Estrogen receptor- α mediates estrogen-inducible abnormalities in the developing penis

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Abstract

Previously, we reported an association between estrogen receptor- α (ER α) upregulation and detrimental effects of neonatal diethylstilbestrol (DES) exposure in the rat penis. The objective of this study was to employ the ER α knockout (ER α KO) mouse model to test the hypothesis that ER α mediates DES effects in the developing penis. ER α KO and wild-type C57BL/6 mice received oil or DES at a dose of 0.2 μ g/pup per day (0.1 mg/kg) on alternate days from postnatal days 2 to 12. Fertility was tested at 80–240 days of age and tissues were examined at 96–255 days of age. DES caused malformation of the os penis, significant reductions in penile length, diameter, and weight, accumulation of fat cells in the corpora cavernosa penis, and significant reductions in weight of the bulbospongiosus and levator ani muscles in wild-type mice. Conversely, ER α KO mice treated with DES developed none of the above abnormalities. While nine out of ten male mice sired pups in the wild-type/control group, none did in the wild-type/DES group. ER α KO mice, despite normal penile development, are inherently infertile. Both plasma and intratesticular testosterone levels were unaltered in the DES-treated wild-type or DES-treated ER α KO mice when compared with controls, although testosterone concentration was much higher in the ER α KO mice. Hence, the resistance of ER α KO mice to developing penile abnormalities provides unequivocal evidence of an obligatory role for ER α in mediating the harmful effects of neonatal DES exposure in the developing penis.

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Introduction

It is well known that androgens are critical in development of male reproductive organs in all mammals studied to date (George & Wilson 1994). It is also known that dihydrotestosterone, a 5 α -reductase reduced testosterone metabolite, is more critical than testosterone in development of those organs which are derived from the urogenital sinus, genital tubercle, and genital swelling in males, including the penis (Anderson & Clark 1990, George & Wilson 1994). Notably, the early differentiation and morphogenesis of male reproductive organs corresponds to the testosterone surge by fetal Leydig cells that occurs at about 12 weeks of gestation in humans (Williams-Ashman & Reddi 1991, George & Wilson 1994, Klonisch *et al.* 2004) and at late gestation and early neonatal period in rodents (Ward & Weisz 1984, El-Gehani *et al.* 1998). Alterations in androgenic activity during the critical period of differentiation, resulting from abnormalities in testosterone,

5 α -reductase, or androgen receptor, cause maldevelopment of internal and external male genitalia, including hypospadias and shorter penis (Gray *et al.* 2001, Sultan *et al.* 2001, Kim *et al.* 2002, Foster & Harris 2005). In a situation where androgen receptors are inherently lacking, such as in androgen insensitivity syndrome, external genitalia develop as those of females (Wiener *et al.* 1997, Regadera *et al.* 1999, Hiort 2000).

Unlike the case with androgens, the role of estrogen in development of male reproductive organs, especially in the penis, remains largely unknown; although both estrogen receptors (ERs) and/or aromatase enzyme have been localized in the developing penis of a number of species, including humans (Crescioli *et al.* 2003, Schultheiss *et al.* 2003, Dietrich *et al.* 2004) and rodents (Jesmin *et al.* 2002, 2004), and rabbits (Srilatha & Adaikan 2004). Additionally, epidemiological studies have suggested links between inappropriate estrogen exposure and higher frequency of reproductive

abnormalities in men and wild animals (Toppari *et al.* 1996, McLachlan *et al.* 2001, Safe *et al.* 2001, Mosconi *et al.* 2002, Fisher 2004, Vidaeff & Sever 2005, Storgaard *et al.* 2006, reviews). In this context, it is noteworthy that male offspring of women exposed to diethylstilbestrol (DES) during pregnancy have higher incidence of epididymal cysts, cryptorchidism, hypospadias, and smaller penis (Gill *et al.* 1979, Swan 2000, Klip *et al.* 2002), as well as higher incidence of hypospadias in sons of women exposed to DES in fetus, implying a transgenerational effect (Klip *et al.* 2002, Brouwers *et al.* 2006); pregnant mothers with high intake of phytoestrogens as a result of vegetarian diet are more likely to give birth to boys with hypospadias (North & Golding 2000); laboratory animals exposed neonatally to estrogen develop hypospadias (McLachlan *et al.* 1975, Kim *et al.* 2004, Newbold 2004); neonatal exposure to estrogen at low doses enlarges the adult prostate gland, while higher doses have the opposite effect (vom Saal *et al.* 1997, Gupta 2000, Putz *et al.* 2001, vom Saal & Hughes 2005); and perinatal estrogen exposure predisposes the prostate gland to a precancerous growth at adulthood by an epigenetic mechanism (Ho *et al.* 2006). Hence, prenatal and/or neonatal exposure to estrogens can have permanent, and even transgenerational, deleterious effects on the development of male reproductive organs; however, the mechanism underlying estrogen-inducible abnormal phenotypes in the male reproductive tract, as well as in the penis, remains unknown.

Recently, we reported permanent dysmorphogenesis of the penis and loss of fertility in adult rats-treated neonatally with DES or estradiol valerate (Goyal *et al.* 2004a,b, 2005a,b). Specifically, these studies showed significant reductions in penile length and weight, maldevelopment of the os penis, and accumulation of fat cells and loss of cavernous spaces and smooth muscle cells in the body of the penis. Furthermore induction of these novel phenotypes is dose-dependent, requires a critical window of exposure, and is associated with decreased plasma testosterone and upregulation of ER α expression in the body of the penis. These observations lead us to hypothesize that a functional ER α -mediated pathway is required in induction of aberrant penile development.

Hence, the objective of this study is to test the above hypothesis by treating ER α knockout (ER α KO) mice neonatally with DES because they inherently lack ER α and, therefore, should be resistant to DES-inducible penile abnormalities, which should develop in the wild-type mice treated similarly.

Materials and Methods

Animals and treatments

Heterozygous breeding pairs for the ER α null allele were crossed to generate ER α KO and wild-type C57BL/6 mice

in a continuous breeding scheme at the University of Illinois at Urbana-Champaign. Pups were treated with DES at a dose of 200 ng (0.1 mg/kg) in 25 μ l olive oil, per pup, every other day, from postnatal days 2 to 12 (note, the same dose regimen was used in our previous studies in the rat, Goyal *et al.* 2004a,b). Controls received olive oil only. DNA was isolated between 8 and 12 days of age, from tail tissues using DirectPCR Lysis Reagent (Viagen Biotech Inc., Los Angeles, CA, USA) and used to determine genotype. Animals were shipped to Tuskegee University at adulthood for further study. Animals at both universities were maintained at 22–23 °C ambient temperature, 55–60% relative humidity, and 12 h light:12 h darkness cycle, and had free access to food (Rodent Chow 5001; Purina Mills, St. Louis, MO, USA) and water for 24 h. Animals were handled in accordance with the guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington, DC, 1996). All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Illinois at Urbana-Champaign and Tuskegee University.

Body and organ weights

All animals were weighed and terminated at 96–255 days of age. The seminal vesicles were weighed as a marker for DES effects on the accessory gland because estrogens are known to have an inhibitory effect on this gland (Goyal *et al.* 2003, 2004a,b). The testes and epididymides were also weighed, but the data are not included in the study because the weight of both organs in the wild-type/DES mice and that of the testes in the ER α KO/oil or ER α KO/DES mice varied widely among animals. These variations resulted from retention of the testis and epididymis of one or both sides in the inguinal canal in more than 70% of the wild-type/DES mice and from accumulation of the testicular fluid in the testis of one or both sides of the ER α KO mice, which worsened with age.

Penis and penile skeletal muscles

The penis was measured for length, diameter, and weight and was processed as described previously (Goyal *et al.* 2005a,b). Briefly, the penis was exposed up to the ischial arch, and its stretched length was measured from the tip of the glans penis to the midpoint of the ischial arch, and the diameter was measured from the middle of the body of the penis. After removing the free loose connective tissue, the entire penis was weighed. Two to three millimeter long sections from the middle of the body were processed for histopathology and histochemistry ($n \geq 5$ per group). For histopathology, tissues were fixed in formaldehyde, and 5 μ m thick paraffin sections were

stained with hematoxylin and eosin (H&E) for general morphology and with Masson's Trichrome stain for smooth muscle. For histochemical demonstration of fat, formaldehyde-fixed tissues were en block stained for 8 h with 1% osmium tetroxide dissolved in 2.5% potassium dichromate solution, and then processed for paraffin embedding. In addition, epoxy sections (1 μ m thick) of glutaraldehyde-fixed tissues were stained with toluidine blue. For evaluating the development of os penis, 1 penis from each group was radiographed with a cabinet radiograph system (Faxitron series, Hewlett-Packard) as described previously (Goyal *et al.* 2004b).

The rodent penis is surrounded by three pairs of skeletal muscles: ischiocavernosus extends from the ischial arch to the middle of the dorsal surface of the body of the penis; bulbospongiosus surrounds the ventro-lateral surface of the bulb of the penis; and levator ani forms a sling-like band around the anus and is connected dorsally to the bulbospongiosus. The latter two muscles were isolated, freed from connective tissue and fat, and weighed separately.

Digital images of paraffin and epoxy sections, as well as of the radiograph, were captured with a Leitz Orthoplan microscope (Vashaw Scientific Inc., Norcross, GA, USA) and the Kodak Microscopy Documentation System 290 (Eastman Kodak Company), and were assembled with the use of Adobe Photoshop 7.0.

Plasma and intratesticular testosterone

For plasma testosterone, one blood sample was collected by cardiac puncture from each animal just prior to necropsy; and for intratesticular testosterone, a part of the right testis was collected from each animal at the time of necropsy. Both plasma and testicular parenchyma were frozen at -20°C until assayed. Testes were processed in accordance with the protocol described by Park *et al.* (2002). Briefly, ~ 50 mg testicular tissue was homogenized in PBS. Eight volumes of ether were added to the homogenate and vortexed vigorously. The aqueous phase was snap-frozen, and the organic supernatant was transferred to a secondary tube and air dried. Just prior to assay, samples were resuspended in PBS. Testosterone in plasma and testicular tissue was measured using a COAT-A-COUNT testosterone RIA (Diagnostic Products Corporation, Los Angeles, CA, USA) according to manufacturer's protocol. The sensitivity of the assay was 0.2 ng/ml. The intra- and interassay coefficients of variations were 6 and 12% respectively.

Fertility

Eight to ten adult male mice from each group were transferred to mating cages and cohabited with untreated, 70- to 80-day-old females (1:2) for 12 days. At the end of cohabitation, females were separated and

allowed to deliver at term. The number of pups was recorded for each litter.

Statistical analysis

Statistical analyses were performed using Sigma Stat statistical software (Jandel Scientific, Chicago, IL, USA). Two-way ANOVA was performed on all parameters. Treatment groups with means significantly different ($P < 0.05$) from controls were identified using the Student–Newman–Keuls (SNK) test. When data were not distributed normally, or heterogeneity of variance was identified, analyses were performed on transformed data or ranked data.

Results

Body and organ weights

The mean body weight in the adult wild-type and ER α KO control mice was 25.88 and 26.53 g respectively, and was not altered as a result of DES treatment (25.68 vs 26.82 g). Seminal vesicles were weighed as a marker for DES effects in the male accessory sex organs. Their weight was reduced by almost 70% in the wild-type/DES mice when compared with that of wild-type controls, but was not significantly ($P < 0.05$) different between the ER α KO/DES and ER α KO/control mice (Fig. 1). Generally, seminal vesicles were one, one and half or two times heavier in the ER α KO mice than in the wild-type/control mice.

Figure 1 The paired weight of the seminal vesicles (SV) in wild-type and ER α KO adult mice treated neonatally with oil (control) or DES. An asterisk indicates a significant ($P \leq 0.05$) reduction in weight in the wild-type mice, as a result of DES treatment. Bars with different superscripts indicate significantly higher weight in the ER α KO/control mice than in the wild-type/control mice ($P \leq 0.05$). Data are expressed as mean $\pm$ S.E.M.

Penile measurements

The mean measurements of the penis, including weight, length, and diameter, were significantly ($P < 0.05$) lower in wild-type/DES mice when compared with those of wild-type/control mice (Fig. 2); and the decrease was more pronounced for weight than length (56% vs 25%). Conversely, DES exposure failed to exhibit any of the above detrimental effects in the penis of ER α KO mice. All penile measurements in the ER α KO/DES or ER α KO/control mice were similar to those in the wild-type/control mice.

Penile skeletal muscles

The weight of both bulbospongiosus and levator ani muscles was significantly ($P < 0.05$) lower in the wild-type/DES mice when compared with that of the wild-type/control mice (Fig. 3), although the percent reduction was much higher for the former muscle (68% vs 35%). In contrast, DES failed to affect the weight of either muscle in the ER α KO mice. The weight

Figure 2 The weight, length, and diameter of the penis in wild-type and ER α KO adult mice treated neonatally with oil (control) or DES.

*Significantly ($P \leq 0.05$) different from controls. Data are expressed as mean $\pm$ S.E.M.

Figure 3 The paired weight of the bulbospongiosus (BS) and levator ani muscles in wild-type and ER α KO adult mice treated neonatally with oil (control) or DES. *Significantly ($P \leq 0.05$) different from controls. Bars with different superscripts are significantly different ($P \leq 0.05$). Data are expressed as mean $\pm$ S.E.M.

of both muscles was almost 10% higher in both the ER α KO/DES and ER α KO/control mice than in the wild-type/control mice; however, the increase was significant ($P < 0.05$) only in the case of the bulbospongiosus muscle.

Penile gross morphology, histopathology, and histochemistry

Gross morphology

The mouse penis, similar to the rat penis (Goyal *et al.* 2004a), consists of a root, a cylindrical body, and a bulbous glans penis. An os penis extends from the distal end of the body to the tip of the glans penis and is responsible for the right angle bend between the body and the glans penis in a non-erect penis (Fig. 4). Based on gross and radiographic examinations, striking effects of neonatal DES exposure in the wild-type mice included a reduction in the right angle and malformations of the os penis, including its underdevelopment and less calcification. Conversely, these effects were completely lacking in the penis of DES-treated ER α KO mice, which was morphologically similar to that of the ER α KO/control and wild-type/control mice (Fig. 4).

Figure 4 Radiographs of the penis in wild-type control (WT-C), wild-type DES (WT-DES), knockout control (KO-C) and knockout DES (KO-DES) adult mice treated neonatally with oil (control) or DES. Note abnormal morphology of the penis, including underdevelopment and undercalcification of the os penis, in DES-treated wild-type mice only.

Histopathology and histochemistry

The body of the penis in both wild-type and ER α KO mice consists of three erectile bodies: two corpora cavernosa that are located dorso-lateral to the urethra and are partly separated by an intercrural septum that brings blood vessels and nerves to the crura and a corpus spongiosus that is located ventrally and surrounds the urethra (Fig. 5A–D). The corpora cavernosa contain endothelial-lined cavernous spaces (also called sinusoids), thin strands of smooth muscle cells surrounding the endothelium, and connective tissue trabeculae between cavernous spaces. In addition, a thick tunica albuginea capsule, consisting of an outer fibrous layer and an inner cellular layer, surrounds the corpora cavernosa.

An examination of paraffin sections stained with hematoxylin and eosin or Masson's Trichrome (distinguishes smooth muscle cells from collagen fibers) revealed that, among various histological components of the penis, the tunica albuginea

capsule and connective tissue trabeculae were less developed in the DES-treated wild-type mice when compared with those of the wild-type/control mice (Fig. 6A and B). In addition, an examination of epoxy sections stained with toluidine blue (Fig. 6C and D) and of undeparaffinized sections stained with osmium tetroxide (Fig. 7A–D) showed an abnormal accumulation of fat cells in the DES-treated wild-type mice. Again, none of the above histopathological abnormalities resulted in the penis of DES-treated ER α KO mice, which was structurally similar to that of the wild-type/control or the ER α KO/control mice.

Plasma and intratesticular testosterone

Neonatal DES exposure did not significantly ($P < 0.05$) alter the mean concentration of plasma or intratesticular testosterone in the wild-type or the ER α KO mice at adulthood (Fig. 8); however, both concentrations were almost three- to four-fold higher in the DES- or oil-treated ER α KO mice than in the wild-type/control mice.

Fertility

While nine out of ten males in the wild-type/control group sired pups, with a mean litter size of 6.5 pups (ranging from 4 to 11 pups/l), no male sired a pup in the DES-treated wild-type group or ER α KO groups treated with oil or DES ($n = 8/\text{group}$).

Discussion

The objective of this study was to test the hypothesis that ER α is essential in mediating detrimental effects of

Figure 5 Micrographs of the body of the penis in wild-type (A and B) and ER α KO (C and D) adult mice treated neonatally with oil (A and C) or DES (B and D). Note, different parts of the body of the penis, including a paired corpora cavernosa (CC), a corpus spongiosus (CS), and an intercrural septum (IC) containing blood vessels and nerves are morphologically developed in both the wild-type and ER α KO mice, regardless whether they are treated with oil or DES. However, the corpora cavernosa in the case of the wild-type DES group only is dramatically reduced in size. Hematoxylin and eosin.

Figure 6 Micrographs of the body of the penis in wild-type adult mice treated neonatally with oil (A and C) or DES (B and D). (A and B) In these micrographs, note a significant reduction in thickness of the tunica albuginea (TA) surrounding the corpora cavernosa and the trabeculae (TR) separating cavernous spaces, as a result of DES treatment. In addition, arrows indicate brownish-stained smooth muscle cells in the trabeculae, which were mainly seen in control mice. (C and D) In these micrographs, arrows indicate fat droplets, which are aggregated in large numbers in DES-treated mice, in contrast to only a few in control mice. (A and B), Masson's Trichrome; (C and D), toluidine blue.

neonatal estrogen exposure in the developing penis. To test this hypothesis, we employed the ER α KO mouse model because it lacks ER α genetically. We reasoned that if ER α expression is obligatory then ER α KO mice should be resistant to the harmful effects of neonatal DES exposure. Results of the present study showed for the first time that ER α KO males were completely resistant to the harmful effects of neonatal DES exposure, which were consistently observed in the penis of wild-type C57BL/6 mice, providing unequivocal evidence of an obligatory role for ER α in mediating estrogen-inducible abnormalities in the developing penis.

The penis is not the only reproductive organ in which the presence of ER α is crucial for estrogen-inducible developmental deformities. The prostate gland in the ER α KO males treated neonatally with DES on postnatal days 1, 3, and 5 and examined at different ages up to 18 months of age were normal in

all age groups, while that of the wild-type mice treated similarly developed alterations at the cellular and molecular levels, including epithelial hyperplasia and dysplasia, which worsened with age (Prins *et al.* 2001). Adult ER α KO female mice treated with DES neonatally failed to develop pathological abnormalities that were observed in the uterus, oviduct, and vagina of similarly treated wild-type mice (Couse *et al.* 2001). On the other hand, mice lacking ER β (ER β KO) and treated with DES neonatally developed abnormalities in the prostate (Prins *et al.* 2001) and female genital organs (Couse & Korach 2004), similar to those observed in the DES-treated wild-type mice. Thus, the above studies, as well as our present study, underscore an essential role of ER α in mediating detrimental effects of neonatal estrogen exposure not only in the penis but also in other reproductive organs of both sexes.

Figure 7 Micrographs of the corpora cavernosa penis in wild-type (A and B) and ER α KO (C and D) adult mice treated neonatally with oil (A and C) or DES (B and D). Note accumulation of fat cells in wild-type DES mice, in contrast to a few, isolated fat cells (arrows) in all other groups. Fat stain, undeparaffinized sections stained en block with osmium tetroxide.

Similarities in penile abnormalities presently observed in DES-treated wild-type mice with those previously reported from our laboratory in DES-treated rats (Goyal *et al.* 2005a,b), including malformations of the os penis; reductions in weight, length, and diameter of the penis; reductions in thickness of the tunica albuginea capsule and connective tissue trabeculae in the corpora cavernosa penis; and abnormal accumulation of fat cells in the corpora cavernosa penis; clearly identify the penis as a target organ for harmful effects of neonatal estrogen exposure. Similarly, other studies also reported a smaller penis in laboratory animals exposed neonatally to estrogen (McLachlan *et al.* 1975, Zadina *et al.* 1979, Newbold 2004); and the rabbit penis treated with bisphenol A (Moon *et al.* 2001) or tetrachlorodibenzo-dioxin (Moon *et al.* 2004) had subcutaneous deposition of fat in the corpora cavernosa. Additionally, alligators

from Lake Apopka, FL, contaminated with environmental pollutants had smaller phalluses (Guillette *et al.* 1994, 1996, Milnes *et al.* 2005); and male offspring of women exposed to DES during pregnancy had smaller penises (Gill *et al.* 1979, Swan 2000).

However, unlike in the DES-treated rat penis, which also showed loss of cavernous spaces (sinusoids) and smooth muscle cells (Goyal *et al.* 2004a,b, 2005a,b), both structures were present in the corpora cavernosa penis of wild-type mice treated neonatally with DES. Reasons for this difference may be attributed to differences in the species, or to the developmental stage of the penis at the time of estrogen exposure, because, even within the rat, the severity of loss of smooth muscle cells and cavernous spaces and the degree of accumulation of fat cells was higher in the penis exposed to estrogen from postnatal days 1 to 6 than that exposed from postnatal days 7 to 12

Figure 8 Plasma testosterone and intratesticular testosterone in wild-type and ER α KO adult mice treated neonatally with oil (control) or DES. Bars with different superscripts are significantly different ($P \leq 0.05$). Data are expressed as mean $\pm$ S.E.M.

(Goyal *et al.* 2005b). Considering that the length of gestation is shorter in mice than rats (19–21 days vs 21–23 days), it is logical to suggest that stromal cells at birth are at a more advanced stage of differentiation in the mouse penis than the rat penis and thus this difference may be a factor in the differential response observed in the penis of these two species.

An important observation of the present study that the penis of ER α KO and wild-type control mice is morphologically similar implies that ER α is not required for normal development of the penis. Similarly, reports of fertility in ER β KO (Couse & Korach 1999) and aromatase knockout (Fisher *et al.* 1998) male mice indicate that neither ER β nor estrogen is essential in normal penile development. This is despite the fact that ER α and ER β (Jesmin *et al.* 2002), as well as aromatase enzyme (Jesmin *et al.* 2004, Mowa *et al.* 2006), are present in the neonatal rat penis. Hence, based on the available data, it appears that neither ER α nor ER β nor estrogen has a significant role in normal development of the penis. On the contrary, the present data provide unequivocal evidence for a crucial role of ER α in mediating estrogen-driven abnormal development of the penis.

One potential mechanism by which neonatal estrogen exposure induces developmental abnormalities may involve an activation of ER α -mediated signaling because

we previously found an association between ER α upregulation and penile abnormalities in the developing rat penis treated neonatally with DES (Goyal *et al.* 2004b, 2005b). Similarly, ER α upregulation is associated with abnormal development of rodent female reproductive tract (Yamashita *et al.* 1990, Markey *et al.* 2005), mammary gland (Tekmal *et al.* 2005), male reproductive tract (Sato *et al.* 1994), prostate gland (Prins & Birch 1997, Prins *et al.* 2001), and seminal vesicles (Williams *et al.* 2001). In addition, ER α overexpression is shown to inhibit growth and angiogenic factors in the endometrial carcinoma cell line Ishikawa (Ali *et al.* 2004); and ER α is the main regulator of estrogenic effect on adipose tissue since an alteration in estrogen/ER signaling during development results in dramatic changes in adipocyte number (Cooke & Naaz 2004).

Another mechanism of estrogen-inducible, developmental penile abnormalities may involve alterations in androgen action because androgen receptors are ubiquitously present in the rat penis (Goyal *et al.* 2004b), and their concentration is maximal prior to puberty (Rajfer *et al.* 1980, Takane *et al.* 1990). Additionally, neonatal estrogen exposure downregulates androgen receptor level in male reproductive organs (Prins & Birch 1995, McKinnell *et al.* 2001, Williams *et al.* 2001, Woodham *et al.* 2003) and lowers plasma testosterone (Sharpe *et al.* 1998, Atanassova *et al.* 2000). Testosterone coadministration with estrogen mitigates developmental abnormalities affecting the male reproductive tract (Rivas *et al.* 2003). *In vitro* treatment with androgens promotes smooth muscle differentiation and inhibits adipocyte differentiation in C3H 10T1/2 pluripotent mesenchymal cells (Bhasin *et al.* 2003, Singh *et al.* 2003), and castration induces subcutaneous fat deposition and loss of smooth muscle cells in the corpora cavernosa of the rabbit penis (Traish & Kim 2005, Traish *et al.* 2005). Taken together, these observations raise the possibility that lower androgen action via estrogen-induced decrease in testosterone or androgen receptor or both as potential mechanisms contributing to penile abnormalities.

Although neonatal DES exposure in the present study did not significantly alter the adult level of plasma or intratesticular testosterone in wild-type mice, the neonatal level of intratesticular testosterone in rat pups treated with DES for 1–3 postnatal days was reduced by 90% at postnatal days 5–8 (Goyal *et al.* 2005b), the developmental period when stromal cells start differentiation in the rat penis (Murakami 1986, 1987), thus suggesting a role for estrogen-induced lower androgen action in inducing penile abnormalities by reprogramming stromal cell differentiation. In this context, observations that endogenous estrogens or DES exposure to fetal or neonatal Leydig cells decreased testosterone secretion in wild-type mice, but not in ER α KO mice, implied an ER α -mediated inhibitory role of estrogen in testosterone secretion (Delbes *et al.* 2005). Supporting

this role and, in agreement with the previous studies (Eddy *et al.* 1996, Akingbemi *et al.* 2003), both plasma and intratesticular testosterone levels are much higher in ER α KO mice than in wild-type mice. Hence, it is hypothesized that the perinatal testosterone surge, typical for rodents from late gestation to first week of life, may be a natural mechanism of downregulating ER α expression in penile stromal cells and thereby safeguarding their normal differentiation at a critical time of penile development. In other words, estrogen-induced downregulation of the perinatal testosterone surge results in ER α upregulation in penile stromal cells and, consequently, abnormal development of the penis in the wild-type mice. Alternatively, the lack of effect in the ER α KO mice could be due to the increased testosterone, which provided protection against DES effects in the same way that testosterone administration protected against DES effects in the neonatal rat (Rivas *et al.* 2003).

In agreement with our previous observations in the rat (Goyal *et al.* 2005b), the weight of both penile skeletal muscles, bulbospongiosus and levator ani was not only significantly decreased but also was differentially decreased, with the former muscle undergoing a much higher decrease, as a result of neonatal estrogen exposure to wild-type mice. The mechanism of this differential decrease may lie in differences in androgen receptor concentration because both muscles are androgen dependent (Breedlove & Arnold 1983, Hadi Mansouri *et al.* 2003) and, interestingly, both muscles are more developed in ER α KO mice than in wild-type/control mice. Likewise, a dramatic weight reduction observed in the seminal vesicle in the DES-treated wild-type mice is consistent with our previous data in the rat (Goyal *et al.* 2005a,b), as well as from other laboratories (Atanassova *et al.* 2000, Putz *et al.* 2001, Hendry *et al.* 2006); and a significantly higher weight of the seminal vesicles in ER α KO mice when compared with wild-type/control mice is also in agreement with previous observations (Eddy *et al.* 1996, Couse & Korach 1999), and is probably attributed to higher testosterone levels in these animals.

In conclusion, ER α KO mice are resistant to estrogen-inducible penile abnormalities present in wild-type mice, implying an unequivocal role for ER α in mediating maldevelopment of the penis.

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