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Mouse hypospadias: A critical examination and definition

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ABSTRACT

Hypospadias is a common malformation whose etiology is based upon perturbation of normal penile development. The mouse has been previously used as a model of hypospadias, despite an unacceptably wide range of definitions for this malformation. The current paper presents objective criteria and a definition of mouse hypospadias. Accordingly, diethylstilbestrol (DES) induced penile malformations were examined at 60 days postnatal (P60) in mice treated with DES over the age range of 12 days embryonic to 20 days postnatal (E12–P20). DES-induced hypospadias involves malformation of the urethral meatus, which is most severe in DES E12–P10, DES P0–P10 and DES P5–P15 groups, and less so or absent in the other treatment groups. A frenulum-like ventral tether between the penis and the prepuce was seen in the most severely affected DES-treated mice. Internal penile morphology was also altered in the DES E12–P10, DES P0–P10 and DES P5–P15 groups (with little effect in the other DES treatment groups). Thus, adverse effects of DES are a function of the period of DES treatment and most severe in the P0–P10 period. In "estrogen mutant mice" (NERKI, βERKO, αERKO and AROM+) hypospadias was only seen in AROM+ male mice having genetically-engineered elevation is serum estrogen. Significantly, mouse hypospadias was only seen distally at and near the urethral meatus where epithelial fusion events are known to take place and never in the penile midshaft, where urethral formation occurs via an entirely different morphogenetic process.

1. Introduction

In a previous paper the "window of susceptibility" of the developing mouse penis to the adverse developmental effects of diethylstilbestrol (DES) was analyzed at 10, 15 and 20 days postpartum in the following groups: (DES E12–E18, DES P0–P10, DES E12–P10, DES P5–P15 and DES P10–P20) (Sinclair et al., 2016a). The rationale of this previous study was to examine the mouse penis shortly after the last DES injection to define the effects of DES during developmental periods. Penises of all 5 groups of DES-treated mice were reduced in size, and the most profound effects were seen in the DES E12–P10, DES P0–P10, and DES P5–P15 groups, thus defining a DES "programming window" for adverse effects centered in the early neonatal period. The most mild of effects on penile development were seen in the DES E12–E18 and DES P10–P20 groups. In the DES E12–P10 and DES P0–P10 groups adverse effects of DES on the MUMP cartilage and erectile bodies were observed shortly after the last DES injection and persisted as enduring adult abnormalities. In contrast, in the DES P5–P15 group abnormalities in the MUMP cartilage and erectile bodies observed immediately after the last DES injection reverted to normality at 60 days postpartum. Thus, the induction of irreversible effects by DES also exhibited a "window of susceptibility" in the early neonatal period.

Expanding on our earlier study in which DES effects were examined in the neonatal period (P10–P20) shortly after the DES treatment period, the current study follows cohorts of mice treated with DES developmentally (DES E12–E18, DES P0–P10, DES E12–P10, DES P5–P15 and DES P10–P20) and analyzed in adulthood (60 days postnatal) to precisely define the resultant enduring DES-induced penile malformations by morphology and morphometric analysis. In so doing, a clearer understanding of estrogen-induced mouse hypospadias has been achieved.

Hypospadias is the second most common urogenital anomaly in boys occurring in approximately 1:200 to 1:300 male births (Baskin, 2000). The incidence of hypospadias in the USA has doubled in recent times (Paulozzi, 1999; Paulozzi et al., 1997).

Abbreviations: DES, Diethylstilbestrol; E, embryonic; P, postnatal; OPT, optical projection tomography; MUMP, male urogenital mating protuberance

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While the etiology of hypospadias in the majority of patients remains undefined, it is thought to involve both genetic susceptibility and environmental exposure to endocrine disruptors (Baskin and Ebbers, 2006; Kalfa et al., 2011; Willingham and Baskin, 2007; Skakkebaek et al., 2016; Wang and Baskin, 2008b). Surgery is the established treatment for hypospadias, and multiple surgeries are often required for a functionally acceptable reconstruction (Lee et al., 2013). In this regard, patients with severe hypospadias are at risk for surgical complications that may lead to life long difficulties with urination, sexual function and psychological problems. Thus, hypospadias is a significant medical condition that consumes substantial health care resources.

Hypospadias results from perturbation of normal penile development (Baskin et al., 1998), and can only be understood in the context of normal penile development. In humans, hypospadias consists of 3 related anomalies: (a) a urethral defect, (b) a preputial defect and (c) chordee (abnormal curvature of the penis). The abnormal urethral orifice in human hypospadias may be situated distally in the glans, at midshaft, or in the perineum (Cunha et al., 2015). Associated with the defect in the urethral meatus is local absence or hypoplasia of the corpus spongiosum (Baskin et al., 1998).

Substantial differences in anatomy and development of the human versus the mouse penis invariably translate to profound differences in hypospadias in these two species (Cunha et al., 2015). While obvious abnormalities in the positioning of the urethral meatus are seen on physical examination in humans, the morphology of murine hypospadias is subtle. Midshaft malformations similar to human hypospadias have not been observed in mice treated perinatally with exogenous estrogens (Sinclair et al., 2016a; Blaschko et al., 2013; Mahawong et al., 2014b, a; Rodriguez et al., 2012) and may be due to the fact that mouse penile urethral development is substantially different from human penile development. Indeed, most of the murine penile urethra develops within the embryonic genital tubercle via canalization of the urethral plate to directly form most of the penile urethra, especially the midshaft region of the mouse urethra (Hynes and Fraher, 2004a, b; Seifert et al., 2008). In contrast, the mouse urethral meatus forms via an entirely different mechanism. The urethral plate of the mouse is not involved in formation of the urethral meatus since the urethral plate does not extend into the distal aspect of the genital tubercle (Sinclair et al., 2016a; Mahawong et al., 2014a; Schlomer et al., 2013). Instead, the mouse urethral meatus forms via fusion of the male urogenital mating protuberance (MUMP) with the MUMP ridge (Blaschko et al., 2013; Mahawong et al., 2014b, a; Rodriguez et al., 2011; Yang et al., 2010; Sinclair et al., 2016a). Consequently, estrogen-induced mouse hypospadias is an event restricted to the distal aspect of the urethra characterized by (a) altered patterning of elements constituting the urethral meatus, namely the male urogenital mating protuberance (MUMP) and MUMP ridge, (b) altered patterning of internal penile elements such as the os penis and urethral flaps relative to the urethral meatus and (c) absence or hypoplasia of the corpora cavernosa urethrae, the homolog of the human corpus spongiosum (Cunha et al., 2015). Whether such malformations seen in estrogen-treated mice are also relevant to mouse penile malformations elicited by other classes of agents (progestins, antiandrogens, phthalates) remains to be determined.

The current study describes the types of adult penile malformations induced developmentally by DES (hypospadias) in cohorts of mice treated with DES over the age range of E12–P20, but examined in adulthood when enduring malformations are present.

2. Materials and methods

2.1. Animals

Animal care and research protocols were approved by the Animal Care and Use Committee of the University of California, San Francisco (UCSF). Adult wild-type CD-1 and C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA, USA) and their offspring were housed in polycarbonate cages ($20 \times 25 \times 47\ cm^3$) with laboratory grade pellet bedding in the UCSF Pathogen Specific Barrier facility. Mice were given water *ad libitum* and fed LabDiet 5058 (PMI Nutrition International, P.O. Box 66812, St. Louis, MO 63166), whose content of phytoestrogen is incapable of eliciting vaginal cornification in ovariectomized adult mice (Buchanan et al., 1998). The following mutant mice were also used: estrogen receptor beta knockout (βERKO) (Paul Cooke, University of Florida, Gainsville, FL), estrogen receptor alpha knockout (αERKO) (Paul Cooke, University of Florida, Gainsville, FL), DNA binding mutation in estrogen receptor-α (NERKI) (Ellis Levin, University of California, Irvine, CA), and aromatase over-expresser (AROM+) (Gail Risbridger, Monash University, Melbourne, Australia). For all mutant mice, formalin fixed mouse rear ends containing the external genitalia were shipped to UCSG for processing. This study is based upon the analysis of 119 CD-1 and mutant mice.

2.2. Hormonal treatments

Pregnant CD-1 dams were weighed and injected subcutaneously on days 12, 14, 16, and 18 of gestation with DES at a concentration of 200 ng/g body weight in ~5 μl sesame oil vehicle. Control group dams were injected with 5 μl sesame oil. Separate Hamilton syringes were used for sesame oil and DES. For postnatal DES treatment, the day of birth was counted as day 0, and pups were weighed and injected subcutaneously with either DES (200 ng/gbw) or oil (5 μl) on days 1, 3, 5, 7, 9 (DES P0–P10 and DES E12–P10), on days 5, 7, 9, 11, 13 (DES P5–P15) or on days 10, 12, 14, 16, 18 (DES P10–P20). For the DES E12-P10 treatment group, the pregnant mice were also treated on days embryonic days 12, 14, 16 and 18.

2.3. Specimen preparation and analysis

DES- or oil-treated CD-1 mice were euthanized at the postnatal ages specified in Table 1. Sex was confirmed by gonadal inspection. External genitalia were dissected and fixed in 10% buffered formalin for a minimum of 24 h. Seven micrometer thick sections were stained with hematoxylin and eosin as described previously (Sinclair et al., 2016a).

2.4. Scanning electron microscopy

Surface details of adult mouse penises were elucidated using scanning electron microscopy (SEM) as described previously (Blaschko et al., 2013).

2.4.1. Optical projection tomography

Genital tubercles of mice at 16 days of gestation were fixed in 10% formalin, bleached with hydrogen peroxide, stained using a whole-mount immunofluorescence protocol (Abcam EP700Y anti-E-Cadherin monoclonal antibody, Alexa fluor 488 anti-rabbit secondary), optically cleared in benzyl alcohol and benzyl benzoate, embedded in agarose and imaged using a Bioptonics OPT scanner 3001M as described (Li et al., 2014). Projected images from each channel were constructed into 3D voxel data sets with in-house software, which were then visualized using the Volocity software suite from PerkinElmer.