. These are enduring defects that persist into adulthood. Based on these observations, the distal most portion of the mouse penis is particularly sensitive to exogenous estrogen.

A key lesson from these observations is the imperative of objective criteria defining “mouse hypospadias,” which persists through the end of puberty and into adulthood when urethral abnormalities are permanent and irreversible. The objective criteria for diagnosing

prenatally-administered estrogen-induced mouse hypospadias are: (a) exposed urethral flaps, (b) an exposed os penis," (c) an elongated ventral cleft in the MUMP ridge, and (d) any abnormality in the shape or position of the elements (MUMP and MUMP ridge) that constitute the urethral meatus. The first three criteria are best judged in serial histological sections, while the form of the urethral meatus can be determined in adult specimens by simple observation with a dissecting microscope, scanning electron microscopy, and/or 3D reconstruction (Figs. 11, 13, 15, 18, 19 and 21).

Mild effects of prenatal exposure to estrogen are exacerbated by neonatal and postnatal DES exposure, further inducing a constellation of malformations of the external genitalia (Mahawong et al., 2014b) (Figs. 13 and 15). Neonatally and postnatally DES-treated mice exhibit severe truncation of the prepuce and glans penis, an abnormal urethral meatus, ventral penile tethering, a reduced os penis length, impaired MUMP growth, impaired MUMP cartilage differentiation, abnormal urethral flaps, and impaired differentiation of the penile erectile bodies. The adverse effects of DES appear to correlate with the expression of estrogen receptors within the affected tissues (Blaschko et al., 2013b), though the many gaps in the ontogeny of ESR1 and ESR2 in developing mouse external genitalia make it difficult to determine much more in detail. Many of DES' effects are due to impaired growth and tissue fusion events during development, indicating that the timing of DES exposure is critical, as the greatest severity of deleterious effects are elicited by neonatal exposure. Expanding on this concept, mice treated with DES during the embryonic time period (E12–E18), early postnatal period (P0–P10), embryonic and postnatal period (E12–P10), postnatal period (P5–P15), and late postnatal period (P10–P20) all exhibited reduced penile size, based on morphometric analysis of the internal penile anatomy. However, the most profound effects on male mouse external genitalia were observed when the period of DES treatment extended from P0–P10 (Sinclair et al., 2016b).

5. Conclusion

Both the human and mouse developing external genitalia express ESR1 and ESR2 along with the androgen receptor. Human clinical data suggests that exogenous estrogens can affect normal penile and urethral development, resulting in hypospadias. Experimental mouse data also definitively demonstrates that exogenous estrogen administered during critical developmental windows causes penile and urethral defects. Timing of estrogen exposure or the "window of susceptibility" is an important concept in both humans and mice. Even though the manifestations of mouse and human hypospadias are distinctly different, estrogen-induced "mouse hypospadias" exhibits certain morphogenetic homologies to human hypospadias, due to shared developmental processes, that can provide further valuable insight into the mechanisms of external genitalia development and how these mechanisms respond to abnormal hormonal environments (Liu et al., 2018b; Cunha et al., 2015; Mahawong et al., 2014a, 2014b; Sinclair et al., 2016c).

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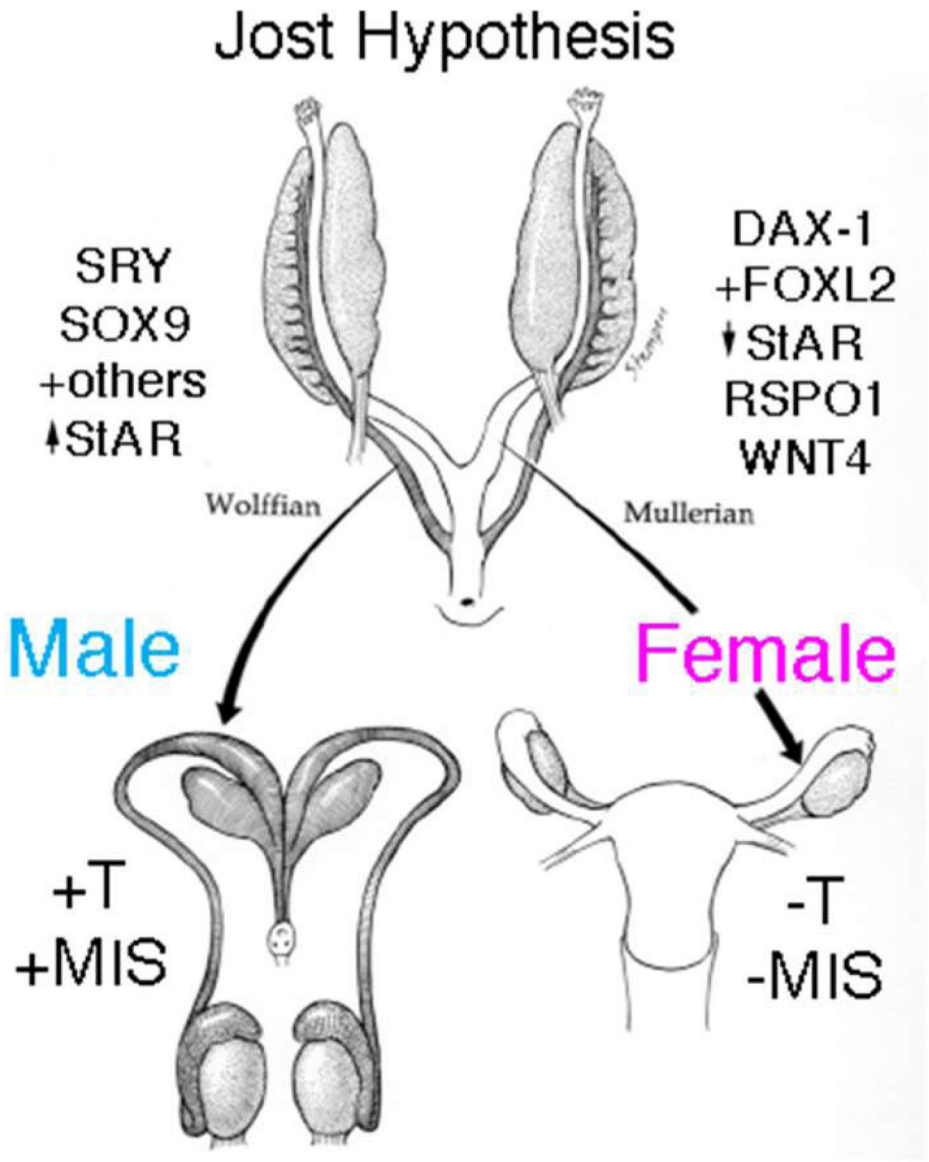

Fig. 1.

Jost Hypothesis of Sexual Differentiation. T = Testosterone, MIS = Mullerian inhibiting substance.

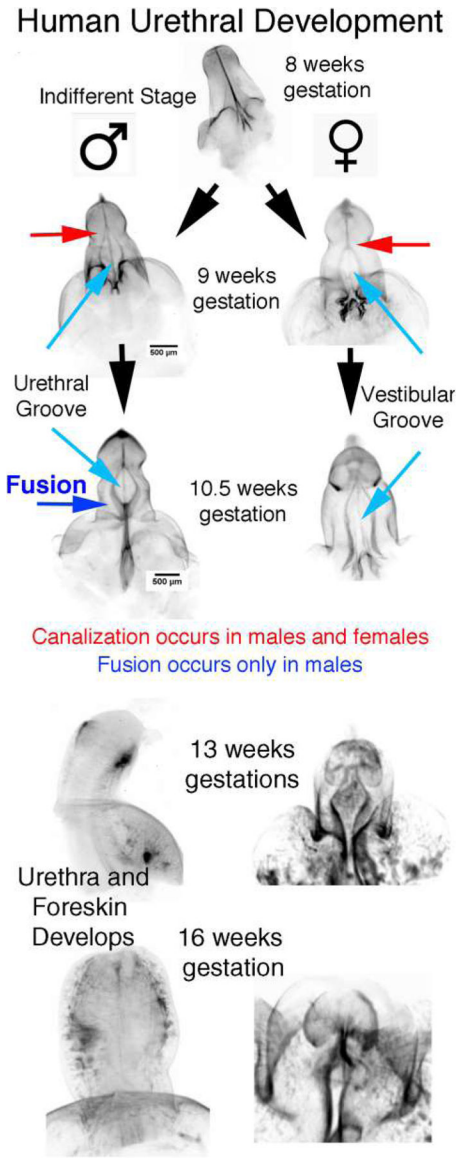

Fig. 2.

Human Urethral Development from the Indifferent Stage at 8 weeks Gestation to Mature Development at 16 weeks gestation Using Optical Projection Tomography. Note complete formation of the tubular urethra in the penile shaft by fusion of the urethral folds and formation of the glanular urethra extending to the tip of the penis (left column). In contrast, the vestibular groove remains open in females and forms the labia minora (right column) (Overland et al., 2016; Li et al., 2015).

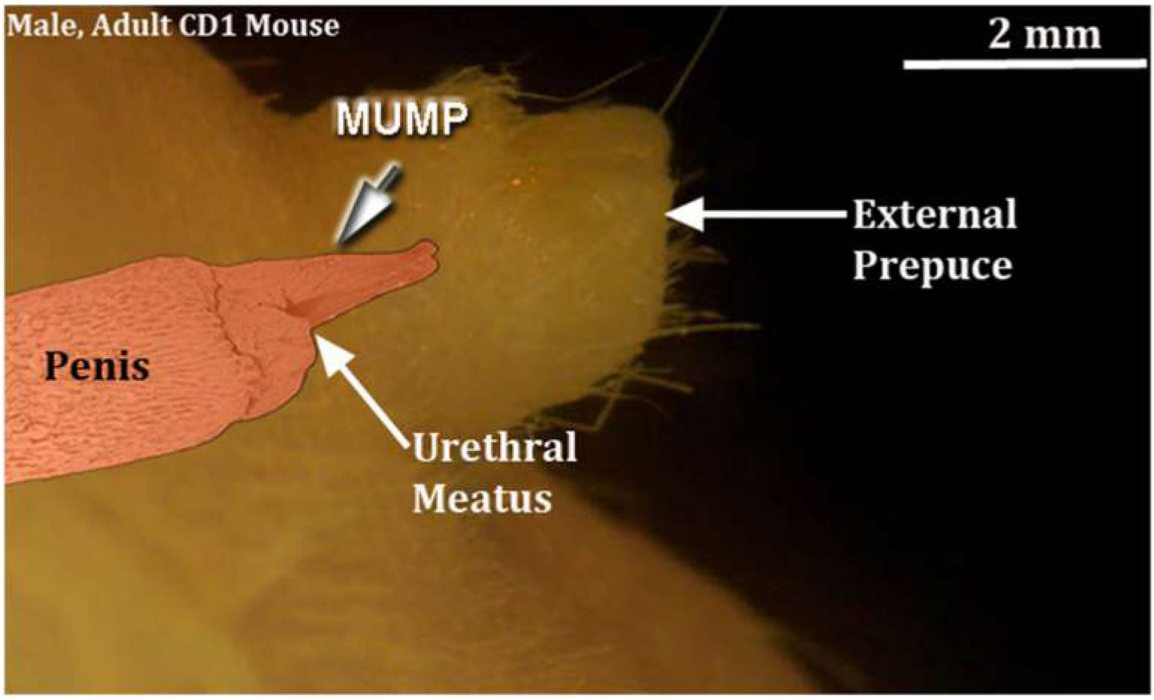

Fig. 3.

Adult CD1 Mouse Penis. Note the penis in the non-erect state is an internal structure housed well within the external preputial space. Position of the tip of the MUMP in the resting state is accurately placed (Cunha et al., 2020c).

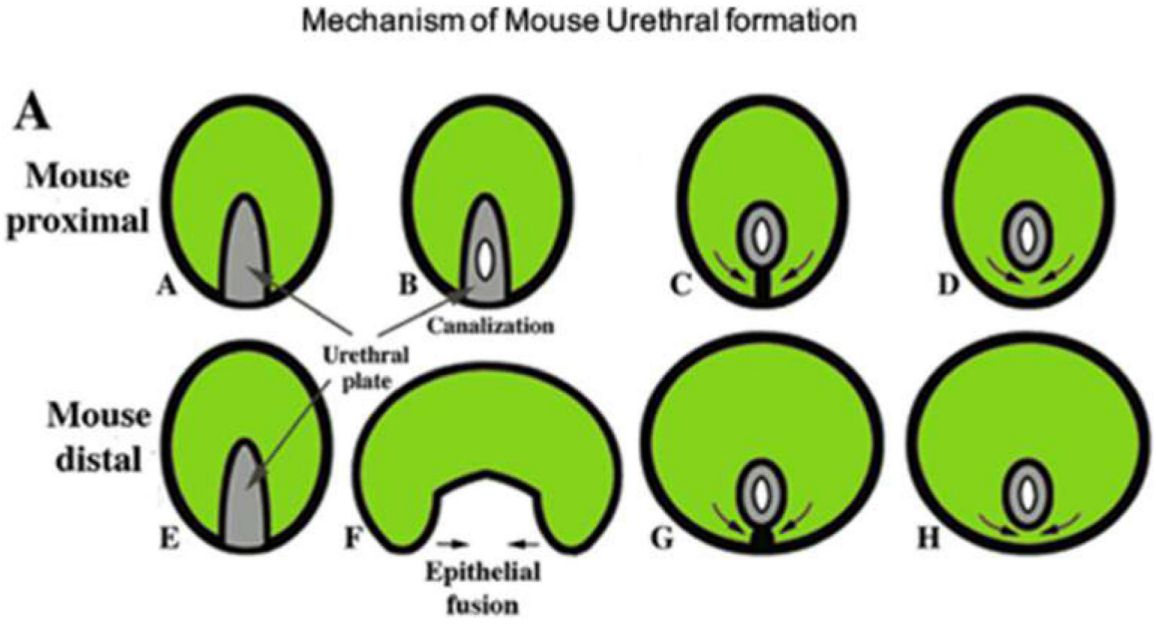

Fig. 4.

Mechanism of Mouse Urethral Formation. The proximal portion of the urethra within the glans develops via direct canalization of the urethral plate (A–D), while the distal aspect of the mouse penile urethra and the urethral meatus form via epithelial fusion (E–H). Finer details of these morphogenetic process can be found in Liu et al. (2018) (Liu et al., 2018b).

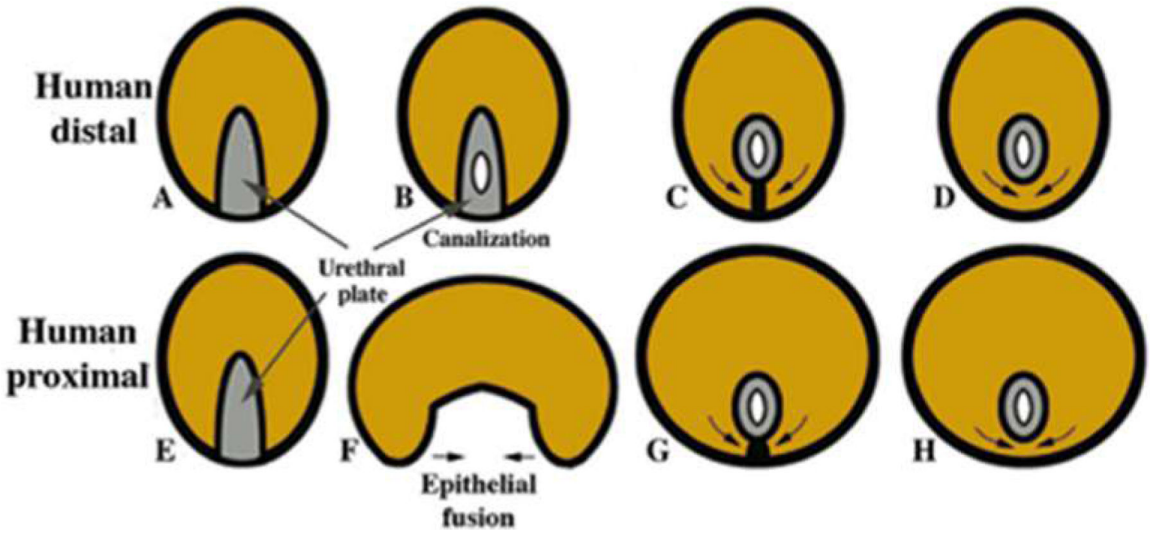

Fig. 5.

Mechanism of Human Penile Urethral Formation. The distal portion of the human penile urethra (within the glans) develops via direct canalization of the urethral plate (A–D), while the proximal portion of the human penile urethra within the shaft forms via canalization of the urethral plate to form the urethral groove, followed by fusion of the urethral folds (E–H). Finer details of these morphogenetic process can be found in Baskin et al., Shen, et al., Cunha, et al., and Liu et al., 2018 (Baskin et al., 2018a; Liu et al., 2018b; Cunha et al., 2019b; Shen et al., 2016).

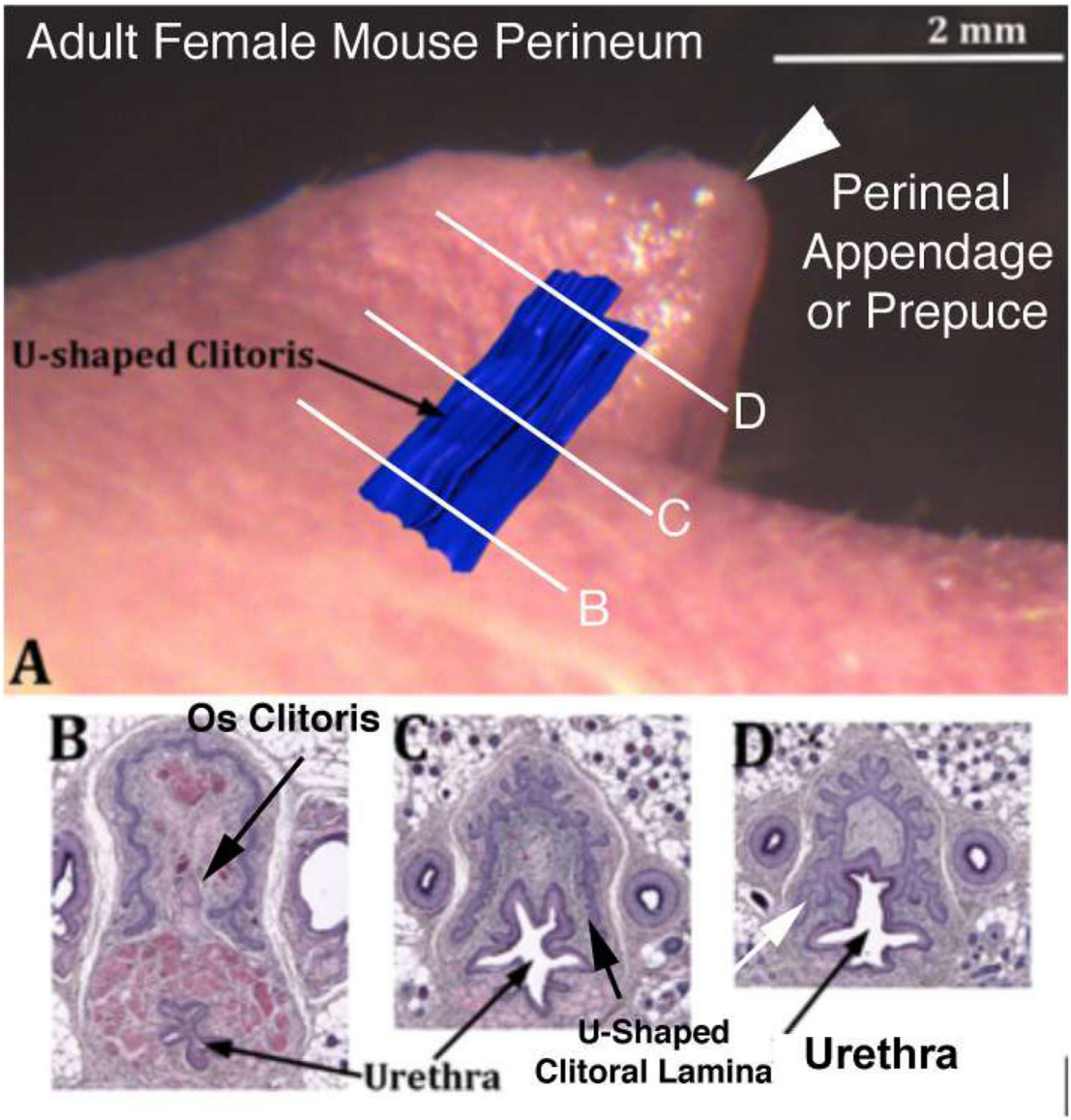

Fig. 6.

(A) Gross micrograph, lateral view of an adult CD-1 mouse perineum. Note the female prepuce (white arrowhead) and the overlaid position of the reconstructed mouse clitoris in blue positioned accurately. Representative transverse histologic sections of adult mouse clitoris: (B) proximal, (C) and (D) distal as indicated. (B) The os clitoris is dorsal and completely separate from the urethra. (C) The U-shaped clitoral lamina is separated from the urethral epithelium stroma (small black arrow). (D) The clitoral epithelial lamina defining the clitoral epithelium is tethered to urethral epithelium (Cunha et al., 2020b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

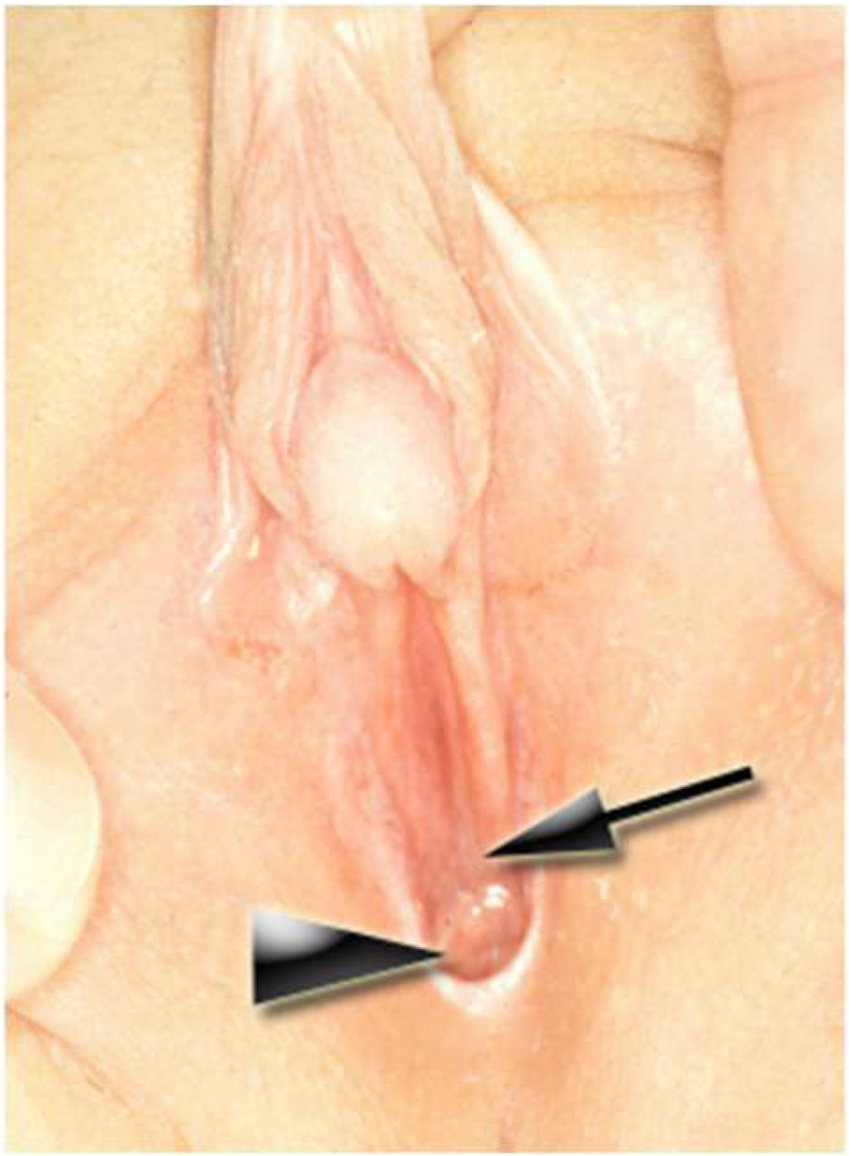

Fig. 7.

Child with 5α reductase type 2 deficiency. Note severe hypospadias (arrowhead = pseudo vagina, arrow = ectopic urethral meatus).

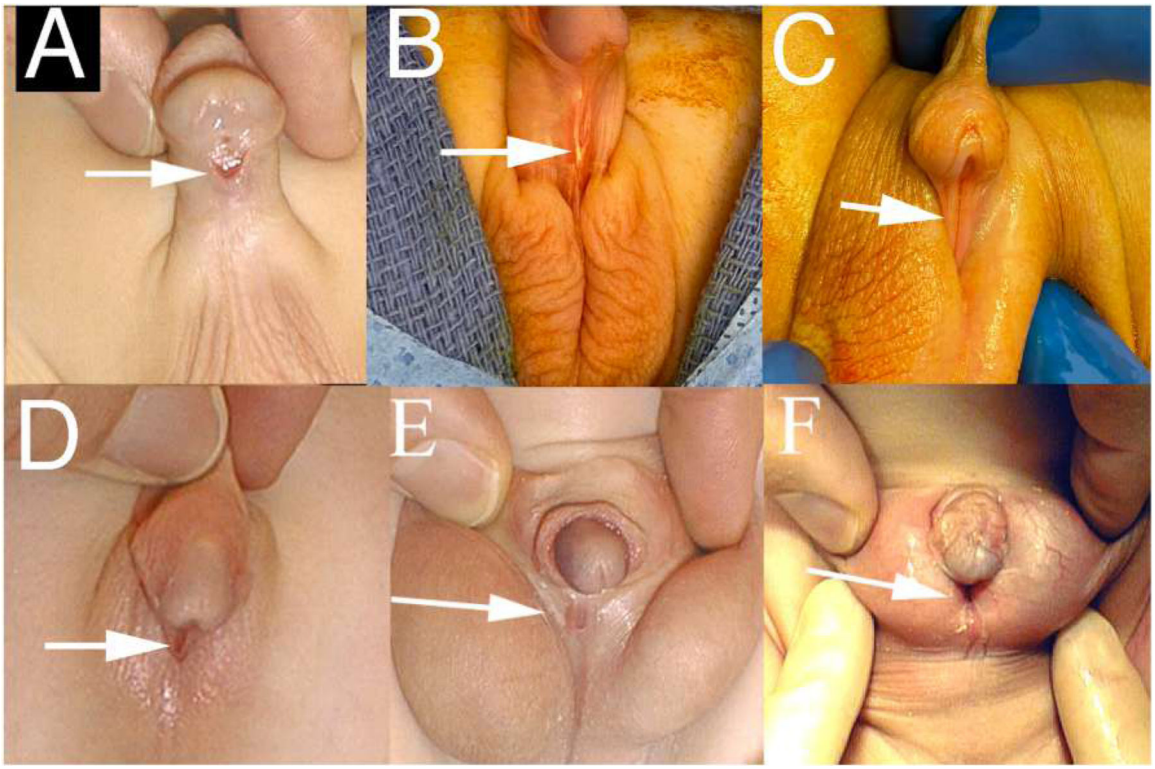

Fig. 8.

Human Hypospadias occurs on a spectrum from mild to severe forms (panel A is least severe through panel F, which is most severe), based on the location of the ectopic urethral meatus (white arrows), associated penile curvature, and dorsal hooded asymmetric foreskin.

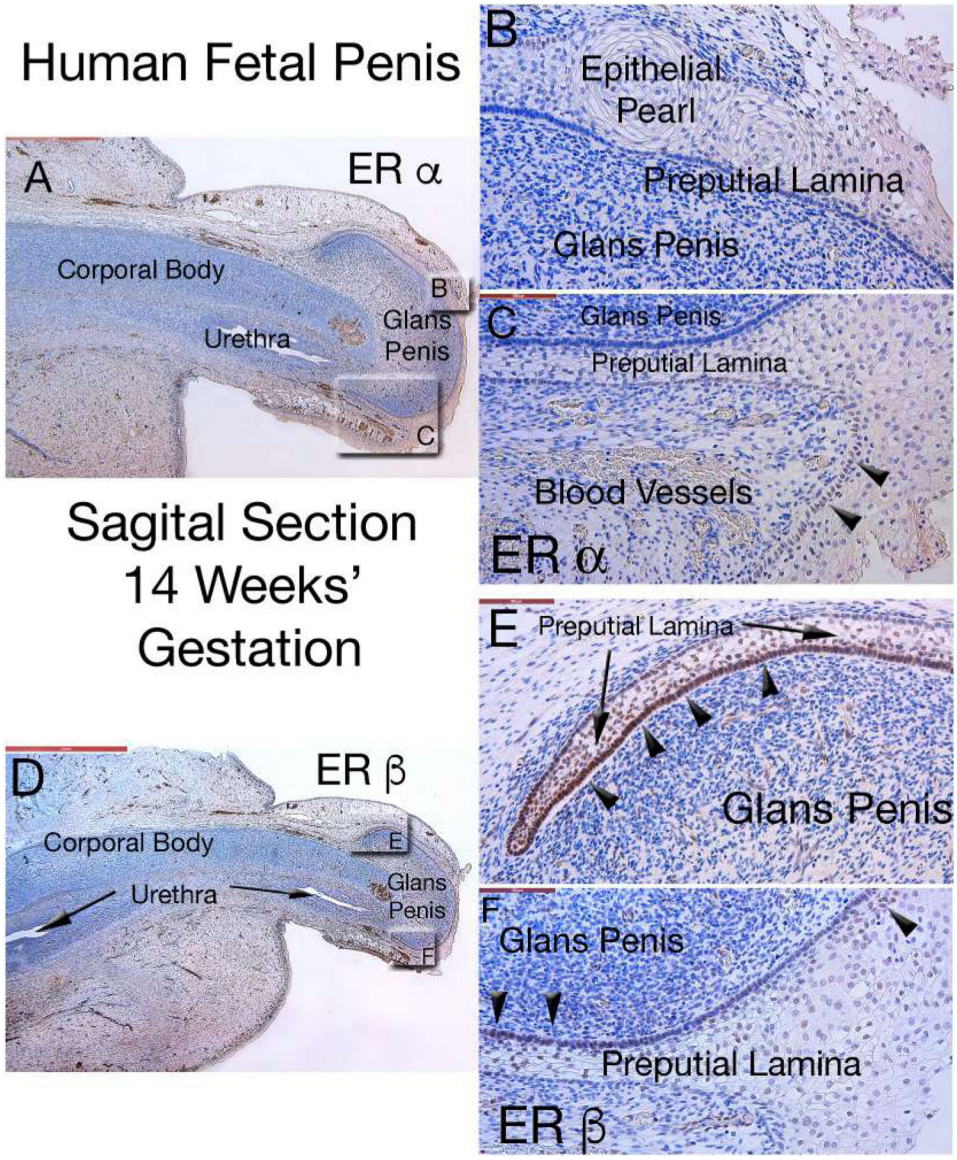

Fig. 9.

Example of ERα (ESR1) and ERβ (ESR2) expression in the human fetal penis. Note the more prominent expression of ESR2 see text for details.

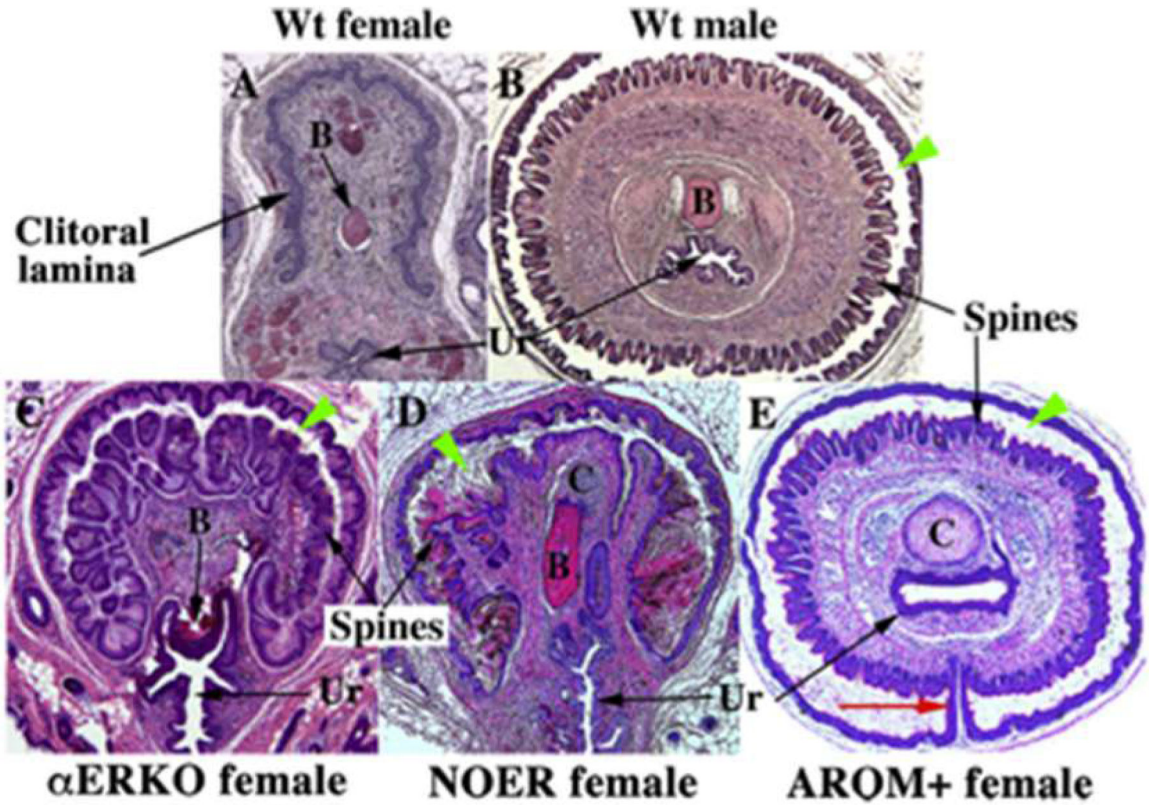

Fig. 10.

Morphology of the adult penis and clitoris in several strains of mice as indicated in transverse histologic sections. Clitori of estrogen receptor α knock-out (ESR1-KO; panel C) and estrogen receptor nuclear-only (NOER; panel D) females are substantially masculinized, while clitori of aromatase knock-in (AROM+; panel E) female mice are almost completely masculinized and exhibit morphology similar to that of the wild-type penis, with the exception of the ventral tethering (frenulum, red arrow). B = bone, C = cartilage, Ur = Urethra, Wt = wild-type. Note the preputial space (green arrowheads) in all specimens except the wild-type clitoris (A). From Cunha et al. (Rainey et al., 2004) with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

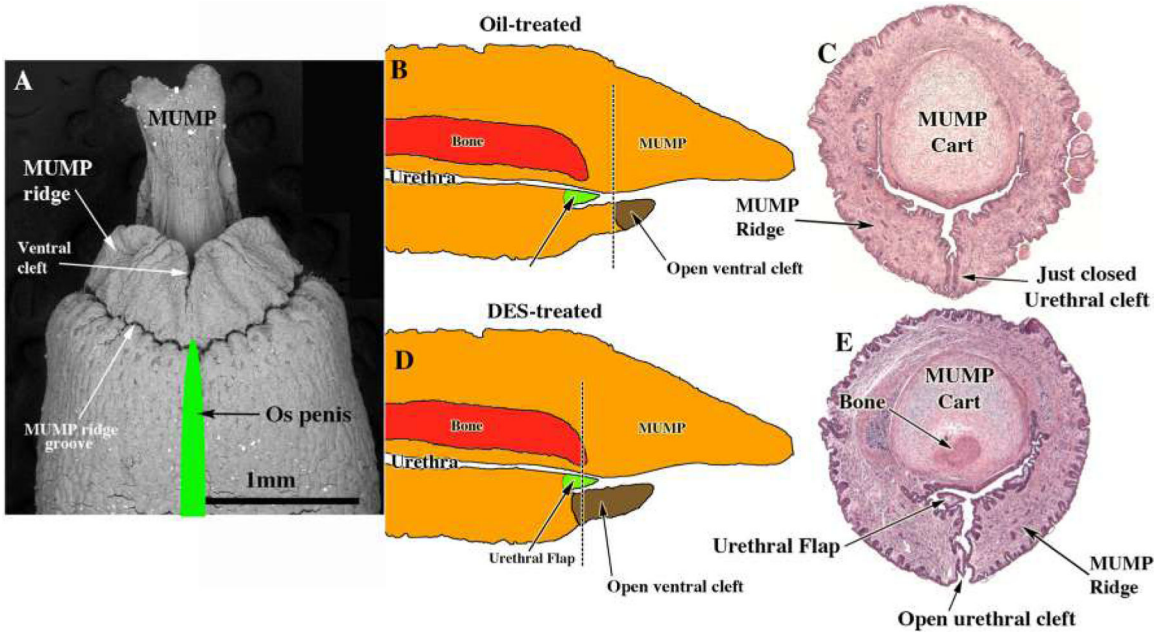

Fig. 11.

Mild Mouse Hypospadias. (A) Scanning electron micrograph of the penis of an adult prenatally oil-treated mouse with the position of the os penis (green) superimposed. Note that the os penis is positioned proximal to the ventral cleft in the male urogenital mating protuberance (MUMP) ridge (B) and in the corresponding histological section (C). The region of the MUMP ridge containing the open ventral cleft in the diagrams is colored brown. In mild hypospadias in the mouse, the ventral cleft extends more proximally than in the control penis, and thus the os penis and urethral flaps are seen in sections with an open ventral cleft (compare panels B and C to D and E, respectively). Panels C and E are transverse H&E-stained sections taken where indicated by the vertical lines in panels B and D. Note the mild hypospadias in (E). Adapted from Mahawong et al. (Mahawong et al., 2014a). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

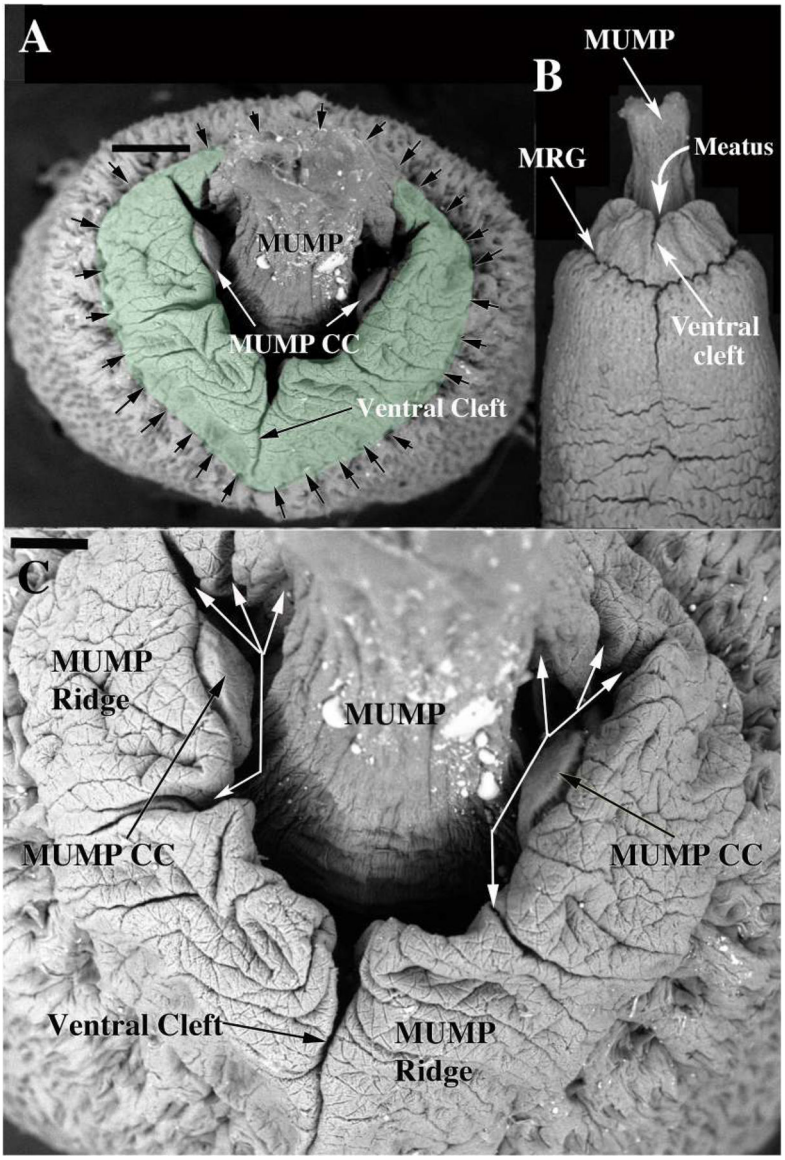

Fig. 12.

Scanning electron micrographs of a normal adult mouse penis. (A) Distal end-on view. The urethral orifice is Y-shaped, with the ventral cleft forming the ventral stem of the “Y”. The male urogenital mating protuberance (MUMP), which is projecting towards the viewer, is dorsally situated and is fused laterally with the MUMP ridge (colorized light green in panel A). The MUMP ridge is demarcated peripherally by the circumferential MUMP ridge groove (small black arrows in A) and labelled MRG in (B). The MUMP corpora cavernosa (MUMP CC) are also seen (C). The MUMP along with the MUMP ridge define the urethral orifice. (B) Scanning electron micrograph of the ventral adult mouse penis. Note the bifid MUMP, the MUMP ridge groove (MRG, small arrows in panel A), and the position of the urethral meatus. Scale bars = 200 μm for (A) and (B), 100 μm for (C). From Blaschko et al. (2013) with permission (Blaschko et al., 2013b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

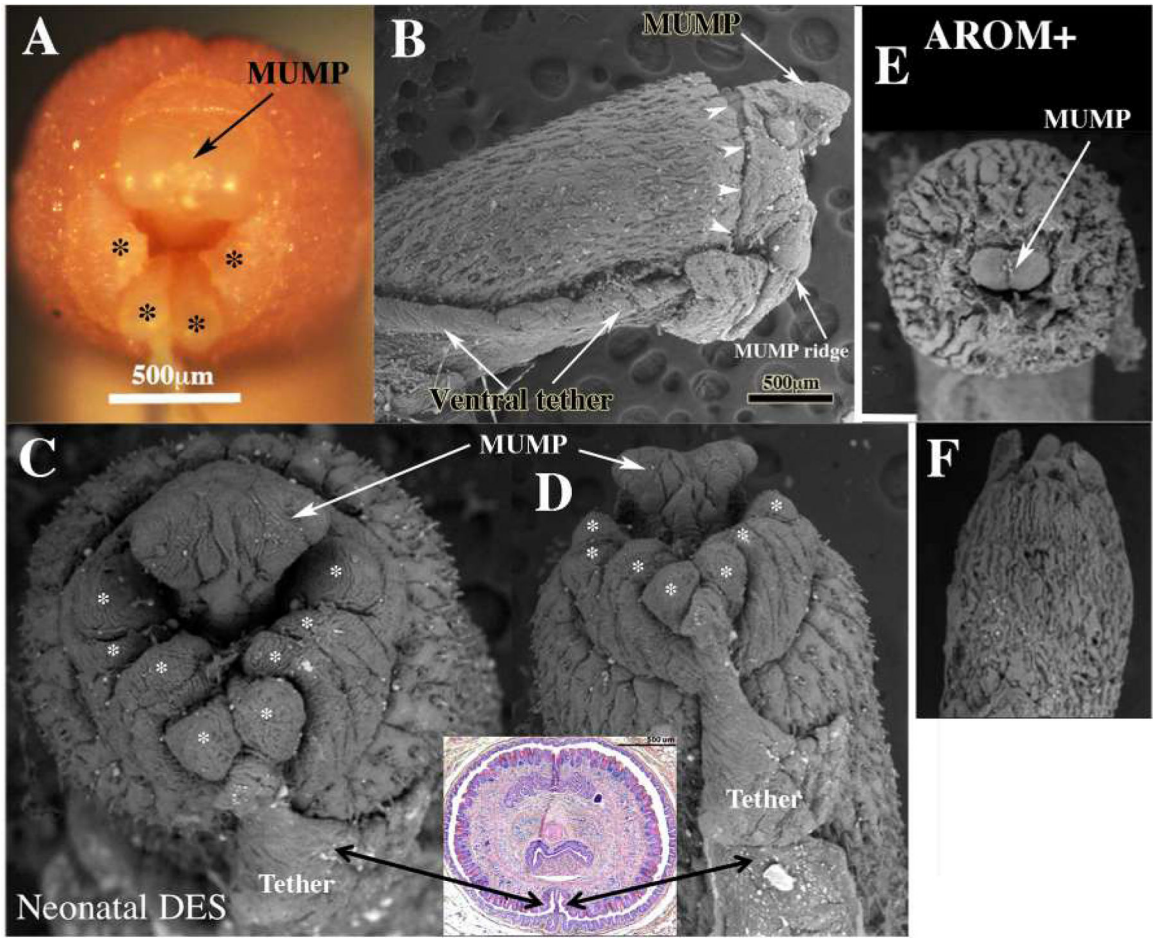

Fig. 13.

Severe Mouse Hypospadias. Wholemount (A) and scanning electron micrographs (B-F) of adult mouse penises. (A–D) Penises of adult CD-1 mice treated daily with diethylstilbestrol (DES; 200 mg/g of body weight) from the day of birth (P0) to postnatal day 10 (P10) (Mahawong et al., 2014b). (A) Adult neonatally DES-treated penis. (B) Side view of an adult neonatally DES-treated penis. (C) End-on view of an adult neonatally DES-treated penis. (D) Ventral view of an adult neonatally DES-treated penis. (A–D) Note truncated male urogenital mating protuberance (MUMP), multiple prominent processes (*) separated by grooves, the prominent groove separating the MUMP from the MUMP ridge (white arrowheads in B), and the ventral tether. (E & F) Adult AROM + penis in end-on view (E) and lateral view (F). From Cunha et al. (2015) with permission (Cunha et al., 2015).

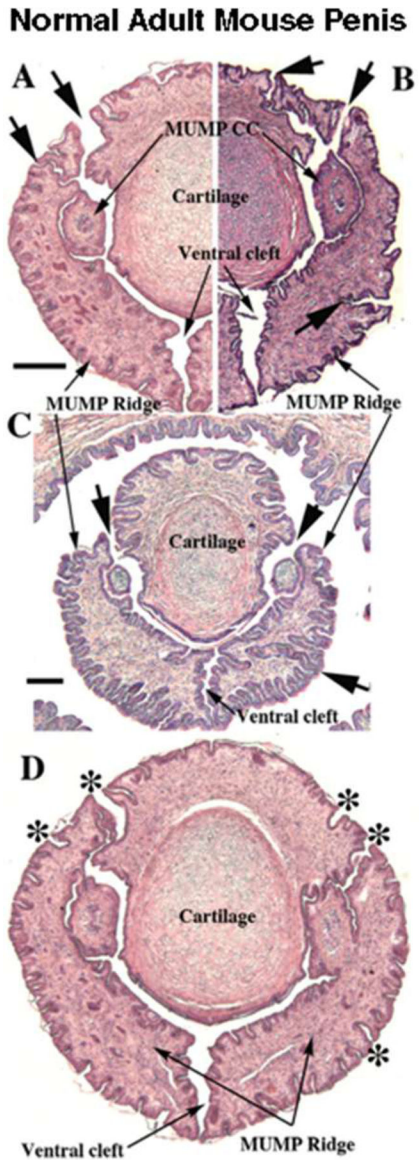

Fig. 14.

Histology of Normal Adult Male Penis. Sections through the glans penis of three adult untreated mice illustrating the clefts (large arrows in A-C) in the male urogenital mating protuberance (MUMP) ridge. In all cases, the prominent ventral cleft is open to the preputial space. Minor clefts penetrate from the penile surface into the incipient urethral lumen in dorsal-lateral positions adjacent to the MUMP, whose core contains cartilage. In all specimens, minor clefts demarcate individual processes on the epithelial surface, which proximally fuse with adjacent structures (the MUMP or other processes) to complete the MUMP ridge. Note minor clefts (black arrows in B and C), which penetrate from the exterior and end blindly within the interior of the MUMP ridge at the 3 to 4 o'clock positions. (D) is a more proximal section in which clefts shown in (A–C) have fused and have become shallow grooves (*). Scale bar = 250 μm for A, B, and D, and 100 μm for C. Adapted from Blaschko et al. (2013) with permission (Blaschko et al., 2013b).

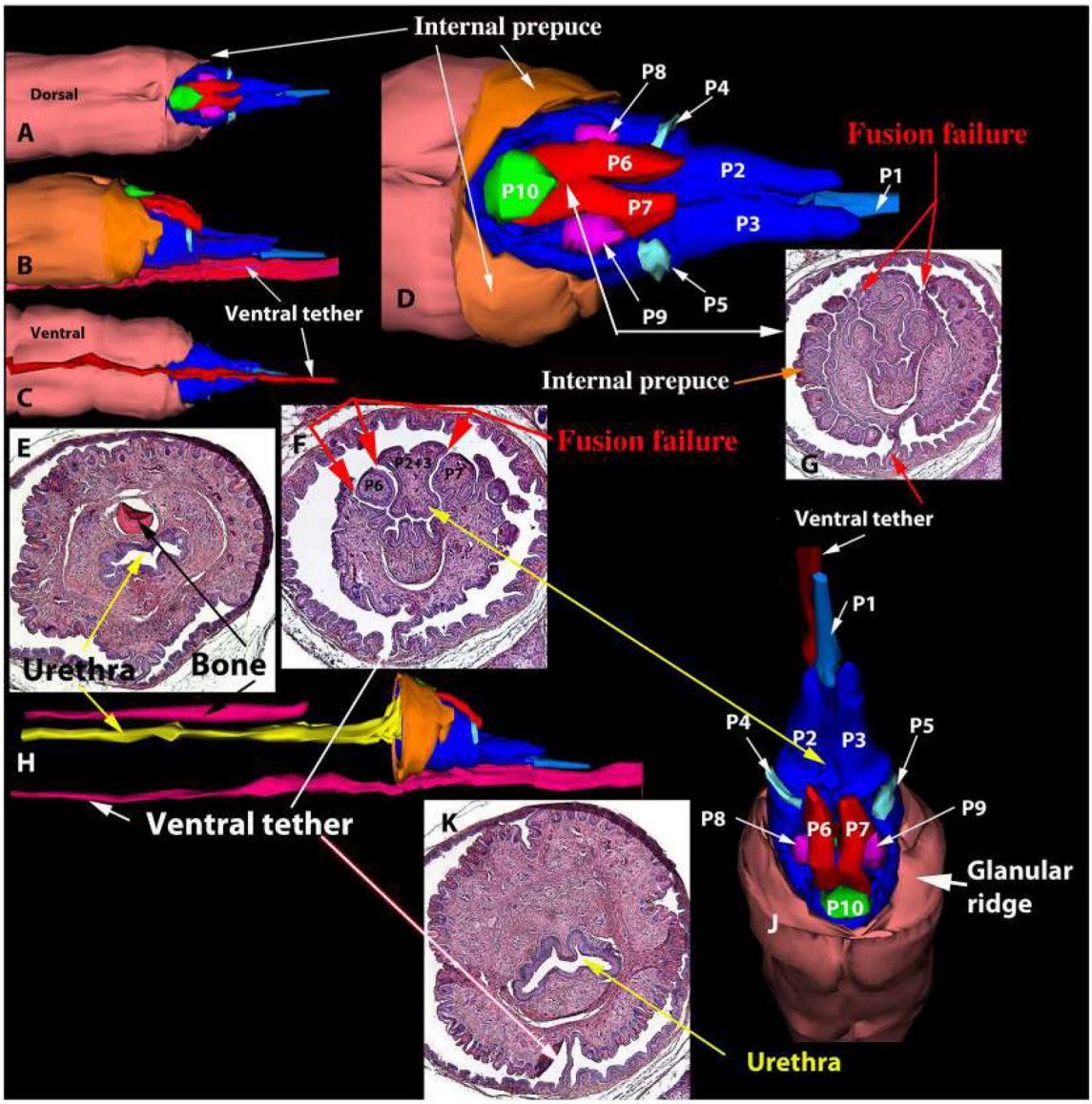

Fig. 15.

Three-dimensional reconstructions (3DR) (A–D, H, J) of the penis of an adult wild-type mouse treated neonatally (P0–P10) with diethylstilbestrol (DES). 3DRs are presented in a variety of orientations with accompanying tissue sections (E–G, K). The patterns of mesenchymal processes across the epithelial surface (p1–p10 at bottom right) denoted in light and dark blue, red, turquoise, magenta, and green are vastly different from that seen in untreated wild-type mice (compare with Fig. 12). From Blaschko et al. (2013) with permission (Blaschko et al., 2013b).

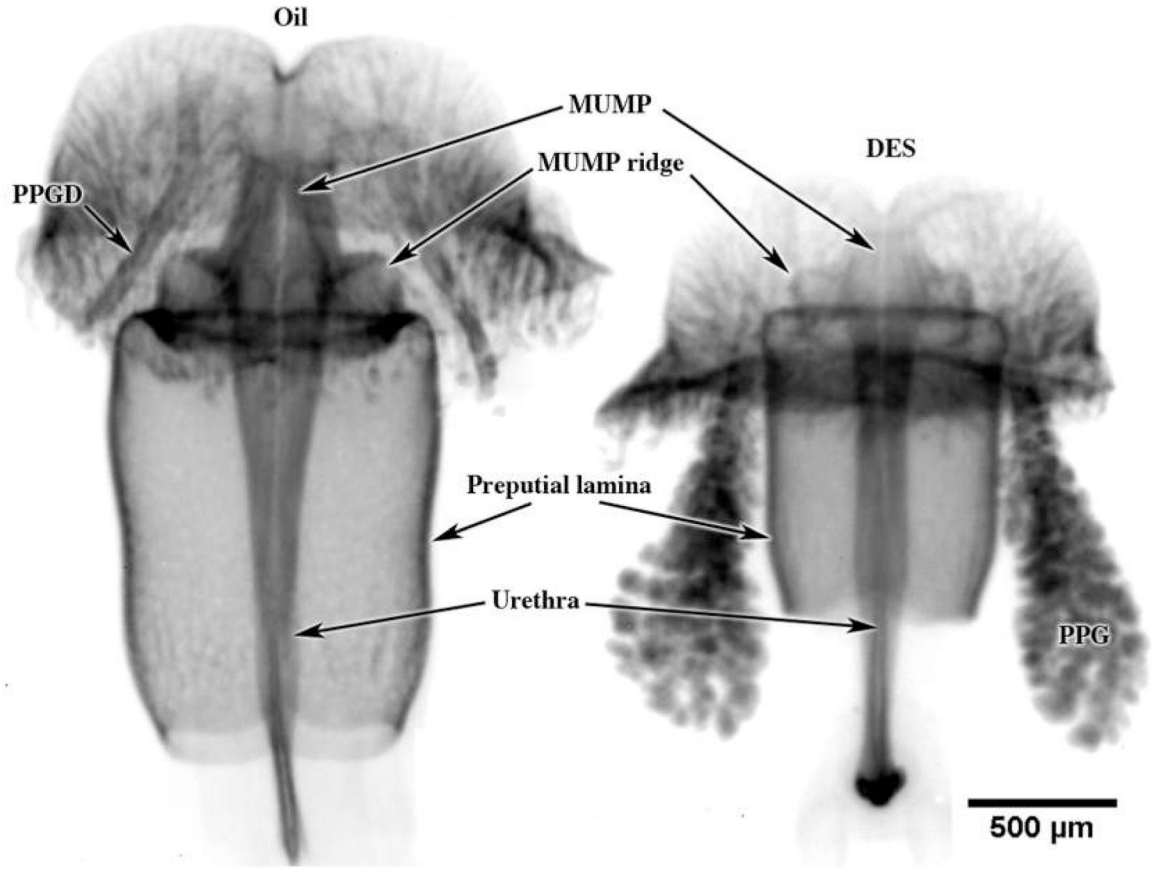

Fig. 16.

DES Affects Penile Growth. Optical projection tomography images of P5 CD-1 external genitalia derived from male mice injected on days 1 and 3 with oil or diethylstilbestrol (DES) stained with anti-E-cadherin. Note the marked reduction in size of all structures, especially that of the preputial lamina, and the truncation of distal structures destined to form the penile urethral meatus. PPG = preputial gland, PPGD = preputial gland duct. From Mahawong et al. (2014) with permission (Mahawong et al., 2014b).

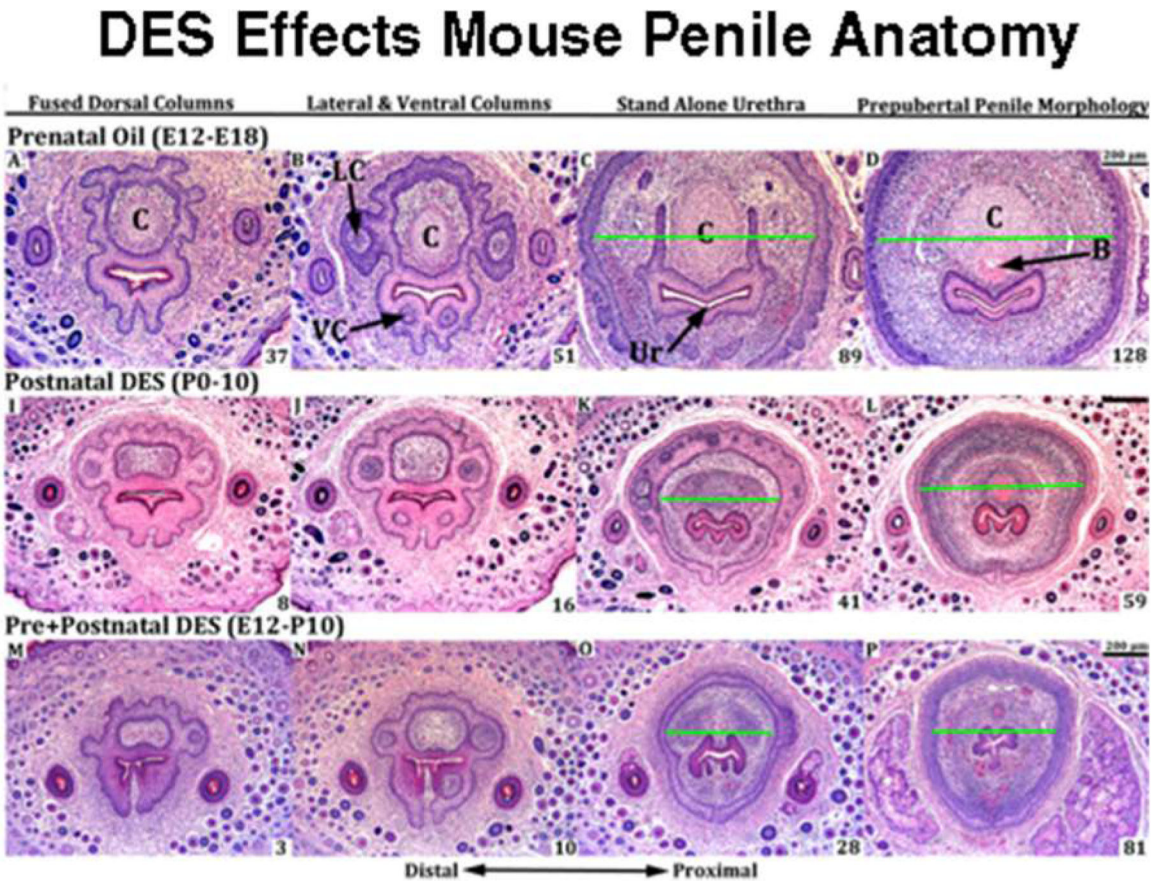

Fig. 17.

Diethylstilbestrol (DES) Affects Penile Growth. Images from transverse serial section sets of P10 CD1 mouse penises treated with oil or DES (Oil, DES P0–P10, and DES E12–P10) assessed at P10. Sections proceed from distal to proximal (left to right). (A–D represent normal prepubertal penile morphology, in which the penis is defined circumferentially by the external preputial lamina and contains a stand-alone urethra (Ur), os penis (B), and male urogenital mating protuberance (MUMP) cartilage (C). Note the smaller penile diameter (defined by the preputial lamina) of the DES P0–P10 and DES E12–P10 groups, as compared to oil control. The green lines emphasize the differences in diameter between the oil group (C–D) versus the DES P0–P10 (K–L) and DES E12–P10 (O–P) groups. Note that the distance from (A) to (D) in the oil treated spans 91 sections (section thickness is 7 μm, for a total of 637 μm), whereas this distance is reduced to 51 sections (for a total of 357 μm) in the DES P0–P10 group and to 78 sections (total of 546 μm) in the DES PE12–P10 group. The lack of developing MUMP cartilage (I, J, M, N) and impaired development of the MUMP corpus cavernosum (K, L, O, P) is visible in the DES P0–P10 and DES E12–P10 groups. All images are at the same magnification. LC = lateral mesenchymal column, VC = ventral mesenchymal column. Adapted from Sinclair et al. (2016) with permission (Sinclair et al., 2016b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

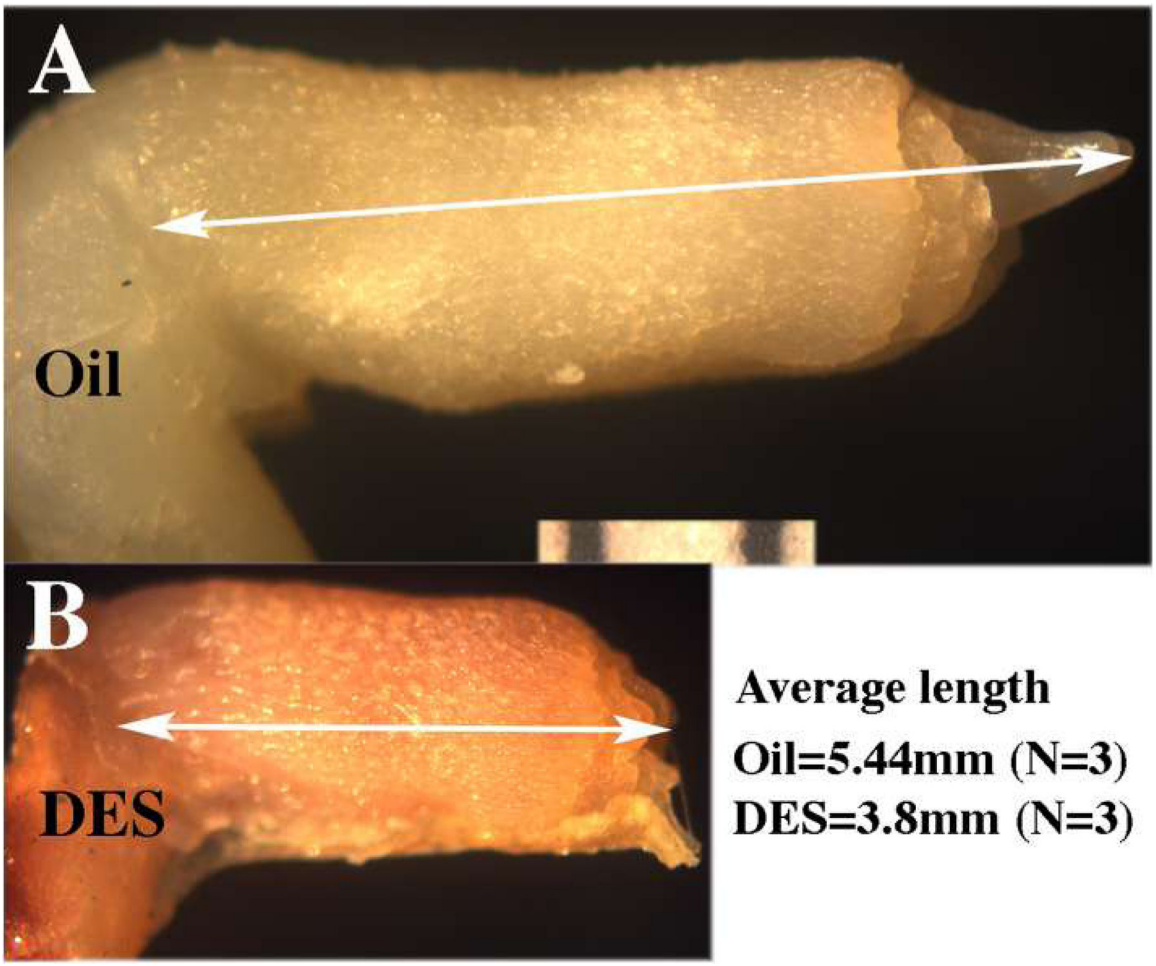

Fig. 18.

Penile wholemounts of adult neonatally oil-treated (A) and P0–P10 diethylstilbestrol (DES)-treated (B) CD-1 mice. Note reduction in length of the glans penis and truncation of the male urogenital mating protuberance (MUMP) in the DES-treated specimen (B). Arrows indicate that penile glans length was determined from depth of the preputial space to the tip of the MUMP.

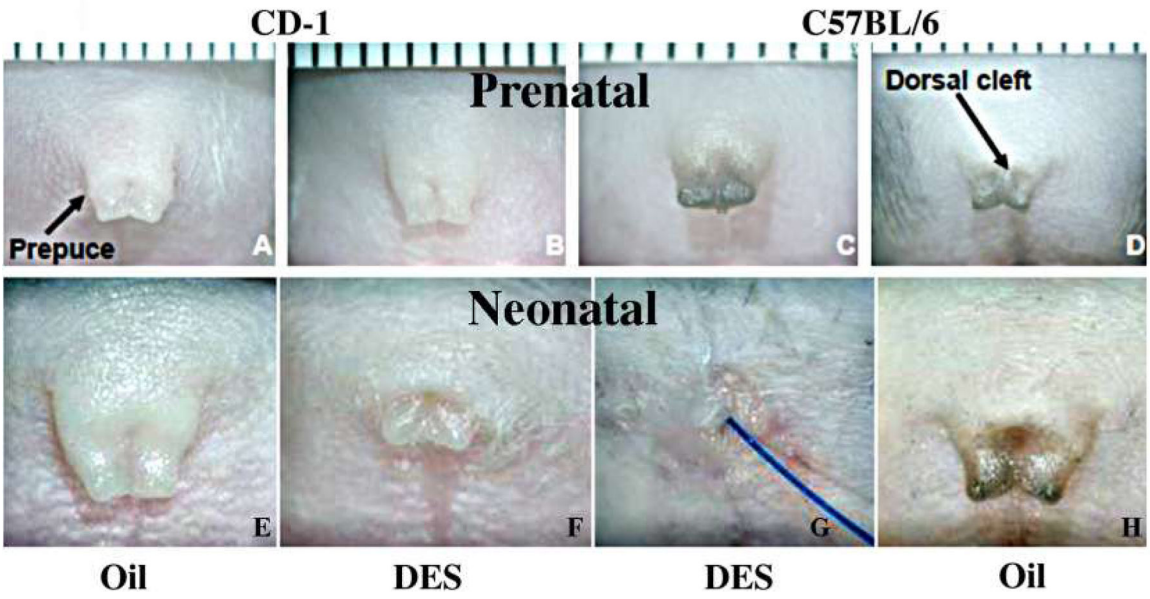

Fig. 19.

Wholemount photos of adult male external prepuce from prenatally and neonatally oil-and diethylstilbestrol (DES)-treated mice. Prenatal DES treatment (upper row) minimally affects the external prepuce, whereas neonatal DES treatment (lower row) elicits substantial malformation of the external prepuce. Note the fine blue suture in G indicating the urethral meatus. From Mahawong et al. (2014) with permission (Mahawong et al., 2014a, 2014b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

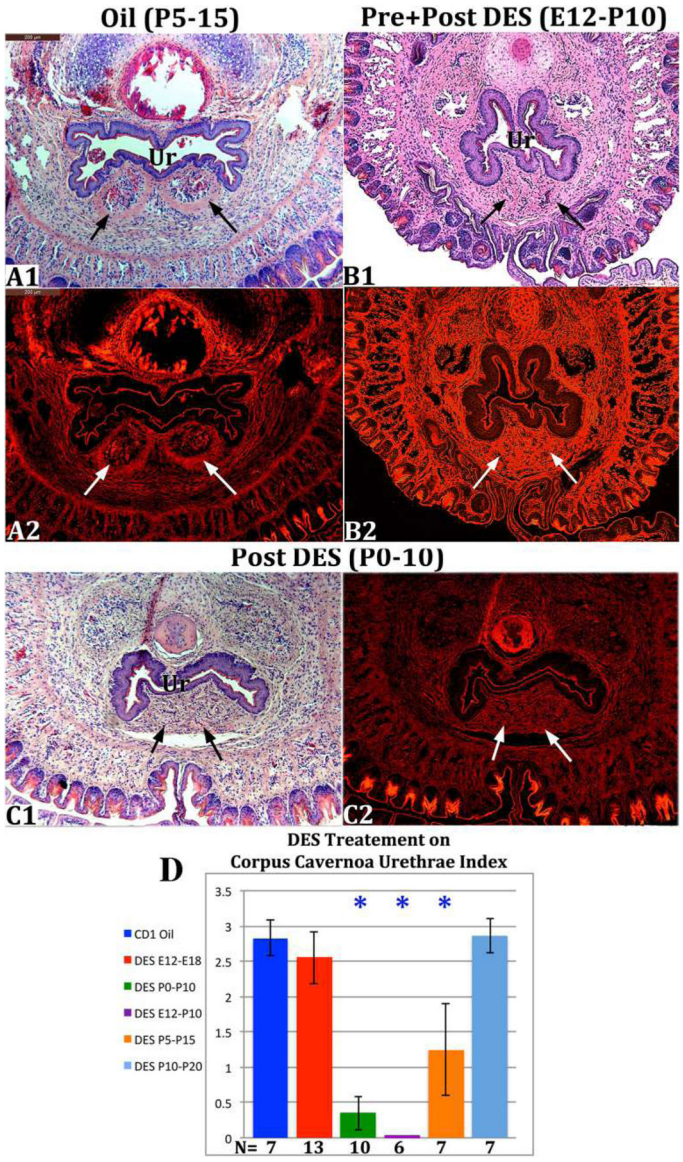

Fig. 20.

Effects of Diethylstilbestrol (DES) on the Corpora Cavernosa Urethrae. Transverse histologic sections at the level of the corpora cavernosa urethrae (arrows). In oil-treated mice (A1–A2), the capsules of the corpora cavernosa urethrae are well-demarcated (white and black arrows in A1 and A2). In both DES groups, E12–E18 (B1–B2) and P0–P10 (C1–C2), the corpora cavernosa urethrae are indistinct. Ur = urethra. (D) Corpus cavernosa urethrae index results of the oil and DES-treated groups showing a marked effect of timing of DES treatment. * = $p \leq 0.0001$. From Sinclair et al. () with permission (Sinclair et al., 2016b).

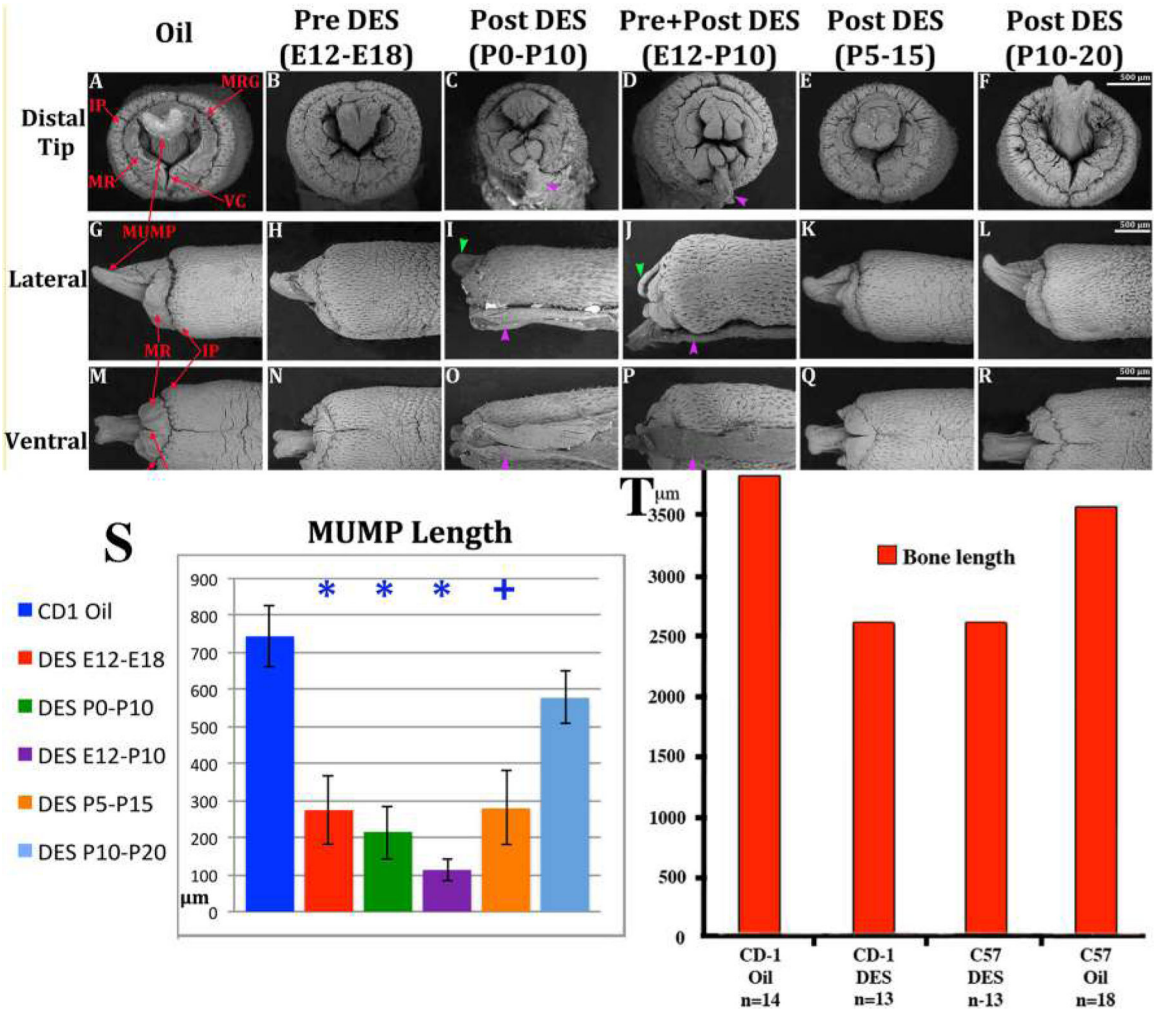

Fig. 21.

Scanning electron micrographs of adult (P60) mouse penises treated with oil vehicle or diethylstilbestrol (DES) at the ages specified. (A–F) end-on views, (G–L) lateral views, and (M–R) ventral views. Severe truncation of the male urogenital mating protuberance (MUMP) (green arrowheads in I and J), malformation of the urethral meatus with abnormal clefting patterns in the MUMP ridge (C and D), and a ventral tether (purple arrowheads in I, J, O, and P) are observed in the P0–P10 and E12–P10 groups. Other treatment groups show less severe malformations or minimal departure from the oil-treated specimens. IP = internal prepuce, MR = MUMP ridge, MRG = MUMP ridge groove, VC = ventral cleft in the MUMP ridge. (S) Morphometric analysis of MUMP length in perinatally DES-treated mice. Significance symbols: **+** = $p \leq 0.05$, ***** = $p \leq 0.0001$. (A–S) from Sinclair et al. (2016) with permission (Sinclair et al., 2016b). (T) Bone length of the adult mouse penis of neonatally oil- or DES-treated CD-1 and C57BL/6 (C57) male mice assessed at age P60, with DES treatment from P0–P10. Os penis length was significantly reduced by DES treatment in both CD-1 and C57BL/6 mice ($p < 0.001$ for both). From Mahawong et al. (2014) with permission (Mahawong et al., 2014b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

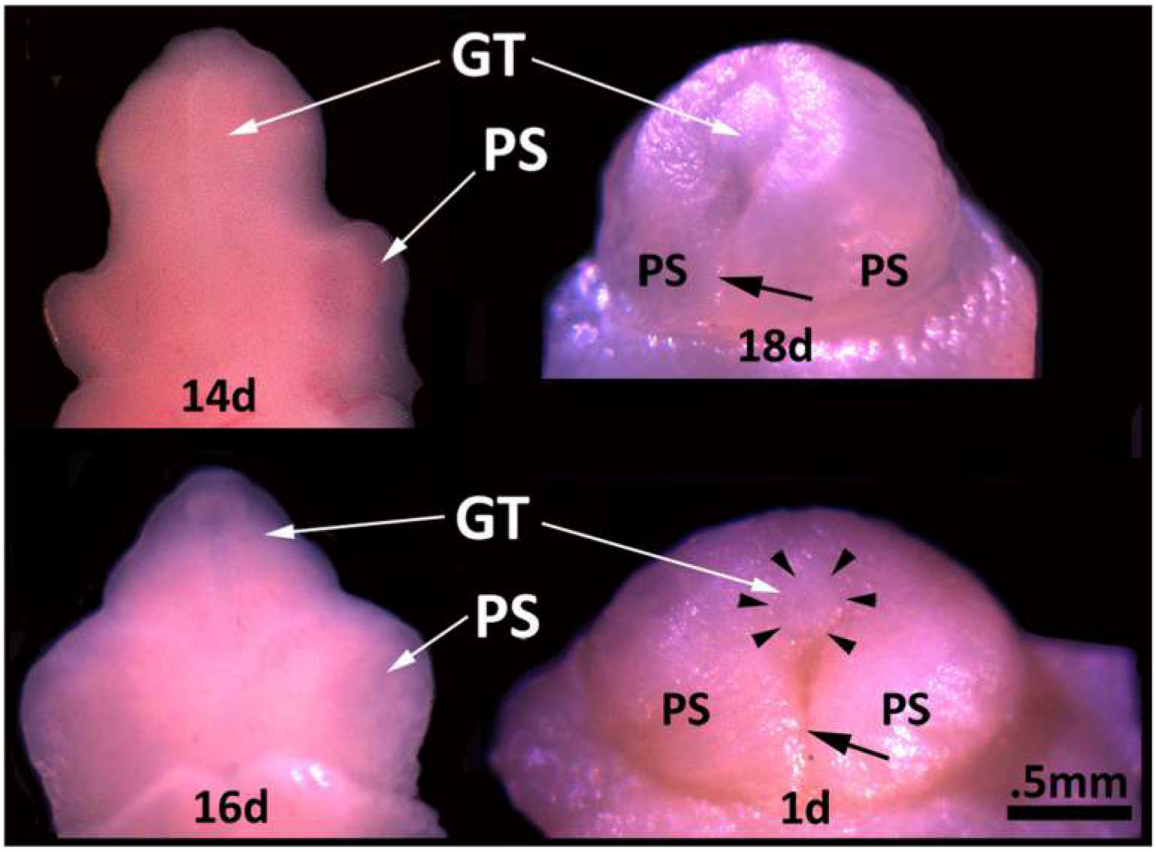

Fig. 22.

Wholemount images of normal female mouse genital tubercles (GTs) at E14, E16, E18, and at P0 (1d) as ventral views. The preputial swellings enlarge and fuse in the midline to form the prepuce, which extends distally to eventually completely cover the GT. Arrowheads outline the distal tip of the P0 GT, which is almost completely covered by the preputial swelling (PS). At E18 and P0, the preputial-urethral groove (PUG) can be seen where the preputial swelling are fusing in the midline (large black arrows). From Cunha et al. (2020) with permission (Cunha et al., 2020a).

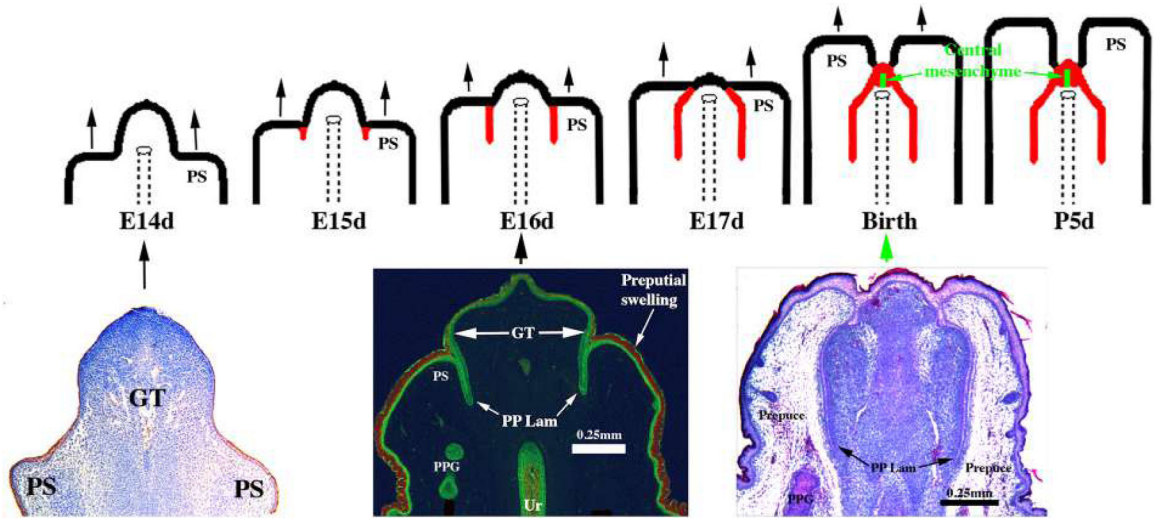

Fig. 23.

Diagrammatic representations of development of the prepuce and the preputial lamina (red) from the preputial swellings. Coronal histologic sections depict the process at E14, E16, and P0. Note that as the prepuce grows distally, the preputial lamina is “left in its wake” and therefore grows in length. Black arrows indicate distal growth of the prepuce and dotted lines represent the urethra. At birth, the central core of mesenchyme (green in top row), which is the precursor of the male urogenital mating protuberance (MUMP), penetrates the complex central epithelium. GT = genital tubercle, PPG = preputial gland, PP Lam = preputial lamina, PS = preputial swelling. From Liu et al. (2018) with permission (Liu et al., 2018b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

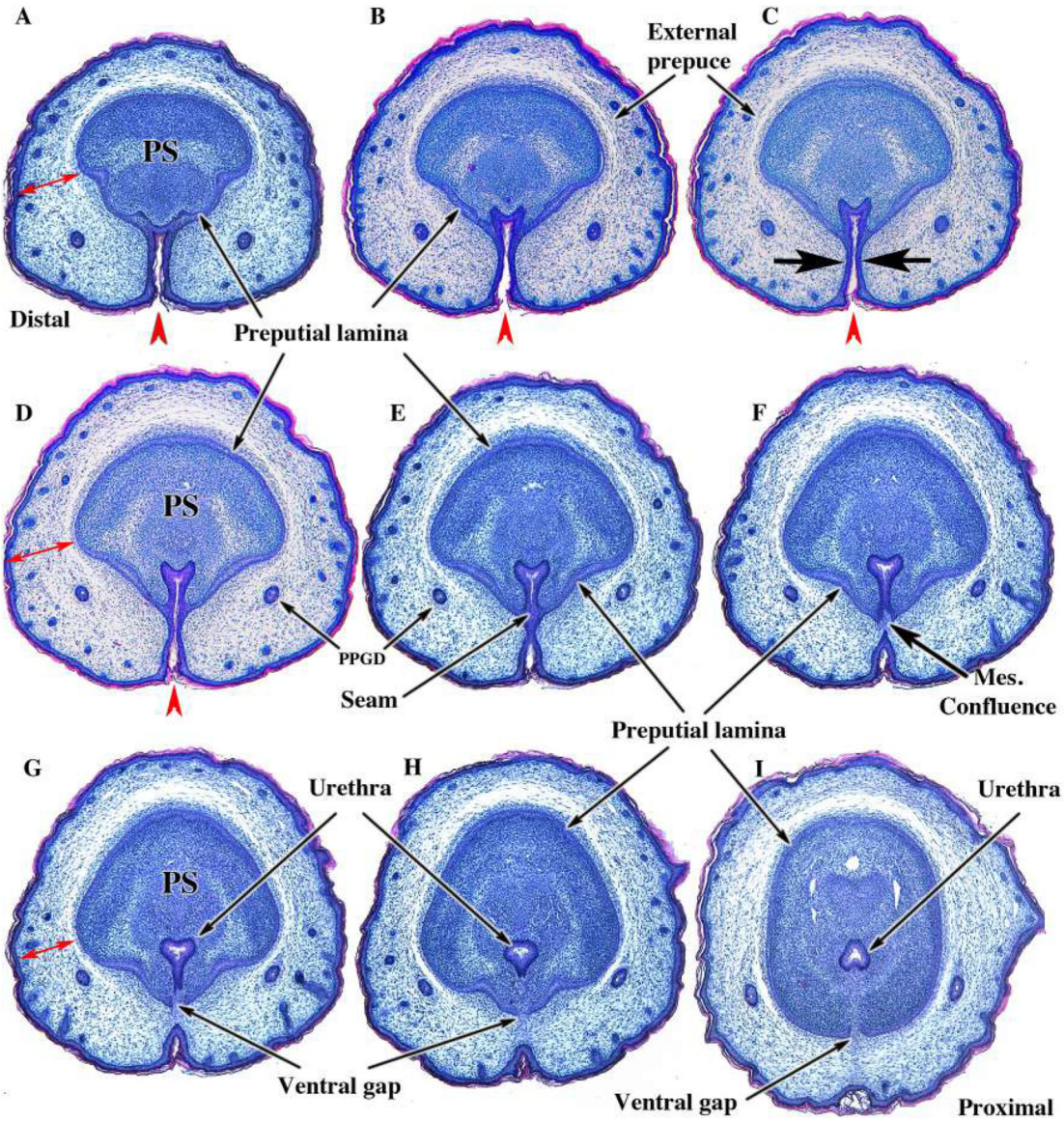

Fig. 24.

Transverse sections of the external genitalia of a normal newborn male CD-1 mouse. Sections are arranged from distal (A) to proximal (I). Distally (A), the right and left preputial swellings are approaching the ventral midline, forming the preputial-urethral groove (PUG). Note the growth and incipient fusion in the midline (large black arrows in C). The prepuce (double-headed red arrows in A, D, and G) consists of a thick wall of loose mesenchyme (PPM). The penis is surrounded by the preputial lamina; A–I) and consists of dense penile stroma. The PUG fuses ventrally (D and F) to complete the prepuce and form the preputial-urethral canal (PUC). A secondary fusion (E) forms a transitory seam, which disappears (F and G) to segregate the urethra and establish the midline mesenchymal confluence. Note that as the urethra is separating from the preputial lamina, a ventral gap in the preputial lamina is observed. Adapted from Sinclair (2016) with permission (Sinclair et

al., 2016c). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

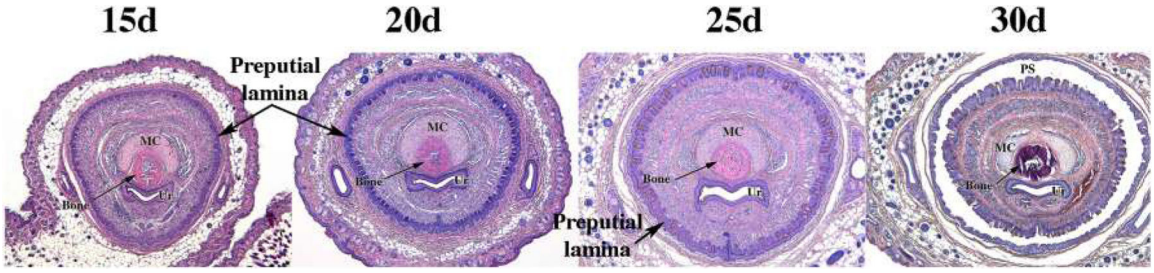

Fig. 25.

Canalization of the external preputial lamina and formation of the penile surface. Transverse sections of the developing CD-1 mouse penis at the postnatal days specified. PS = preputial space, Ur = urethra, MC = MUMP cartilage. From P0–P25, the preputial lamina remains solid. At P30, the preputial lamina is fully canalized, creating the preputial space that houses the penis. From Mahawong et al. (2014) with permission (Mahawong et al., 2014b).

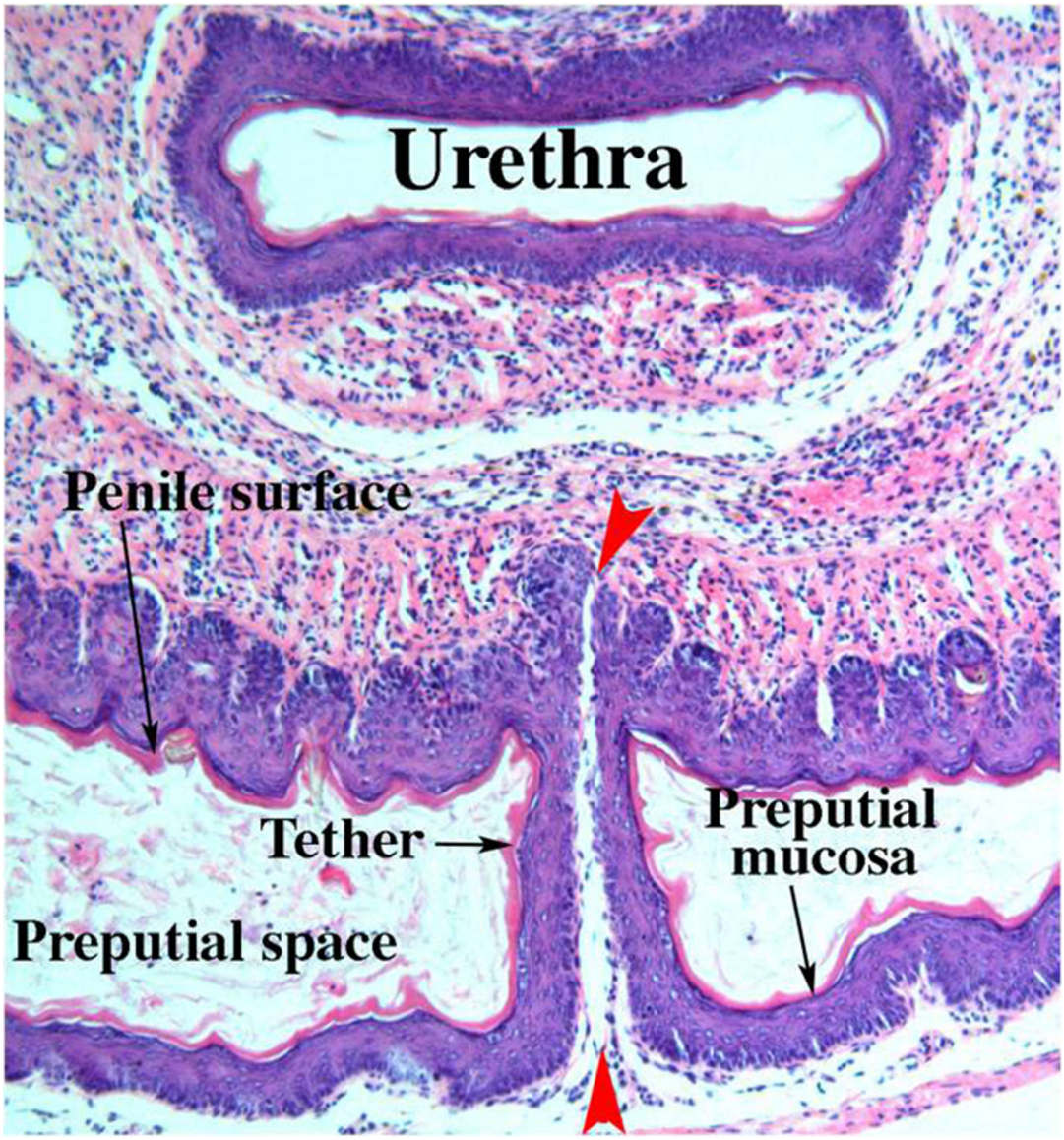

Fig. 26.

Ventral tethering in the mouse penis. Note the continuous stromal channel (large red arrows) linking the connective tissue of the preputial mucosa with the penile stroma. Likewise, note the continuity of epithelium of the preputial mucosa and the penile surface epithelium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

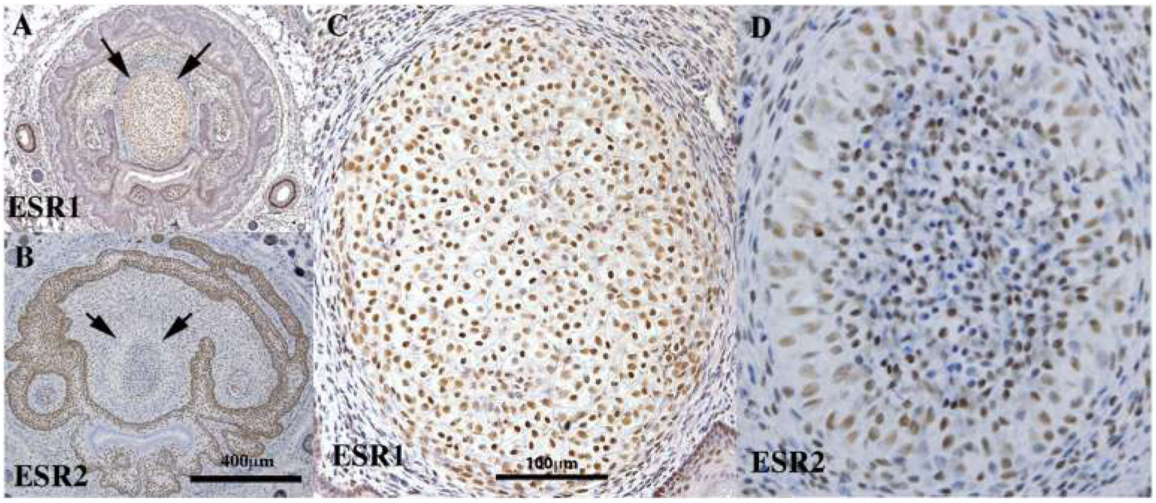

Fig. 27.

Immunohistochemistry of developing penises of P10 mice stained for estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) as indicated. The male urogenital mating protuberance (MUMP) cartilage (arrows in A–B) expresses both receptors. (C–D) are higher magnifications of the MUMP cartilage in (A–B). Scale bar for (A) and (B) is in the bottom right of (B) and scale bar for (C) and (D) is in the bottom center of (C). From Blaschko et al. (2013) with permission (Blaschko et al., 2013b).

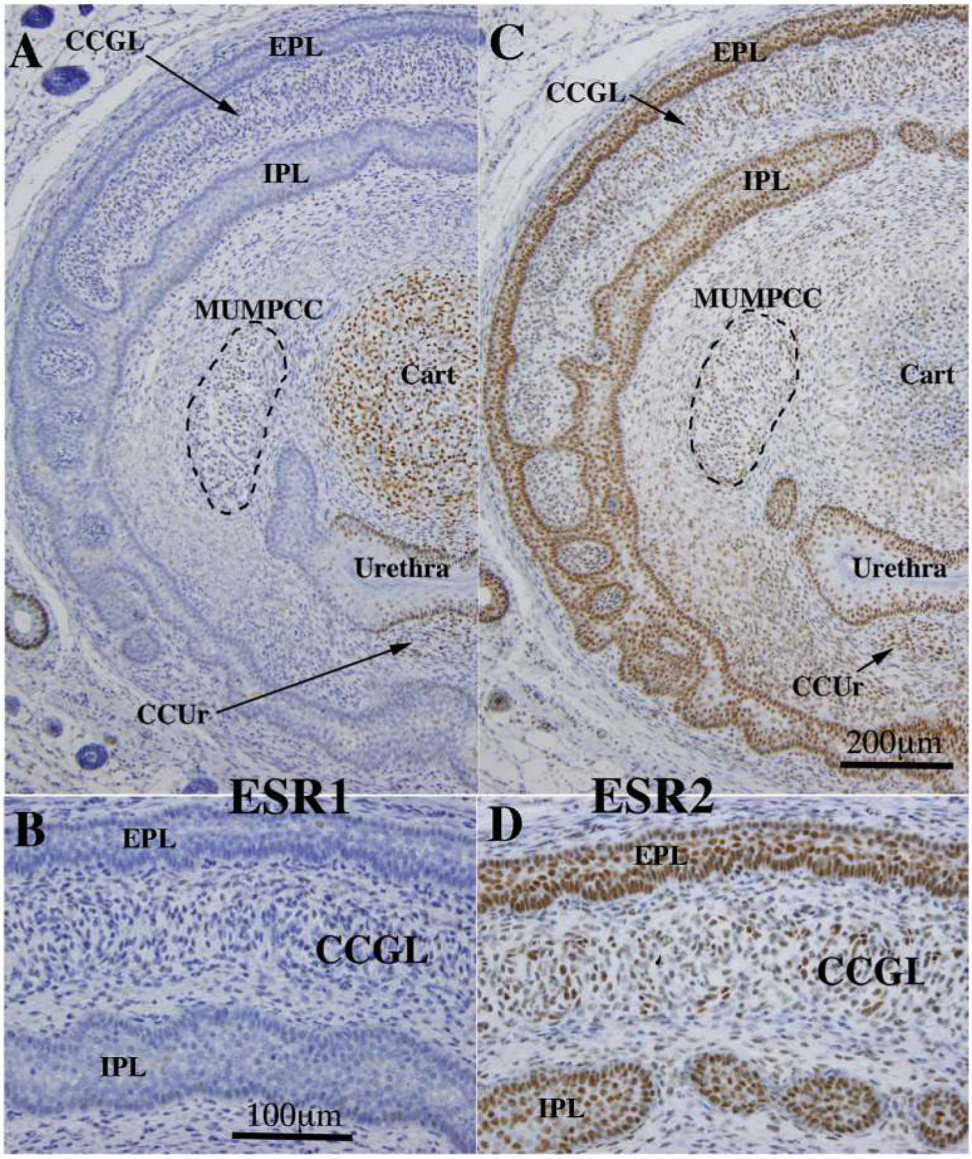

Fig. 28.

Immunohistochemistry of developing penises of P10 mice stained for estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) as indicated. Abbreviations: EPL = External preputial lamina, IPL= Internal preputial lamina, MUMPCC = MUMP corpus cavernosum, CCUr = corpus cavernosum urethrae, CCG = corpus cavernosum glandis, Cart = MUMP cartilage. Scale bar for (A) and (C) is in the bottom right of (C) and scale bar for (B) and (D) is in the bottom center of (B). From Blaschko et al. (2013) with permission (Blaschko et al., 2013b).

Table 1

Masculinization index of anatomic features in penis and clitoris of adult wild-type and mutant mice.

Wild-type clitoris	Wild-type penis	αERKO clitoris	NOER clitoris	AROM + clitoris
U-shaped lamina (0)	Circular profile (Jost, 1971)	Intermediate (0.5)	Intermediate (0.5)	Circular profile (Jost, 1971)
Tethering/immobile (0)	No tethering, mobile organ (Jost, 1971)	Tethering/immobile (0)	Tethering/immobile (0)	Tethering/immobile (0.5)
Small os clitoris (0)	Large os penis (Jost, 1971)	Large os penis (Jost, 1971)	Large os penis (Jost, 1971)	Large os penis (Jost, 1971)
Not located in epithelial-lined space (0)	Located in preputial space (Jost, 1971)	Located in preputial space (Jost, 1971)	Located in preputial space (Jost, 1971)	Located in preputial space (Jost, 1971)
No spines (0)	Penile spines (Jost, 1971)	Penile spines (Jost, 1971)	Penile spines (Jost, 1971)	Penile spines (Jost, 1971)
Small organ size (0)	Large organ (Jost, 1971)	Large organ (Jost, 1971)	Large organ (Jost, 1971)	Large organ (Jost, 1971)
No MUMP (0)	MUMP (Jost, 1971)	MUMP (Jost, 1971)	MUMP (Jost, 1971)	MUMP (Jost, 1971)
0.0 = score	7.0 = score	5.5 = score	5.5 = score	6.5 = score

Table 2

Effects of Diethylstilbestrol (DES) on the MUMP cartilage, erectile bodies, and penile size.

Comparison to Oil-Treated Group				
Harvest Age	**Treatment Periods**	**MUMP Cartilage**	**Capsules of Erectile Bodies**[a]	**Penis Size**
10 day	Prenatal DES (E12–E18)	Near Normal	Near Normal	Smaller
10 day	Postnatal DES (P0–P10)	Absent	Absent	Much Smaller
10 day	Pre- & Postnatal DES (E12–P10)	Absent	Absent	Much Smaller
15 day	Postnatal DES (P5–P15)	Absent	Absent	Smaller
20 day	Postnatal DES (P10–P20)	Near Normal	Near Normal	Near Normal

[a]Corpora cavernosa urethrae and MUMP corpora cavernosa. From Sinclair et al. (2016) with permission (Sinclair et al., 2016b).

Table 3

Effects of prenatal Diethylstilbestrol (DES) treatment on the external genitalia of CD-1 and C57Bl/6 Mice. CD-1 C57BL/6.

Specimens/Results	Oil	DES	Oil	DES
Urethral flap associated with ventral cleft	1/23 (4%)	11/28 (39%)	1/11 (9%)	11/13 (85%)
Os penis associated with ventral cleft	1/23 (4%)	5/28 (18%)	1/11 (9%)	4/13 (31%)

Note Effects more severe in the more sensitive strain (**C57BL/6)**.