Published in final edited form as:

Differentiation. 2014 ; 88(0): 51–69. doi:10.1016/j.diff.2014.09.005.

Prenatal diethylstilbestrol induces malformation of the external genitalia of male and female mice and persistent second-generation developmental abnormalities of the external genitalia in two mouse strains

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Abstract

Potential trans-generational influence of diethylstilbestrol (DES) exposure emerged with reports of effects in grandchildren of DES-treated pregnant women and of reproductive tract tumors in offspring of mice exposed in utero to DES. Accordingly, we examined the trans-generational influence of DES on development of external genitalia (ExG) and compared effects of in utero DES exposure in CD-1 and C57BL/6 mice injected with oil or DES every other day from gestational days 12 to 18. Mice were examined at birth, and on 5 to 120 days postnatal to evaluate ExG malformations. Of 23 adult (60 days) prenatally DES-exposed males, features indicative of urethral meatal hypospadias (see text for definitions) ranged from 18 to 100% in prenatally DES-exposed CD-1 males and 31 to 100% in prenatally DES-exposed C57BL/6 males. Thus, the strains differed in the incidence of male urethral hypospadias. Ninety-one percent of DES-exposed CD-1 females and 100% of DES-exposed C57BL/6 females had urethral-vaginal fistula. All DES-exposed CD-1 and C57BL/6 females lacked an os clitoris. None of the prenatally oil-treated CD-1 and C57BL/6 male and female mice had ExG malformations. For the second-generation study, 10 adult CD-1 males and females, from oil- and DES-exposed groups, respectively, were paired with untreated CD-1 mice for 30 days, and their offspring evaluated for ExG malformations. None of the F1 DES-treated females were fertile. Nine of 10 prenatally DES-exposed CD-1 males sired offspring with untreated females, producing 55 male and 42 female pups. Of the F2 DES-lineage adult males, 20% had exposed urethral flaps, a criterion of urethral meatal hypospadias. Five of 42 (11.9%) F2 DES lineage females had urethral-vaginal fistula. In contrast, all F2 oil-lineage males and all oil-lineage females were normal. Thus, prenatal DES exposure induces malformations of ExG in both sexes and strains of mice, and certain malformations are transmitted to the second-generation.

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Keywords

Prenatal; diethylstilbestrol; external genitalia; hypospadias; mice; trans-generational effect

INTRODUCTION

Hypospadias is one of the most common human congenital anomalies with an incidence 1: 250 to 1: 300 live male births (Baskin, 2000). While its cause has not been established, the most likely etiology of hypospadias is an interaction of genetic susceptibility with exogenous endocrine disruptors (Kalfa et al., 2011b). Diethylstilbestrol (DES) is a synthetic estrogen that was used in the mid-twentieth century to prevent miscarriage, even though shortly after FDA approval DES was shown to be ineffective (Dieckman et al., 1953). After decades of treatment of pregnant women with DES, epidemiological studies of children born to DES-treated mothers revealed teratogenic effects in DES sons and daughters (offspring of pregnant women treated with DES) and vaginal cancer in DES daughters (Giusti et al., 1995; Herbst and Bern, 1981; Jefferies et al., 1984; Herbst et al., 1971). Among the identified outcomes in DES sons is an increased incidence of hypospadias (Gill et al., 1981; Kalfa et al., 2011a). In addition, grandsons of DES-treated women also have an increased incidence of hypospadias (Brouwers et al., 2007). Thus, prenatal DES exposure has been implicated in affecting not only the children of the exposed mother but also her grandchildren. Findings from animal studies also suggest a trans-generational effect, with epigenetic mechanisms offered as a possible explanation (Newbold et al., 2006; Prins, 2008).

Given the nature of human hypospadias, in which urethral meatal defects from the midshaft to the perineum are seen in ~ 50% of the cases (Baskin and Ebbers, 2006), the term, hypospadias, when applied to mice, carries with it the expectation of malformations of comparable severity. Unfortunately this has not been the case. For many decades mouse models have been used to elucidate the mechanism of estrogen-induced malformations of the reproductive tract including malformations of the external genitalia. Since the human penile urethra develops as a result of midline fusion of the urethral folds, the common observation in mice of an open “urethral” groove observed at the end of gestation treated prenatally with estrogens (Kim et al., 2004) has been interpreted as hypospadias with the tacit (but unproven) assumption that such genital tubercle malformations observed in late gestation fetuses are irreversible and will result in enduring penile malformations in adulthood similar to those seen in humans. This unproven assumption must now be questioned (Cunha et al., 2014). Iguchi et al described hypospadias at the end of gestation in male mice treated prenatally with a 5 α -reductase inhibitor (Iguchi et al., 1991), which resolved to normality at 90 days postnatal. Thus, at least some abnormalities observed at the end of gestation may reflect developmental retardation, which given sufficient time will become normal. In our previous studies prenatal estrogen exposure resulted in “hypospadias” (an open preputial/urethral groove) at the end of gestation with an incidence of 40 to 57% (N=134) (Kim et al., 2004). However, in recent studies utilizing the Kim et al protocol, 57 male mice were treated in utero with DES from 12 to 18 days of gestation, but at 60 days postnatal malformations comparable to human penile shaft hypospadias were not

observed (0/57 male mice) (Cunha et al., 2014). There are three lessons to be derived from these observations: (a) Malformations seen at the end of gestation may not lead to enduring adult malformations, but instead may represent retardation of development capable of resolving to normality. (b) Post-puberty to adulthood is the best time to assess adverse enduring ExG malformations elicited by exposure to endocrine disruptors in mice (Cunha et al., 2014). (c) The types of malformations elicited by prenatal DES treatment may be subtle, easily overlooked and requiring a detailed knowledge of normal as well as abnormal mouse penile anatomy and associated accurate terminology to convey results.

Since an open preputial/urethral groove observed in late gestation male mouse fetuses may not be reflective of enduring hypospadias, it is evident that objective criteria are required to accurately diagnose estrogen-induced adult male hypospadias in mice. Potential relevance of mouse hypospadias to human hypospadias (a misplaced abnormal urethral meatus, a preputial defect and/or abnormal penile curvature) must also be assessed (Cunha et al., 2014). In a broader sense, male urethral hypospadias is a perturbation of morphologic patterning within the penis, which is a particularly complex organ containing several types of epithelium (penile skin, urethral epithelium and preputial epithelium), several erectile bodies, connective tissue, smooth muscle, nerves, and cartilage and bone in the case of the mouse, all arranged in a precise pattern required for function. Abnormal penile patterning, when affecting the urethra, may result in an abnormally situated urethral meatus located distally, within the penile shaft or in the perineum, or alternatively may involve abnormality in the form of the urethral meatus. All types of male urethral defects, whether proximal or distal qualify as hypospadias, but should be verified by objective criteria. Due to the pervading image of human hypospadias, the term “mouse hypospadias” connotes a urethral defect anywhere from the glans to the perineum and especially midshaft hypospadias. Since estrogen-induced defects of the external genitalia are subtle in mice, it is imperative to specify the precise type of malformation observed: preputial hypospadias, meatal hypospadias (and abnormal urethral meatus), or midshaft hypospadias. Without these qualifiers the term, hypospadias, as applied to mice, lacks clarity. One of the goals of this paper is to present objective criteria of male mouse urethral hypospadias diagnosed in adulthood, when it is certain that enduring malformations are fully and irreversibly established.

Given the differences in the morphogenetic mechanisms of penile development in mice versus humans, another critically important issue is whether penile shaft or proximal penile urethral malformations are to be expected (or even possible) in mice treated prenatally with exogenous estrogens. This comment is based upon the idea that urethral development within the penile shaft appears to be due to canalization of the urethral plate (Hynes and Fraher, 2004; Seifert et al., 2008), whereas formation of the urethral meatus appears to be due to fusion of the elements forming the urethral meatus (Blaschko et al., 2013; Cunha et al., 2014). As our knowledge of mouse penile development has matured over the years, we now question whether prenatal estrogen can elicit penile shaft hypospadias, and accordingly will devote attention to this question in this paper along with a detailed description of DES-induced malformation of the mouse penile urethral meatus.

With regard to prenatally DES-induced malformations in human females, clinical reports of human urethral-vaginal fistula are rare and to our knowledge not associated with prenatal DES exposure (Bhat et al., 2010). In contrast, studies in mice have shown that neonatal exposure of female mice to exogenous estrogen results in urethral/vaginal fistula in adulthood (Miyagawa et al., 2002; Forsberg and Lannerstad, 1968; Takasugi and Bern, 1962). The developmental anatomy of the emerging mouse vagina (composed of Mullerian epithelium cranially and urogenital sinus epithelium caudally) is complex and changes temporally as the urogenital sinus component divides into the female urethra ventrally and the “sinus vagina” dorsally, which is subsequently replaced by Mullerian epithelium (Kurita, 2010). Accordingly, the developmental mechanisms involved in generating male versus female hypospadias (urethral-vaginal fistula) are very different.

In addition to its putative role in increasing of incidence of male and female external genitalia (ExG) malformations, DES also serves as the reference estrogen of choice for modeling vertebrate endocrine disruption, especially in mouse models (Newbold, 2004; McLachlan, 1981; Bern, 1992). One potential variable in such studies, however, might be mouse strain because different strains of mice differ in their responsiveness to estrogens (Spearow et al., 1999). The outbred CD-1 mouse is a strain commonly used for biomedical research as well as for study of ExG development because of its vigor, ease of breeding, and large litter size. However, CD-1 mice are less sensitive to estrogen relative to C57BL/6 mice (Spearow et al., 1999), a strain used for many genetic mouse models. Thus, difference in strain sensitivity to estrogen is another focus of this paper. Finally, we examined the effects of prenatal DES exposure in two generations: prenatally DES-exposed mice (F1) and the offspring (F2) of prenatally DES-exposed male mice, with male and female ExG malformations as the primary endpoint.

MATERIALS AND METHODS

Animals and housing

All animal protocols were approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee. Adult CD-1 and C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA, USA) were housed in polycarbonate cages (20×25×47 cm³) with laboratory-grade pellet bedding in the UCSF Pathogen Specific Barrier housing facility. The mice were given Purina lab diet and tap water *ad libitum* and acclimated to 20° to 23°C and 40% to 50% humidity on a reversed light schedule (14 hours light and 10 hours dark). After mating, pregnant female mice were separated from the males and allowed to deliver. This study is based upon the analysis of 426 untreated, prenatally oil- and DES-treated mice.

Interventions

CD-1 and C57BL/6 dams were used in this study. Pregnant dams were weighed and injected subcutaneously on days 12, 14, 16, and 18 of gestation with DES (100 ng/gbw in ~5μl in sesame oil vehicle). For the control groups, CD-1 and C57BL/6 dams were injected with sesame oil (~5μl/g). Separate 50μl Hamilton syringes were used for sesame oil and DES. For developmental studies pregnant dams were injected as above with DES or sesame oil, and

ExG were harvested at birth (n=16), and on days 5 (n=6), 10 (n=14), and 60 (n=366) postnatal. Additional untreated CD-1 mice were harvested at 15 (n=6), 20 (n=5), 24 (n=7) and 30 (n=6) days postnatal.

Specimen preparation and analysis

At 60 days postnatal, some of the adult CD-1 and all of the C57BL/6 prenatally oil- and DES-treated mice were euthanized (some CD-1 were retained for the second-generation study). After hair removal with Nair®, the ExG were photographed using a digital camera mounted on a dissecting microscope for identification of ExG surface characteristics. ExG were dissected and fixed in 10% buffered formalin followed by paraffin embedding and serial sectioning (transversely or longitudinally) at 7µm for histological staining with hematoxylin and eosin.

Metrics of pertinent key morphological features were obtained via direct microscopic measurement of transverse or longitudinal sections or by counting the number of serial transverse sections containing the object of interest. Penile width was measured at mid-glans. Clitoral width was determined at the mid point of the U-shaped clitoral lamina. Our morphological analysis of ExG focused on the so-called glans penis and the U-shaped clitoral body as described previously (Rodriguez et al., 2011; Weiss et al., 2012)

Scanning electron microscopy

Surface details were elucidated via scanning electron microscopy (SEM). For these studies, ExG were dissected and fixed in 2% glutaraldehyde/0.1 M sodium cacodylate buffer at pH 7.2 for 6 hours. The specimens were then post-fixed in 2% osmium tetroxide for 2 hours, subsequently dehydrated in serial alcohol solutions, and critical point-dried in a Tousimis AutoSamdri 815 Critical Point Dryer (Tousimis, Rockville, MD). The specimens were then mounted on a stub with carbon tape, and images were obtained using a Hitachi TM-1000 Scanning Electron Microscope (Hitachi High Technologies America, Inc., Pleasanton, CA).

Three-dimensional reconstruction

Three-dimensional computer reconstructions were created from serial 7µm transverse sections utilizing SURF driver 3.5 (SURF driver, University of Hawaii and University of Alberta) software. Sections were digitized to achieve linear tracings of relevant structures. Digital linear tracings were oriented using Photoshop software (Adobe, Inc. San Rafael, CA 94903) and then were exported into SURF driver software for three-dimensional reconstruction.

Optical projection tomography

Dissected mouse genital tubercles were fixed in 4% paraformaldehyde (PFA) for 2h, and then stored in 70% methanol. When ready for staining, samples were bleached overnight in a H₂O₂ solution composed of 15 parts of 30% H₂O₂ and 17 parts DMSO in 100% methanol. Samples were then incubated in acetone at room temperature (RT) for 3h, moved sequentially to 100% methanol, and frozen at -80°C for 1h and sequentially thawed 5 times. Following washing in Tris-buffered saline containing 1% Triton X-100 (TBST), samples were incubated in antibody block (TBST plus 10% goat serum) overnight at RT on a rocker.

After blocking, the primary antibody (mouse anti-E-cadherin diluted 1:100, BD Transduction Laboratories, San Jose, CA) was applied for 48–72 h at RT with rocking. Samples were washed for two days in a 5–10 ml volume of TBST with media changes every 2 h (min 5 changes per day). The secondary antibody, Alexa Fluor 555 goat anti-mouse (Life Technologies, Foster City, CA), was diluted in TBST and applied for 48–72 h at RT before finally washing in TBST for 3 days as previously described. Finally, samples were post-fixed in 4% PFA for 2 h at 4°C, and moved to test tubes containing 1% PFA for storage until ready for imaging. All solutions, except for methanol, were filtered through a 0.2 µm filter. Imaging was performed by the Histology and Light Microscopy Core at the Gladstone Institute, UCSF, which then provided the computer files and Volocity software for analysis.

The second-generation (F2) study

Dams at the beginning of the study were defined as the F0 generation, and their pups were defined as the first-generation mice (F1), either oil- or DES-exposed. Ten adult male and ten female F1 CD-1 mice at ~60 days of age from both DES- and oil-treated groups were housed in mating cages with untreated male and female CD-1 mice of known fertility (1:1) for 30 days. Mice were checked once daily for the presence of vaginal plugs. The plug-positive females were separated and evaluated for offspring. Pup numbers from successful breedings were recorded. These litters were defined as second-generation mice (F2) and sacrificed on day 60 of life so that their ExG could be prepared and analyzed as described above.

Statistics

Comparison of length and width measurements was done using Student's *t* tests, with a *p* value < 0.05 considered significant. For analysis of frequency data, Fisher's exact tests were used with a *p* value for significance set at 0.05, as well.

RESULTS

Definition of terms

Dorsal versus ventral—The convention for defining dorsal versus ventral for the penis is based upon the human erect penis. Accordingly, human hypospadias has been described as affecting the ventral penile surface, namely the surface of the penis closest to the anus. This human convention has been historically applied to mouse ExG anatomy and will be used in this paper. The human convention for defining dorsal versus ventral in female genitalia is different and corresponds with true dorsal-ventral body position. Accordingly, in humans the arrangement of structures from ventral to dorsal is: clitoris, urethra, vagina and anus. This convention will be used in this paper for the mouse. The implications of this convention, based upon human anatomical literature, are that the surface of the mouse prepuce closest to the anus is designated as ventral for males and dorsal for females.

Mouse glans penis—As reported previously, the mouse penis consists of an internal segment defined by the attachment of corporal body elements to the pubic bones and is traditionally designated as the body of the penis. The external component of the penis projects into the preputial space and is called the glans (Rodriguez et al., 2011). The so-

called glans has a shaft as well as a complicated distal extremity constituting the cartilagenous MUMP and the urethral meatus. This report focuses upon the distal aspect of the glans and especially on the urethral meatus.

Preputial/urethral groove—It is important to recognize that the open “urethral” groove observed at the end of gestation and exacerbated by prenatal DES treatment (Kim et al., 2004) is actually involved in the development of both the prepuce and distal urethra, and thus will be designated specifically as preputial/urethral groove/cleft/or fold.

Defining hypospadias in the adult mouse

Given the ambiguity in the literature concerning prenatally estrogen-induced mouse hypospadias, we propose the following objective criteria for hypospadias. First, it is important to realize that hypospadias in male mice involves two structures: (a) the prepuce and (b) the penile urethra, and thus hypospadias should be specifically designated as preputial hypospadias, urethral hypospadias or meatal hypospadias to avoid confusion. Preputial hypospadias is simply an abnormality in the form of the external prepuce, typically an exaggerated cleft in the external prepuce as will be illustrated below. [Note that the mouse has two prepuces, external and internal (Blaschko et al., 2013)]. Fundamentally, male mouse urethral hypospadias is typically an abnormality in the urethral meatus either in shape, position or both that departs significantly from normal morphology. Detailed accurate descriptions of normal penile anatomy have been reported recently (Weiss et al., 2012; Yang et al., 2010; Blaschko et al., 2013; Rodriguez et al., 2011). The urethral meatus in adult male mice is Y-shaped and defined by the fusion of the MUMP (male urogenital mating protuberance) with the MUMP ridge, which is partially bisected by a ventral cleft (Fig. 1A) as described previously (Rodriguez et al., 2011; Blaschko et al., 2013). Accordingly, the tubular urethra of the male mouse opens to the exterior at the urethral meatus, which is the point of closure of the ventral cleft in the MUMP ridge (Fig. 1A). In this study, we define urethral hypospadias in several ways: (a) An abnormality in the morphological patterning of the elements (the MUMP and MUMP ridge) that form the urethral meatus as judged in end-on photographs or SEMs of the mouse penis (Fig. 4). (b) Since normally the urethral meatus (closure of the ventral cleft in the MUMP ridge) is distal to the urethral flaps and distal to the os penis (Figs. 2B & C) (Rodriguez et al., 2011), presence of urethral flaps and os penis in transverse sections containing an open urethral cleft is also designated as urethral hypospadias (Fig. 2D & E). Such malformations are accordingly called “exposed urethral flaps” or “exposed os penis”, respectively, and constitute a diagnostic element of urethral hypospadias. Note that the presence of “exposed urethral flaps” or “exposed os penis” in histological sections is in part affected by the precision of orientation of the specimen within the paraffin block, and thus “exposed urethral flaps” or “exposed os penis” may be seen in a small fraction of untreated or oil-treated specimens, which represents a background level to which DES-treated specimens are compared. Accordingly, the incidence of “exposed urethral flaps” or “exposed os penis” may vary from study to study even though the incidence of this abnormal configuration is elevated in DES- versus oil-treated mice. Such mal-position of the urethral flaps and os penis relative to the urethral meatus could be accounted for by an elongated ventral cleft within the MUMP ridge (Figs. 1A, 5B2–B4 &

C2–C4). Accordingly, we monitored length of the ventral cleft in the MUMP ridge in prenatally oil- and DES-exposed males (Fig. 5) and found it to be elongated.

The major malformation elicited in females by prenatal DES is urethral-vaginal fistula (sometimes called female hypospadias), an abnormal opening of the urethra into the vaginal lumen as opposed to separate orifices for the urethra and vagina.

External genitalia in first-generation (F1) adult prenatally oil- and DES-treated CD-1 and C57BL/6 male mice

Table 1 gives an overview of the results for first generation DES- and oil-treated F1 mice. In oil-treated F1 males, all gross morphological ExG features (prepuce and penis) were normal and identical for both mouse strains. Figures 3A–D are views of the prepuce of prenatally oil- and DES-treated CD-1 and C57BL/6 mice with hair removed. The normal bifid nature of the distal aspect of the prepuce of oil-treated male mice is readily apparent for both CD-1 and C57BL/6 mice (Figs. 3A & D) as described previously (Yang et al., 2010). The ducts of the male preputial glands open distally into the preputial cleft, which more proximally closes to define a tubular space called the preputial space housing the penis (Figs. 2A & 6B) (Weiss et al., 2012). Effects of DES on the external form of the CD-1 prepuce are subtle/imperceptible in gross photos (compare Figs. 3A & B). However, the prepuce of DES-treated C57BL/6 mice exhibited a blunt distal surface and was more deeply clefted in contrast to the angularity of the oil-treated controls and shallow clefting (compare Figs. 3C & D).

The tip of the mouse penis lies within the preputial space and is formed by the distally bifid MUMP (Figs. 1A, 2A), which is somewhat truncated in prenatally DES treated CD-1 and C57BL/6 mice (compare Figs. 3E & H versus 3F & G). This overall truncation of the penis was verified in serial sections in which internal penile structures were seen at lower section numbers (Fig. 5). Other effects involving the distal urethral meatus cannot be evaluated in ventral views of these gross specimens, but are evident in end-on views (Fig. 4).

The urethral meatus of untreated or oil-treated mice is Y-shaped (Fig. 4A) (Blaschko et al., 2013; Yang et al., 2010; Rodriguez et al., 2011) and is constituted by the fusion of the MUMP with the MUMP ridge, which is normally only partially bisected by the ventral cleft (Fig. 1A). In contrast, in prenatally DES-treated adult mice the ventral cleft frequently completely bisects the entire MUMP ridge and extends proximally to partially bisect the internal prepuce (Fig. 1B & 4C). Alternatively, the MUMP ridge may be so distorted that the ventral cleft is not apparent (Fig. 4B). The effect of DES in lengthening the ventral cleft of the MUMP ridge (Fig. 4C) was verified in serial sections through the adult penis of both CD-1 and C57BL/6 mice, thus “exposing” the urethral flaps and os penis (Figs. 5B4, C3 & C4). Figure 5 depicts histologic transverse sections through adult penises of CD-1 and C57BL/6 male mice in comparable distal to proximal regions. Distal penile sections (Figs. 5A1–D1) are located in a region in which the ventral cleft is confluent with the preputial space in oil- and DES-treated CD-1 and C57BL/6 adult male mice. A few sections proximally (Middle sections, Figs. 5A2–D2), the ventral cleft is closed with midline stromal confluence in the oil-treated CD-1 and C57BL/6 mice (arrows in Figs. 5A2–D2). In contrast, in DES-treated specimens the ventral cleft in the MUMP ridge remained open in both mouse

strains long after closure of the ventral cleft in oil-treated mice (Figs. 5B2–B4 and C2–C4). Note that urethral flaps are appropriately seen for the first time in oil-treated CD-1 and C57BL/6 mice in association with a closed ventral cleft (arrowheads in Figs. 5A3, A4, D3 & D4), whereas “exposed urethral flaps” are seen in association with an open ventral cleft in sections of the DES-treated CD-1 and C57BL/6 male mice (Figs. 5B4, C3–C4). Likewise, even more proximally, the os penis is present in association with a closed ventral cleft in oil-treated CD-1 and C57BL/6 mice (Figs. 5A4 & D4), while in DES-treated CD-1 and C57BL/6 mice the os penis is present in association with an open ventral cleft (exposed os penis) (Figs. 5B4–C4). These results are graphically illustrated in Figures 2B & E.

Strain differences in response to prenatal diethylstilbestrol

As shown in Table 1, prenatally DES-treated CD-1 and C57BL/6 males both exhibited a number of urethral anomalies, including exposed urethral flaps, exposed os penis, and an elongated ventral cleft relative to the oil-treated controls. Comparing the effects of DES in the two strains, overall the CD-1 males had a lower rate of DES-induced urethral meatal anomalies than C57BL/6 males (Table 1). In regard to hypospadias as defined by exposed os penis/exposed urethral flaps, the incidence of hypospadias was 18 to 39% in CD-1 mice and 31 to 85% in DES-treated C57BL/6 mice (Fisher’s exact, $p=0.018$) depending on the feature examined. However, the incidence of DES-induced malformation of the urethral meatus was 18/18 (100%) in CD-1 male mice and 10/10 (100%) in C57BL/6 mice. In summary, the first-generation (F1) DES-exposed males of both mouse strains exhibited a variety of malformations with the highest incidence of urethral hypospadias consistently observed in C57BL/6 mice (Table 1). The incidence of exposed urethral flaps and exposed os penis in oil-treated controls was considerably lower than that seen in prenatally DES-treated specimens.

ExG in first-generation (F1) adult CD-1 and C57BL/6 female mice

The elevation in the perineum of female mice is the prepuce and not the clitoris. The clitoris is an “internal” organ composed of an U-shaped epithelial lamina (Figs. 7E & 9A4–D4) (Martin-Alguacil et al., 2008) not visible externally and lying deep to the clefted female prepuce. The os clitoris lies within the concavity of the U-shaped epithelial lamina (Weiss et al., 2012) (Fig. 9A4 & D4). The ducts of the female preputial glands open distally into the preputial cleft, which more proximally closes to define a tubular space that is appropriately called the female preputial space (Fig. 6A). The female preputial space is homologous with its male counterpart (Fig. 6B) based upon the following observations: The female prepuce has a thick wall (a) surrounding the female preputial space (b) and contains the lower ends of numerous hair follicles (c), similar to that of the male counterpart (Fig. 6). Ducts of male and female preputial glands (d) open into the preputial cleft as described previously (Fig. 6) (Weiss et al., 2012). The epithelium lining the female preputial space (e) resembles epidermis and is stratified squamous and cornified (Figs. 6A & B).

Urine escapes to the exterior in females from a dorsal cleft in the preputial meatus, and thus the preputial meatus may be mis-construed as urethral meatus in females, which raises the question as to the junction between female urethra and preputial space. Urine from the female bladder after traversing the urethra enters the preputial space (Fig. 6A) and hence to

the exterior via the female preputial meatus, meaning that the female urethra and preputial space are continuous with each other at some point (Fig. 7A). Continuity of the female urethra with the preputial space occurs “internally” and is difficult to discern, as there is no defining landmark. Taking a logical approach, we propose that the female urethra is appropriately a stand-alone tubular structure (not attached to other epithelia) lined in total or in part by a non-cornified urethral epithelium (Fig. 7E). The female urethra is never entirely within the stroma “circumscribed” by the U-shaped clitoral lamina, but instead is completely or partially dorsal (dorsal means closest to the anus) to the U-shaped clitoral lamina (Fig. 7E) depending on proximal-distal location (Weiss et al., 2012). Examination of serial transverse sections from proximal to distal indicates that as epithelium of the urethra extends distally it fuses with epithelium of the U-shaped clitoral lamina to form a composite epithelium (Figs. 7B–D). As the U-shaped clitoral lamina attenuates distally, this composite epithelium merges with the well-defined stratified squamous, cornified, preputial epithelium (Figs. 6A & 7B–D), which provides the final pathway for urine to the exterior through a dorsal cleft in the preputial meatus. Thus, the pathway for urine includes the “stand alone” urethra (Fig. 7E), a composite preputial/clitoral epithelium (Figs. 7B–D) and more distally the epithelium lining the female preputial space from which urine exits via a dorsal cleft in the preputial meatus (Fig. 6A). Accordingly, we suggest that the demarcation between the female urethra and preputial space occurs at the point where the urethral epithelium is completely separate from the clitoral lamina, namely, at the distal-most point where a “stand-alone” urethra exists (Figs. 7A & E).

External surface morphology of the prepuce of prenatally oil-treated adult female mice is similar in CD-1 and C57BL/6 strains except for pigmentation in C57BL/6 females (Fig. 8A & D). While sub-optimal contrast in photos makes it difficult to assess external surface morphology, it is evident that prenatal DES exaggerates distal cleaving of the female prepuce (Fig. 8B & C). In addition, surface features of the vaginal orifice are distorted in mice of both strains as a result of prenatal DES treatment (Fig. 8).

Examination of serial transverse sections of prenatally oil-exposed CD-1 and C57BL/6 females revealed normal morphology, identical in both mouse strains (Figs. 9A1–A4 & 9D1–D4). All oil-exposed females of both mouse strains had an os clitoris situated within the stroma “circumscribed” by the U-shaped clitoral lamina (Figs. 9A4 & 9D4). Mean clitoral widths also did not differ significantly between the two strains (448 μ m [CD-1] vs. 540 μ m [C57BL/6], $p>0.05$). In oil-treated females, the urethra is partly within the confines of the U-shaped clitoral lamina distally (Figs. 7E, 9A3, 9D3), but more proximally the urethra tracks dorsally (Figs. 9A4 & 9D4) away from the clitoral lamina to lie in association with the vaginal wall. In DES-treated CD-1 and C57BL/6 females the clitoral epithelial lamina remained U-shaped, and did not differ in width in CD-1 and C57BL/6 mice (592 μ m [DES] vs. 585 μ m [oil], $p>0.05$), even though width of the clitoral lamina was slightly larger in DES- versus oil-treated mice (not significant). The os clitoris was not observed in any of the prenatally DES-exposed females of both strains (compare Figs. 9A4 & D4 versus 9B4 & C4). Almost all of the DES-exposed CD-1 females (19/21) and all of the DES-exposed C57BL/6 females (10/10) had urethral-vaginal fistula (Table 1 and Figs. 9B1–2, 9C1–3 and 10).

In summary, first-generation (F1) DES-treated females differed from their oil-treated counterparts by virtue of an almost 100% incidence of urethral-vaginal fistula and absence of the os clitoris. Thus, response of both mouse strains to prenatal DES exposure did not differ in females.

ExG endpoints in the second-generation (F2) adult CD1 males

All DES-treated F1 CD-1 females mated (vaginal plug-positive), but showed no signs of pregnancy and failed to deliver pups after pairing with untreated males for a 1-month period. In contrast, all oil-treated CD-1 F1 male and female mice of both strains bred successfully and produced pups. Nine of the ten DES-treated CD-1 F1 males bred successfully and sired viable young (Table 2). ExG of all of the oil-lineage CD-1 F2 males were completely normal, and none of them had exposed urethral flaps or os penis. None of the 55 DES-lineage CD-1 F2 males had an “exposed os penis”, but 11/55 (20%) had “exposed urethral flaps” (Fig. 11), and 2 of these 11 (3.6%) had an elongated ventral cleft within the MUMP ridge. In summary, the DES-lineage F2 CD-1 males showed some differences from their oil-lineage F2 counterparts suggestive of hypospadias, but at a reduced incidence and severity.

ExG endpoints in the second-generation (F2) adult CD1 females

All oil-lineage F2 CD-1 females had normal external genitalia, and urethral-vagina fistula was never observed (0/60). An os clitoris (average length=235µm) was present in all oil- and DES lineage F2 CD-1 females (Figs. 12A2 & B2). Of 42 DES-lineage F2 CD1 females in the study, five (11.9%) had urethral-vaginal fistula (Figs. 12A1 & B1). In summary, the second-generation (F2) DES-lineage CD-1 females showed a surprising frequency of urethral-vagina fistula but otherwise did not differ from their oil-lineage counterparts. They differed from DES-exposed F1 females in that all of the F2 DES-lineage females had an os clitoris, whereas all DES-exposed F1 females lacked an os clitoris.

Developmental correlates of DES-induced malformation of male external genitalia

As reported previously (Kim et al., 2004), prenatal exposure to exogenous estrogens, including DES, resulted in male “hypospadias”, that is, an abnormal open preputial-urethral groove observed at the end of gestation. In the Kim et al study estrogens were administered daily from days E12 to E18 with harvest on day E19. In the current study DES was administered on days E12, E14, E16, and E18 with harvest at birth. Despite this minor technical variation, the results of the current study mimic the Kim et al study in so far as prenatally DES-treated mice exhibited an open preputial/urethral groove at birth. In newborn CD-1 mice, length of the preputial-urethral cleft was 240µm in DES-treated males (n=4) versus 158µm in oil-treated males (n=4), while in C57Bl/6 mice length of the preputial-urethral cleft was 313µm in DES-treated males (n=4) versus 198µm in oil-treated males (n=4). Based upon this elongated preputial-urethral cleft seen during development in DES-treated mice, we expected severe hypospadias involving the proximal shaft of the glans in adulthood. However, such severe hypospadias was never observed in 57 prenatally DES treated CD-1 and C57BL/6 male mice. Instead, rather mild penile urethral malformations (easily overlooked) were only seen distally in the urethral meatus and involved truncation of the MUMP and distortion of patterning of the elements that constitute the urethral meatus as described above. In order to provide a developmental correlate to the mild hypospadias

malformations seen in adulthood, optical projection tomography (OPT) was performed on 5-day old mice to reveal epithelial structures in E-cadherin-stained ExG of prenatally oil- and DES-treated CD-1 males (Fig. 13). In a general sense OPT demonstrated that prenatal DES elicited reduction in the proximal-distal length of the ExG, with specific reduction of distal epithelial structures, destined to form the urethral meatus, namely the MUMP and the MUMP ridge. In addition, prenatal DES reduced the proximal-distal length of the preputial lamina relative to oil-treated mice. ExG of mice treated prenatally with DES or oil from birth to day 10 were also examined in serial transverse sections paying close attention to selecting sections in comparable areas using well-defined landmarks. In sections located at the beginning of the lateral mesenchymal columns (Schlomer et al., 2013), the distal aspect of the MUMP cartilage is well differentiated in oil-treated, but not in DES-treated specimens (compare Figs. 14A & G). Progressing proximally to the point of appearance of the ventral mesenchymal columns (Schlomer et al., 2013), the MUMP cartilage is present in both DES- and oil-treated specimens, but is distinctly larger in the oil-treated specimens (Figs. 14B & H). More proximally, where the right and left ventral mesenchymal columns are fusing in the midline (confluence of the ventral mesenchymal columns) (Figs. 14C & I), both DES- and oil-treated specimens are similar in appearance, and in both cases the preputial lamina is becoming apparent. Sections containing the beginning of the internal preputial lamina are, likewise, similar in both DES- and oil-treated specimens (Figs. 14D & J). More proximally within the specimens, the internal preputial lamina and the external preputial lamina (Figs. 14E, F, K & L) are normal in both DES- and oil-treated specimens. Thus, malformations were most evident distally versus proximally in prenatally DES-treated mice, which may indicate a temporal sensitivity of distal structures to prenatal DES exposure. Throughout the full set of serial sections, the developing penile shaft (including the urethra) is normal in both prenatally DES- and oil-treated specimens. However, the proximal-distal axis of DES-treated 10-day-old specimens was truncated relative to the oil-treated specimens in that comparable structures in DES-treated specimens were consistently encountered at lower section numbers in comparison to oil-treated specimens as indicated in Figure 14. This observation agrees with the 5-day OPT data (Fig. 13) as well as the 60-day data (Fig. 5) demonstrating substantial DES-induced proximal-distal truncation of the penis. In summary, we examined serial sections encompassing distal structures destined to contribute to the urethral meatus as well as proximal structures destined to form the penile shaft. At days 5 and 10 the observed defects primarily involved impaired morphogenesis/differentiation of distal structures such as the MUMP, which forms part of the urethral meatus. More proximal sections, destined to give rise to penile shaft structures were normal in prenatally DES-treated specimens at day 5 and 10 postnatal (Figs. 13–14). Thus, evidence of penile shaft hypospadias was not evident in prenatally DES-treated ExG in 5- and 10-day specimens. These observations during development of DES-induced malformations are consistent with the manifestation of enduring adult malformations of urethral meatus and other distal structures within the penis, and are incompatible with development of penile shaft hypospadias. Indeed, we now consider the possibility that prenatally estrogen-induced abnormalities of the penile shaft (hypospadias) are unlikely if not impossible in mice. Whether this applies to other agents such as 5 α -reductase inhibitors or anti-androgens or to neonatally DES-treated mice remains to be determined.

Implicit in penile shaft hypospadias is the absence of 3 elements: (a) ventral skin, (b) epithelium of the ventral urethral wall, and (c) associated stroma and an erectile body (the corpus cavernosa urethrae in mice, which is the homologue, the corpus spongiosum in humans) as indicated in Figure 15. Significantly, the ventral surface of the mouse penis and its covering of skin is not formed/revealed during normal development until 25- to 30-days postnatal, fully 4 to 5 weeks after initiation of prenatal DES treatment. The reason that the penile surface does not form until 25 to 30 days postnatal is that prenatally the genital tubercle (penile rudiment) is over grown by the developing prepuce such that the tip of genital tubercle becomes situated deep to the surface of the prepuce (Perriton et al., 2002; Petiot et al., 2005; Baskin et al., 2004) where it remains in adulthood (Fig. 2A). Thus, the future surface of the penis is represented in the neonate by the preputial epithelial lamina (Fig. 16), which canalizes postnatally (~20 to 30 days postnatal) to create the preputial space defined by two epithelial layers (the inner lining of the prepuce and the penile surface epithelium). The process of canalization of the preputial lamina is depicted in Figure 17, and indicates that the surface of the penile shaft forms or is revealed between 24 to 30 days postnatal. In summary, examination of the developing penis of 5- and 10-day-old mice treated prenatally with DES (Figs. 13–14) did not reveal any malformations interpreted as precursors to penile shaft hypospadias, namely absence of ventral penile epidermis, dermis, sub-dermal stroma, corpus cavernosa urethrae or ventral urethral epithelium.

DISCUSSION

Several cohort studies have identified a potential trans-generational effect of in utero DES exposure on ExG development in humans, although prevalence values vary. Klip and colleagues, evaluating a cohort of women with fertility problems, reported a 21-fold increased prevalence of hypospadias among DES-exposed sons (Klip et al., 2002). However, it must be noted that the absolute numbers of hypospadias in both exposed (4/205; 1.95%) and unexposed (8/8729; 0.09%) men were quite low. Later studies examined DES sons and found no association between in utero DES exposure and hypospadias (Palmer et al., 2005; Palmer et al., 2009). In that study, the rates of hypospadias were higher in both groups as compared to the study by Klip et al. In conflict with this apparent disconnect between rates of hypospadias in DES sons are the findings of Kalfa et al., who performed a multi-generational study in DES-exposed families. In their investigation of 529 families, 1000 pregnancies had involved DES exposure, compared to 180 that had not (Kalfa et al., 2011a). In the first generation, 3.5% of boys from the non-DES-exposed mothers had hypospadias. Unlike Palmer et al., Kalfa et al. and Gill et al. identified more hypospadias in DES-exposed boys versus controls (Gill et al., 1981; Kalfa et al., 2011a). Pons et al. in an investigation of hypospadias rates among DES-exposed boys, found a hypospadias rate of 1.23% among DES-exposed sons compared to 0.5% among boys who had not been exposed to DES in utero (Pons et al., 2005). In the second generation, Kalfa et al. found no hypospadias in boys born either to non-DES-exposed males or non-DES-exposed females. However, they found that 8.2% of sons born to DES-daughters had hypospadias, although none of the DES-sons fathered boys with hypospadias. One caveat about the latter finding is that the 552 DES-exposed daughters had 97 sons among them, whereas the 448 DES-exposed sons fathered only 8 sons. None of the sons or grandsons with hypospadias was related. Overlooked or

unreported in these studies are second-generation effects in daughters of DES-exposed sons. We have not found data in the literature on the daughters of DES sons. In any case, the possible trans-generational effect of in utero DES exposure is far from clear in humans.

In contrast, studies in mice support a trans-generational effect of DES exposure. The first report of trans-generational effects of DES came from the work of Newbold et al., who reported a greater frequency of reproductive tract tumors in mice two generations removed from DES treatment of pregnant dams (Newbold et al., 2006). The mechanism by which signals lead to derailed trans-generational urogenital development and other adverse endpoints are unknown but may involve alterations or damage to germ cells (Ma et al., 2009; Newbold et al., 2006; Skinner et al., 2013; Prins, 2008). In spite of these findings in mice, to our knowledge, no studies have examined the potential trans-generational effects of prenatal DES exposure on ExG-related endpoints in mice. Utilizing objective criteria of urethral hypospadias in male mice, we observed a modest trend towards hypospadias (“exposed urethral flaps”) in 20% (11/55) of F2 DES-lineage male mice versus 0% (0/50) in F2 oil-lineage male mice.

Surprisingly, urethral-vaginal fistula was observed in 5/42 (11.9%) of F2 DES-lineage female mice (sired by F1 DES-treated males) versus a complete absence of this malformation in all (0/60) F2 oil-lineage female mice. The 11.9% incidence of urethral-vaginal fistula in F2 DES-lineage female mice was far below that of F1 DES-lineage female mice (which was 91%-100%). Thus, transmission of this malformation to the second (F2) generation is greatly attenuated. Unfortunately, we did not track the individual phenotypes of the F1 breeder males used in the second generation study relative to those second generation (F2) pups that expressed features of either male hypospadias or urethral-vaginal fistula. Thus, an important question to consider in future studies is whether the appearance of ExG malformations in the second generation females is only transmitted from those males having ExG malformations or whether prenatally DES-treated males lacking ExG malformations can transmit urethral-vaginal fistula to their female offspring? In any case, given the many homologies in development of male and female ExG, it is of interest that prenatally DES-treated male mice can confer ExG malformations (urethral vaginal fistula) to their female offspring just as human DES daughters apparently transmit ExG malformations (hypospadias) to their sons (Kalfa et al., 2011a).

Another difference between F1 and F2 DES-lineage female mice is the 100% absence of the os clitoris in F1 DES-lineage female mice versus the universal presence of the os clitoris in F2 DES-lineage female mice. The absence of the os clitoris in F1 prenatally DES-treated females is supported by the absence of the os clitoris in 10-day postnatal female mice ovariectomized at birth and treated from birth to day 10 with DES (Rodriguez et al., 2012). Apparently, DES-impaired development of the os clitoris persists into adulthood in prenatally DES-treated female mice and suggests that formation and possibly growth of the os clitoris is negatively regulated by estrogen. This interpretation is supported by reduction in bone mineral content in adult mice treated with DES (Rowas et al., 2012), as well as reduction in os penis length in mice castrated at birth and treated with DES (Rodriguez et al., 2012). These observations imply the presence of estrogen receptors in the rudiments of the os penis and os clitoris. While estrogen receptors alpha or beta are known to be

expressed throughout the developing mouse clitoris (Blaschko et al., 2013; Rodriguez et al., 2012), the specific expression of estrogen receptors in the developing os penis and os clitoris has not been reported, even though estrogen receptors are known to be expressed in many bones (Borjesson et al., 2013; Windahl et al., 2002).

Clearly, the absence of the os clitoris in the DES-treated F1 generation females did not translate to absence of this bone in F2 DES-lineage females. Formation and initial differentiation of bone within mouse ExG (os penis and os clitoris) are known to be androgen-independent in so far as the os clitoris forms in wild-type female mice, in androgen-insensitive X^{Tfm}/Y male mice and in neonatally castrated oil-treated male mice (Rodriguez et al., 2012). In contrast, growth of the os penis and os clitoris is androgen-dependent (Rodriguez et al., 2012; Glucksmann et al., 1976). Thus, implied reprogramming of the germ line elicited by DES does not affect androgen-independent development of the os clitoris in the second generation. It appears, therefore, that estrogen-induced impairment of bone in ExG requires the actual presence of estrogen during development, presumably at pharmacological levels.

Considerable ambiguity and confusion exists regarding the definition of estrogen-induced mouse hypospadias, and accordingly objective criteria are needed for mouse hypospadias as discussed recently (Cunha et al., 2014). Most reports of mouse “hypospadias” are based upon the observation of an extensive open preputial/urethral cleft in the embryonic genital tubercle, with the tacit assumption that this major embryonic malformation is indicative of enduring (adult) hypospadias. For DES and perhaps other teratogenic agents this assumption must be questioned, as there are now several examples of “embryonic/neonatal ExG malformations” reverting to normality or to surprisingly mild defects in adulthood. In this regard, several reports of “embryonic hypospadias” have come from our laboratory. In particular Kim et al. reported an incidence of “embryonic hypospadias” (an extensive open preputial/urethral cleft) at an incidence of 40% to 59%, in a cohort of 134 mice treated prenatally with either ethinyl estradiol or DES from day 12 to day 19 of gestation (see Figure 4, in Kim et al., 2004). If this extensive “embryonic hypospadias” had endured into adulthood, we would expect the presence of substantial adult penile shaft hypospadias, yet in our current study such penile shaft hypospadias was not seen in 57 adult prenatally DES-treated mice that received a similar dosage and time course of DES treatment, and instead a rather mild form of hypospadias was observed. Clearly, extensive “embryonic hypospadias” did not persist into adulthood as was also the case of 5 α -reductase inhibitor-induced “embryonic hypospadias” (Iguchi et al., 1991). Thus, while some embryonic malformations may persist into adulthood, it is incumbent upon investigators to verify this tacit assumption. The effect of prenatal DES on MUMP cartilage differentiation provides yet another example of a developmental anomaly reverting to normality in adulthood. In a study to be reported elsewhere, mice treated with DES from birth to day 10 exhibited inhibition of MUMP cartilage differentiation at day 10 postnatal. However, allowing these mice to age to 60 days revealed normal MUMP cartilage differentiation (Sinclair and Cunha, unpublished).

A key lesson from these observations is the need for objective criteria for male mouse hypospadias and the realization that the best time to diagnose mouse hypospadias is after attainment of sexual maturity (end of puberty or adulthood [30 to 60 days postnatal]) when

enduring malformations are firmly and irreversibly established. The objective criteria that we advocate for diagnosing prenatally estrogen-induced mouse urethral hypospadias are: (a) “exposed urethral flaps”, (b) “exposed os penis”, (c) an elongated ventral cleft in the MUMP ridge and (d) abnormality in the shape or position of the elements (MUMP and MUMP ridge) that constitute the urethral meatus. The first 3 criteria (a–c) are best judged in serial histological sections, while the form of the urethral meatus can be recognized in adult specimens by simple observation with a dissecting microscope or by SEM. We recommend actual presentation of micrographs illustrating estrogen-induced mouse hypospadias and not presentation of observations by text only, which prevents evaluation of actual findings.

Another critical point in regard to mouse hypospadias is the question of what kinds of malformations are possible in the context of prenatally estrogen-induced perturbations of the normal developmental process. In this regard, it is highly advisable to not only document enduring adult malformations, but also to present data on how the adult malformations emerged. In this report, we demonstrated that prenatal DES exposure elicits mild malformations of the urethral meatus in adulthood with confirmatory observations at day 5- and 10-days postnatal in the primordial structures destined to form distal penile morphology including the urethral meatus (Figs. 13 & 14). Such an approach has the advantage of strengthening one’s interpretation as well as shedding light on the morphogenetic mechanism(s) responsible for the malformations. Finally in the case of male mouse hypospadias, the importance of incorporating developmental features into the mix has raised in our minds the possibility that prenatally estrogen-induced mouse penile shaft hypospadias may be highly unlikely if not impossible for the following reasons: (a) At 5 and 10 days postnatal, developing penises of prenatally DES-treated mice show no malformations in the area of the penis destined to form the penile shaft; the urethra in the “penile shaft” region of prenatal DES-treated mice is normal. (b) Penile surface epithelium, that would be deficient in penile shaft hypospadias (Fig. 2), does not form until 25 to 30 days postnatal (Fig. 17), which is approximately 4 to 5 weeks after initiation of DES treatment and 3 to 4 weeks after the end of DES treatment. It is highly unlikely that initiation of a DES-induced effect would be delayed so long after in utero DES treatment. An apparent contradiction to our interpretation is a definitive example of midshaft hypospadias in mice with genetically impaired ephrin-B2 reverse signaling (Dravis et al., 2004). However, in this germline mutant mouse impaired ephrin-B2 reverse signaling profoundly affected earlier development of the cloaca, and thus the midshaft hypospadias described by Dravis et al. may be a consequence of earlier malformations that have secondary effects on penile development. Thus, we affirm that induction of penile shaft hypospadias by estrogens such as DES is unlikely/impossible, an idea that may also be true for other agents such as 5 α -reductase inhibitors or anti-androgens.

There are two schools of thought regarding the morphogenetic mechanism of penile urethral development in mice. The Cohn lab suggests that the penile urethra forms as a result of distal extension of the solid urethral plate that subsequently canalizes to form the urethral lumen (Seifert et al., 2008). The other view of penile urethral formation comes from the Baskin lab that advocates the view that (like the human) the mouse penile urethra forms as a result of midline fusion of the preputial-urethral folds (Cunha et al., 2014; Kim et al., 2004). The truth may be that both concepts are correct, in that the proximal portion of the mouse

penile urethra may develop through extension and canalization of the urethral plate as suggested by the Cohn group, whereas the distal portion of the penile urethra, and especially the urethral meatus, may develop via midline fusion events. Several malformations seen in the current study can be directly or indirectly attributed to prenatally DES-induced impaired fusion events: (a) “bifid prepuce”, (b) defects in the MUMP (known to develop as a result of fusion of bilateral elements (Schlomer et al., 2013)), (c) an elongated midline ventral cleft in the MUMP ridge and (d) associated “exposed urethral flaps and “exposed os penis”.

Estrogens have a multitude of effects throughout the body, regulating growth and differentiation in many tissues. In adulthood the effects of estrogen are generally transient being dependent upon the actual presence of estrogenic substances. Consequently, after estrogenic compounds are cleared from the body, effects revert to an unstimulated steady state. In contrast, exposure to estrogens during development can have profound irreversible effects on morphogenesis, differentiation and gene expression persisting throughout life. For instance, sexual dimorphism of structure and function of the brain as well as sexual behavior in vertebrates is determined by estrogen action in the perinatal period (Carrer and Cambiasso, 2002; Kudwa et al., 2006). At puberty estrogen promotes closure of epiphyseal growth plates in long bones, and thus early exposure to estrogens can elicit short stature (Grumbach, 2000). In the liver, neonatal estrogen treatment irreversibly alters sexual differentiation of hepatic histidase in adult female rats (Lamartiniere, 1979). Neonatal exposure to genistein reduces expression of estrogen receptors α and β in testes of adult mice and thus alters the normal hormonal regulation of the adult testes (Shibayama et al., 2001). Male and female reproductive tract development and differentiation are also profoundly affected by exposure to exogenous estrogens during the perinatal period. For example, estrogens administered during the neonatal period inhibit rat uterine gland formation (Branham et al., 1985; Hayashi et al., 2011; Hayashi et al., 2004) and inhibit development of the myometrium (Brody and Cunha, 1989). Neonatal estrogen treatment of mice also elicits ovary-independent persistent vaginal cornification in adulthood, a condition in which vaginal epithelium remains thick and cornified despite ovariectomy (Iguchi et al., 1988; Kimura et al., 1967). In males exogenous estrogens elicit life-long deleterious pathogenic effects in the prostate and down regulation of the androgen receptor (McPherson et al., 2008; Prins, 1997; 1992). Dr. Hari Goyal has been a pioneer in the field of effects of estrogens on penile development/differentiation and has focused most of his attention on the body of the rat penis (as opposed to the penile glans, the subject of the current paper). Goyal's group has reported mal-development of the rat penis elicited by neonatal estrogens such as DES or estradiol involving reduction in penile length and weight, replacement of cavernous spaces by fat cells within the penile body, and inhibition of cavernous smooth muscle cell differentiation associated with down-regulation of myosin heavy chain 11 expression (Goyal et al., 2005; Goyal et al., 2007; Okumu et al., 2012; Goyal et al., 2004). Our studies have focused on the mouse glans penis and have demonstrated inhibitory effects on cartilage and bone differentiation (Blaschko et al., 2013; Rodriguez et al., 2012). Observations derived from wholemounts (Fig. 3), OPT images (Fig. 13) and histologic sections (Figs. 5 & 14) demonstrate a substantial truncation of distal penile structures such as the MUMP and MUMP ridge as well as the preputial lamina of prenatally DES-treated mice. While the role of cell proliferation was not examined in the examples given above, it

is likely that these penile hypoplasias elicited by prenatal DES involve inhibition of proliferation in penile target tissues.

Comparison of strain response to prenatal DES exposure in our study was predicated on the fact that C57BL/6 mice are more sensitive to estrogen than CD-1 mice (Spearow et al., 1999). However, F1 females from both of these mouse strains did not differ in the incidence of DES-induced urethral-vaginal fistula. In contrast, malformation rates of certain penile structures (Table 1) in F1 prenatally DES-treated C57BL/6 mice were consistently higher than that observed in CD-1 mice. This difference between females and males may be due to a critical timing window for generating penile versus urethral-vaginal malformations. Alternatively, the pharmacological dose of DES may have elicited the maximum response in females of both strains. In males it is perhaps worth noting that the incidence of prenatally DES-induced exposed os penis and exposed urethral flaps was 18 to 39%, respectively, in CD-1 mice and 31 to 85%, respectively, in C57BL/6 male mice (Table 1). In contrast, the incidence of an abnormal urethral meatus was observed was 100% in both mouse strains treated with a pharmacological DES dose. In addition, physiologic elevation of estrogen levels in aromatase over-expressing mice also leads to an abnormal penile urethral meatus in adulthood (Blaschko et al., 2013). Failure to achieve a near 100% incidence in all abnormal penile parameters in males of both strains may be due to (a) strain differences in sensitivity, (b) strain differences in the timing of the window of sensitivity (missing the critical developmental window of sensitivity), (c) sub-optimal timing of DES exposure, or (d) suboptimal dosage. Clearly our results using prenatal DES treatment should be compared in the future with neonatal DES exposure or a combination of prenatal plus neonatal DES treatment. In this regard, Ma emphasizes in a 2009 review that if the goal is to model human ExG disruption, the neonatal mouse model best represents the developmental stages of potential human exposure, even though in utero events are eliminated (Ma, 2009).

CONCLUSION

This study addresses certain ambiguities in the literature regarding mouse hypospadias and accurately specifies the types of malformations in the male and female ExG elicited by prenatal DES. First, we have examined male and female offspring of prenatally DES-exposed males and found urethral/ExG abnormalities in both sexes in the F2 (second) generation. ExG malformations are more pronounced in the first generation in both sexes, with rates of hypospadias being higher in the F1 (DES-exposed) males, and with rates of urethral-vaginal fistula being higher in F1 versus F2 females. Thus, prenatally DES-induced ExG malformations (male urethral hypospadias and urethral-vaginal fistula) of both sexes are transmitted to the second generation. That prenatally DES-exposed males can transmit ExG malformations to their female offspring suggests that the absence of data on the daughters of DES sons in epidemiological studies might be an important gap. Finally, C57BL/6 males exhibited a higher incidence of prenatally DES-induced ExG malformations than the less estrogen-sensitive CD-1 males. In contrast, females from these strains showed no significant differences in DES-induced urethral-vagina fistula.

Acknowledgments

This work was supported by NSF Grant IOS-0920793 and NIH grant RO1 DK0581050.

ABBREVIATIONS

ExG	external genitalia
DES	diethylstilbestrol
MUMP	male urogenital mating protuberance
SEM	scanning electron micrograph
PFA	paraformaldehyde
DMSO	dimethyl sulfoxide
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
RT	room temperature
OPT	optical projection tomography

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HIGHLIGHTS

1. Objective criteria of prenatally DES-induced adult mouse hypospadias are presented for the first time.
2. The incidence of penile and preputial hypospadias was higher in C57BL/6 versus CD-1 mice consistent with enhanced estrogen sensitivity of C57BL/6 mice.
3. The incidence of urethral-vaginal fistula was similar in prenatally DES-treated mice of both strains.
4. Prenatally DES-induced male hypospadias and urethral-vaginal fistula were transmitted to second-generation mice.

Figure 1.

A scanning electron micrograph (A) and a photo (B) of the penis of an adult prenatally oil-treated (A) mouse and a prenatally DES-treated mouse (B). In (A) note that the ventral cleft only partially bisects in the MUMP ridge of the prenatally oil-treated mouse. Red arrow denotes the beginning of the tubular urethra at the point of closure of the ventral cleft. In (B) note that the ventral cleft (opposed arrowheads) completely bisects the MUMP ridge and extends a considerable distance to partially bisect the internal prepuce.

Figure 2.

Adult mouse penises from untreated (A), prenatally oil-treated (B–C) and prenatally DES-treated mice (D–E) with transverse H&E stained sections (C & E) taken where indicated by the dotted lines. In (A) the lateral wall of the preputial space has been removed by dissection revealing the glans penis within the preputial space. Opposed arrows to the left denote the proximal extent of the prepuce and the junction of the penile body with the so-called glans extending to the right. In (B & C) the area denoted by brown represents the extent of the ventral cleft in the MUMP ridge. Green represents the urethral flaps and red represents the os penis. In untreated or oil-treated mice (B & C) note that sections through the closing ventral cleft (C) shows the Y-shaped urethra and the absence of urethral flaps and os penis. In (D & E) note that the urethral flaps and os penis are observed in sections having an open ventral cleft (brown) in the MUMP ridge.

Figure 3.

Wholemount photos of adult male prepuce (A–D) and penis (E–H, ventral views) from prenatally oil- and DES-treated mice as indicated. Note altered shape of the C57BL/6 prepuce (C). In (E–H) note that the distally bifid MUMP is truncated in both strains as a result of prenatal DES treatment. Blue suture threaded from the bladder to the exterior indicates the urethral meatus.

Figure 4.

Scanning electron micrographs and a photo of the penis of adult mice treated prenatally with oil (A) or DES (B & C). In untreated or oil-treated mice (A) the urethral meatus is Y-shaped and formed by the MUMP dorsally fused with the elements of the MUMP ridge that are separated by clefts including the prominent ventral cleft. As a result of prenatal DES treatment (B & C) the end-on pattern is distorted in several different ways. In (B) the ventral cleft is almost imperceptible, whereas patterning of clefts and MUMP is markedly distorted with absence of the typical Y-shaped urethral meatus. In (C) note distortion of the MUMP ridge, distortion of the urethral meatus and complete bisection of the MUMP ridge by the ventral cleft. Arrowheads denote MUMP ridge groove.

Figure 5.

Transverse sections through penises of adult CD-1 and C57Bl/6 male mice treated prenatally with either oil or DES. In each column the sections read from distal (1) to proximal (4). Numbers indicate section number from the distal tip of the penis, and verify overall penile truncation as suggested in wholemount photos (Figs. 3F & G). All distal sections (A1–D1) depict an open ventral cleft, which in oil-treated mice is closed in “middle sections” (A2 & D2). In the corresponding level of DES-treated specimens (B2 & C2) the ventral cleft remains open (black stars), a situation that persists in more proximal regions (B3, C3, B4, C4). Note in DES-treated specimens that urethral flaps are present (triangles) in sections (C3, B4 & C4) in which the ventral cleft is open. Likewise, the os penis (white arrows) is present in sections (B4 & C4) in which the ventral cleft is open in prenatally DES-treated specimens.

Figure 6.

Transverse sections through the prepuce of adult female (A) and male (B) CD-1 mice demonstrating the homology of the female prepuce to the male prepuce. There are 5 characteristic features of male and female prepuces: (a) the preputial space, (b) stratified squamous cornified epithelium lining the preputial space, (c) preputial gland ducts in the wall of the prepuce which open into the preputial space, (d) an outer hair-bearing epidermis, (e) a thick wall between the epidermis and the lining of the preputial space containing sebaceous gland and the lower ends of hair follicles (double headed arrows). Male and female prepuces share all of these features.

Figure 7.

Three-dimensional reconstruction (A) and serial sections through the junction of the preputial space (PS) (B–D) and the urethra (Ur) (E) of an untreated adult CD-1 female mouse. The three-dimensional reconstruction (A) has been artificially elongated while maintaining proportionality. Note the position of the distal aspect of the urethra (junction of the stand-alone urethra [E] and the composite epithelium [D]). The preputial space begins immediately distal to the “stand-alone” urethra and is illustrated in sections B–D, depicting a transition zone containing the composite stratified squamous cornified epithelium lining the preputial space fused to the U-shaped clitoral lamina. The centrally placed white arrow in (A) denotes the junction of the female prepuce with the ventral body surface. The sections read from proximal to distal (B to E). Positions of sections (B–E) are indicated in the three-dimensional reconstruction. Note that the urethra is a “stand alone” structure, not attached to other epithelia only in (E), while in more proximal sections (B–D), the preputial space is attached (arrows) to the U-shaped clitoral lamina, which is a “stand alone” structure in (E) only. In (E) the epithelium of the urethra is stratified and cornified dorsally, but stratified and non-cornified ventrally. More proximally the urethral epithelium will be completely

non-cornified. Note that for all images ventral is up and dorsal is down. PPGD=preputial gland ducts.

Figure 8.

Wholemount photos of adult female external genitalia (prepuce and vaginal meatus). The prepuce of adult oil-treated female mice (A&D) have a bifid tip as described previously (Yang et al., 2010). The clefting of the prepuce is exaggerated in prenatally DES-treated female mice (B & C).

Figure 9.

Serial transverse sections through ExG of prenatally oil- and DES-treated CD-1 and C57BL/6 adult female mice. In each column the sections read from distal (1) to proximal (4). Arrow in (A2 & D2) indicates normal preputial space. Star indicates abnormal urethral-vaginal fistula (B1–2 & C1–3). Arrowhead indicates os clitoris in (A4 & D4) and double-headed arrows indicate vagina epithelium.

Figure 10.

Mid-sagittal sections through female external genitalia. In prenatally oil-treated (F1) females the urethral and vaginal meatuses open separately to the exterior in both mouse strains (A & B), whereas in prenatally DES-treated females the urethra opens into the vaginal lumen (C) or opens into a common meatus (D). Dotted ellipses denote vagina meatus.

Figure 11.

Transverse sections of penises of two separate second generation (F2) mice generated from breeding a F1 prenatally DES-treated male with an untreated female. Note urethral flaps (arrowheads) and persistence of epithelium of the ventral cleft (arrows).

Figure 12.

Transverse sections of the external genitalia of F2 females (second generation) derived from mating F1 prenatally DES-treated male CD-1 mice with untreated female mice. Star indicates urethral-vaginal fistula. Arrow indicates os clitoris and double headed arrow=vaginal epithelium.

Figure 13. Optical projection tomography images stained with anti-E-cadherin of day 5 postnatal penises derived from mice treated prenatally with oil or DES as indicated. Note overall reduction in size of all structures, specifically reduction in overall length of the preputial lamina and truncation of distal structures destined to form the penile urethral meatus.

Figure 14.

Transverse sections of day 10 postnatal penises derived from mice treated prenatally with oil or DES as described. The only malformation seen at 10-days postnatal in prenatally DES-treated male mice was impaired differentiation of the MUMP cartilage (compare A & B). Section numbers on each image denotes distance from the distal tip of the ExG and indicates substantial truncation in the proximal-distal axis.

Figure 15.

By way of illustrating the defects involved in penile shaft hypospadias, drawings of human penises in transverse sections are given. (A) is a cross section of normal human penis. (E–F) depict what is missing/defective in penile shaft hypospadias, namely absence/defect of ventral skin, ventral urethral epithelium and corpus spongiosum, which in the mouse is the corpus cavernosum urethrae.

Figure 16.

At birth (A), and on days 5 (B) and 10 postnatal (C) the outer surface of the external genitalia is covered by preputial skin. The future outer surface of the penis is represented by the solid epithelial preputial lamina. Accordingly, a free penile surface does not exist as such from birth to day 10 postnatal. (B) is an OPT mid-sagittal image of a 5-day postnatal mouse penis revealed by E-cadherin immunostaining and shows that the penis is defined by the preputial lamina, which has not yet canalized. The preputial space is present only distally.

Figure 17.

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. In the top row the arrows denote the distal bifid tip of the MUMP. The designation urethra (Ur) is based upon a stand-alone tube fully surrounded by penile stroma in turn enclosed within the external preputial lamina. The process of canalization of the external preputial lamina begins distally and progresses proximally and thus penile surfaces are revealed temporally in the following order: distal bifid MUMP (B1), mid-MUMP (C2 & D2), penile shaft (D3 & D4).

Table 1

Effects of Prenatal DES treatment on external genitalia of CD-1 and C57Bl/6 Mice

Specimens/Results	CD-1		C57Bl/6	
	Oil	DES	Oil	DES
Number of F0 Dams	4	8	3	10
Number of F1 Pups	54	57	23	23
Number of Adult F1 Males	27 [*]	32 [*]	11	13
-Exposed urethral flap	1/23 [*] (4%)	11/28 [*] (39%)	1/11 (9%)	11/13 (85%)
-Exposed os penis	1/23 [*] (4%)	5/28 [*] (18%)	1/11 (9%)	4/13 (31%)
-Abnormal urethral meatus	0/15 [*] (0%)	18/18 (100%)	0/9 [*] (0%)	10/10 (100%)
Number of Adult F1 Females	28 ^{**}	25 ^{**}	13	10
-Os clitoris absent	0/24 ^{**} (0%)	21/21 ^{**} (100%)	0/13 (0%)	10/10 (100%)
-Urethral-vaginal fistula	0/24 ^{**} (0%)	19/21 ^{**} (91%)	0/13 (0%)	10/10 (100%)

*
and

**
Note difference in the denominator due to the use of mice for different purposes.

Table 2

Malformations Observed in F2 Male and Female Offspring

Identifiers	Oil-treated F1 Males Mated to Untreated Females	DES-treated F1 Males Mated to Untreated Females
F1 Adult Males	10	10
a. Successful Breeding	10/10 (100%)	9/10 (90%)
b. Number of F2 Male Pups produced	50	55
c. Number of F2 Female Pups produced	60	42
d. Exposed Urethral Flaps in F1 Males	0/10 (0%)	4/10 (40%)
e. Exposed Os Penis in F1 Males*	0/10 (0%)	1/10 (10%)
Malformations in Male F2 pups		
a. Exposed Urethral Flaps	0/50 (0%)	11/55 (20%)
b. Exposed Os Penis	0/50 (0%)	0/50 (0%)
Malformations in Female F2 pups		
a. Urethral-Vaginal Fistula	0/60 (0%)	5/42 (11.9%)
b. Os Clitoris	Present 60/60 (100%)	Present 42/42 (100%)