

Diethylstilbestrol Action on Leydig Cell Function and Testicular Descent

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Abstract: Exposure to environmental chemicals may adversely affect the endocrine system and male sexual development. Testicular descent is regulated hormonally by Leydig-cell derived testosterone and Insulin-like factor 3 (Insl3). While Insl3 is required for transabdominal descent by stimulating the contraction and growth of the gubernaculum, testosterone contributes to the involution of the suspensory ligament and mediates the inguinoscrotal phase of the descent. In rodents, prenatal exposure to estradiol or xenoestrogens such as diethylstilbestrol (DES) leads to cryptorchidism and is associated with a downregulation of Insl3 transcription. In this review, we summarize the molecular and endocrine events responsible for testicular descent and how fetal exposure to xenoestrogens can adversely affect it. Recent data suggest that *in utero* exposure to DES affects fetal Leydig cell endocrine functions, more precisely the steroidogenic function, Insl3 expression and testicular descent, *via* an ER α -dependent mechanism. The expression of Leydig-specific genes such as Insl3, Cyp17a1, Star and Renin1 were profoundly decreased upon exposure to E2 or DES but not affected in mutant testes lacking ER α . Whether these effects of ER α are mediated *via* its classical mode of action or through a non-genomic action remains unclear. Unraveling the molecular events inhibiting the endocrine functions of fetal Leydig cells through an ER α -dependent mechanism will provide important insights into the adverse effects of estrogenic exposure on the male urogenital system and testicular descent.

Keywords: Cryptorchidism · Endocrine disruptors · Expression profiling · Gene regulation · Testis · Uterus

Human Male Reproductive Health Disorders

Clinical and epidemiological studies published since the 1980s have indicated a drastic increase in the incidence of male reproductive health problems. These disorders include cryptorchidism (undescended testes),^[1] hypospadias,^[2] declining semen quality^[3] and testicular cancer,^[4] all of which are grouped under the term *Testicular Dysgenesis Syndrome* (TDS). The rapid increase of TDS over the past 50 years suggests that environmental or lifestyle factors and not genetic factors are most likely involved in

these changes in male reproductive health. Furthermore, basic, clinical and epidemiological studies have come to a consensus in suggesting that the underlying causes of human male reproductive disorders operate in the fetus (for a review see Norgil Damgaard *et al.*^[5]). This seems logical since testicular descent, closure of the urethra along the shaft of the penis (the failure of which causes hypospadias), testicular development and differentiation all occur during fetal life.

Endocrine Disruptors and Xenoestrogens

Any chemical with the ability to interfere with, mimic or antagonize the function and/or the production of testicular hormones may cause adverse effects by disrupting testicular function and subsequent reproductive organ development. Such molecular compounds are called endocrine disruptors (EDs) and are defined by the European Commission as “exogenous substances that alter function(s) of the endocrine system and, as a consequence, cause adverse health effects in an intact organism”. Xenoestrogens are one particular class of EDs characterized by their estrogenic activity. These estrogenic compounds are part of our daily environment since they are found in numerous human-made or

natural products such as in pesticides (*e.g.* DDT derivatives, methoxychlor, kepone), in products associated with plastics (*e.g.* bisphenol-A, nonylphenol), in pharmaceutical agents (*e.g.* DES, tamoxifen, raloxifen), in ordinary household products (breakdown products of detergents and surfactants), in industrial chemicals (PCBs), in UV filters (*e.g.* 4-methylbenzylidene camphor (4-MBC) and octocrylene (OC)) as well as in food (phytoestrogens such as genistein, daidzein). Among these, diethylstilbestrol (DES), a synthetic estrogen, stands apart since it was the first and remains the best-characterized endocrine disruptor. DES was prescribed to more than 5 million pregnant women from the late 1940s to early 1970s to prevent abortion, pre-eclampsia and other pregnancy complications (for review see Jensen *et al.*^[6]). Daughters of women treated with the drug during pregnancy have a higher risk of developing clear cell adenocarcinoma of the vagina, while male children exposed *in utero* to DES have a higher risk of developing the symptoms of TDS.^[7,8]

Male Sexual Development

Male sexual differentiation requires the synthesis and the secretion of three testicular hormones: testosterone, Müllerian inhibit-

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ing substance (MIS) and insulin-like factor 3 (Insl3). MIS, secreted by Sertoli cells, promotes the regression of the Müllerian ducts. Without MIS, the Müllerian ducts would develop and ultimately give rise to the uterus, the oviduct and the upper part of the vagina. Testosterone, synthesized by Leydig cells, directs the differentiation of male accessory organs including the vas deferens, the seminal vesicles, the second phase of testicular descent and the development of the external male genitalia. Finally, Insl3, also produced by Leydig cells, mediates the first phase of testicular descent. Interference with the production or action of these three sexual hormones by EDs during gonadal development can disrupt the sexual differentiation of the male fetus.

Testicular Descent Occurs in Two Hormonally Controlled Phases

Testicular descent is an important aspect of male sexual development. Failure of the testes to descend into the scrotum (cryptorchidism) is one of the most common birth defects in humans affecting approximately 2–3% of newborn males. Because intraabdominal temperature is toxic for male germ cells, cryptorchidism often results in infertility and increases the risk of testicular cancer.^[9]

Gonadal positioning relies on the development of two ligaments that connect the gonad to the abdominal wall: the cranial ligament known as the cranial suspensory ligament (CSL) and the caudal ligament named the gubernaculum, a mesenchymal tissue that connects the developing gonad to the inguinal abdominal wall. In males, outgrowth of the gubernaculum and regression of the CSL descends the testis transabdominally whereas in females, development of the CSL and developmental failure of the gubernaculum positions the ovaries next to the kidneys (Fig. 1A).

Hormonal control of testicular descent occurs in two steps: the transabdominal phase and the inguinoscrotal phase.^[9,10] The transabdominal phase (from E15.5 to E17.5 in mice) relocates the testes from a high abdominal position to the base of the abdomen, close to the inguinal canal. This process is mediated by androgens and Insl3. Testosterone triggers the regression of the CSL, and Insl3 acts on the gubernaculum through the binding to its receptor LGR8 to promote its outgrowth and contraction. The second phase involves the movement of the testes from the base of the abdomen to the scrotum and is mediated by androgens (for a review see Nef and Parada^[11]). In females, the absence of testosterone and Insl3 enables the CSL to develop and maintain the ovaries next to the kidneys, while the gubernaculum remains thin and elongated.

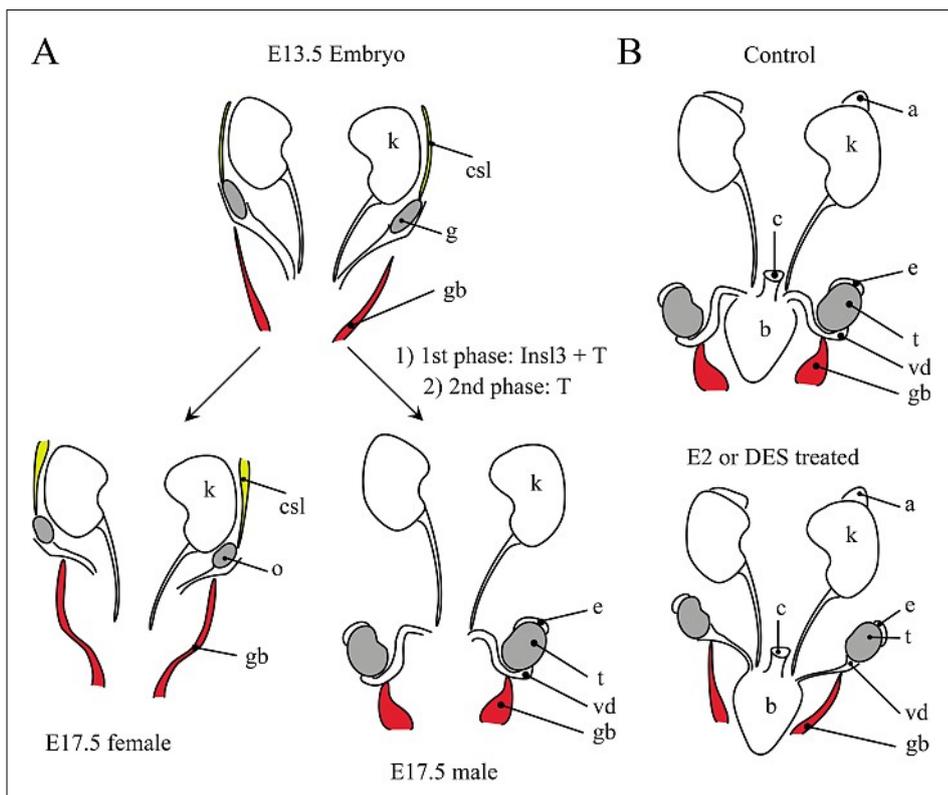


Fig. 1. (A) Schematic drawing describing the first phase of testicular descent in mice. In males, the presence of testosterone induces regression of the cranial suspensory ligaments (CSL), while Insl3 promotes the contraction of gubernacular cords and outgrowth of gubernacular bulbs. These morphological changes in the genital ligaments allow the testes to relocate at the base of the abdominal cavity. In females, the absence of testosterone allows the CSL to develop and retain the ovaries at their original position, while the gubernaculum elongates and does not develop because of the absence of Insl3. (B) Schematic drawing of testicular positioning at E18.5 under normal conditions or after *in utero* exposure to xenoestrogens. At this stage, the transabdominal descent is completed and the testes are located at the level of the bladder neck, and the gubernaculum is contracted and well developed. Estrogen exposure induces cryptorchidism characterized by intra-abdominal testes usually located below the kidneys and a thin elongated gubernaculum. Legend: adrenal (a), kidney (k), coecum (c), cranial suspensory ligament (csl), ovary (o), gonad (g), bladder (b), epididyma (e), testis (t), vas deferens (vd), gubernaculum (gb – in red). Modified from Nef *et al.*^[11]

Insulin like-3: An Essential Hormone for Gonadal Positioning

The evidence that Insl3 plays a role in testicular descent came from the generation of mice lacking Insl3 or its receptor LGR8. These mice were found to exhibit bilateral cryptorchidism, abnormal gubernacular development, spermatogenesis defects, and infertility.^[12,13] The insulin like-3 gene, also known as Leydig insulin-like hormone (Ley I-L), or relaxin-like factor (RLF), is a member of the insulin family which includes insulin, relaxin, and insulin-like growth factors I and II. Like insulin, the mature Insl3 peptide is composed of A and B-chains linked by disulfide bonds^[14] and binds to its receptor LGR8 which is a member of leucine-rich repeat-containing G-protein coupled receptor family.^[15] Insl3 expression during sex determination is sexually dimorphic.^[16] In male mice, Insl3 transcripts are first detected at E13.5 in fetal Leydig cells. Its expression remains constant up until postnatal (P) day 3–5 where a slight decrease

is observed. Post-natal transcript levels of Insl3 increase again as fetal Leydig cells are replaced by mature Leydig cells and reach the highest levels in adult testis. Insl3 gene regulation appears to be regulated differently in fetal and adult population of Leydig cells. In fetal Leydig cells, Insl3 gene expression occurs independently of the hypothalamo-pituitary-gonadal (HPG) axis and is inhibited by estrogenic compounds such as estradiol or DES. In mature Leydig cells in contrast, Insl3 gene expression is under the control of the HPG axis^[17,18] but unaffected by estrogens.^[19] For example, in *hpg* mutant mice that lack a functional HPG axis, Insl3 expression is unaffected in fetal Leydig cells whereas it is fairly absent in mature Leydig cells, suggesting that only postnatal transcription of Insl3 requires signals from the CNS.^[17] Confirming this hypothesis, mice lacking the LH-receptor (LhRKO) display normal transabdominal descent but postnatal bilateral cryptorchidism with impaired inguinoscrotal descent which is rescued by testosterone treatment.^[18] Thus, the expres-

sion of *Insl3* in fetal Leydig cells is crucial for transabdominal descent and development of the gubernaculum.

Xenoestrogens and Cryptorchidism

Insl3 and *LGR8* appear to play similar roles in human sexual development, although mutations in these genes are not a common cause of cryptorchidism in humans.^[20] Still, cryptorchidism has been associated with mutations in the *Insl3* or *LGR8* loci.^[12] Since 90% of cryptorchidism cases are spontaneous, it would seem that environmental factors, rather than genetic factors, are the most plausible cause. Abnormal estrogen action has been hypothesized to be a possible cause for sporadic cryptorchidism in humans. For instance, treatment of pregnant women with DES is associated with undescended testis in male offspring.^[7] A clinical study showed that mothers of cryptorchid children had higher levels of free estradiol during the first trimester as compared to mothers whose offspring had normally descended testes.^[21] Finally, Hadziselimovic *et al.*^[22] demonstrated an increased expression of estradiol in placenta of boys with cryptorchid testes.

Animal studies support the observations in humans. Indeed, for many years the main experimental model for intra-abdominal cryptorchidism was obtained by exposure of pregnant mice to exogenous estrogens.^[23] The effects of estradiol include a reduction of gubernacular outgrowth, the induction of estrogen receptors within the Wolffian ducts, and the stabilization of the Müllerian ducts. In mice, *in utero* exposure to estradiol or DES induces cryptorchidism (Fig. 1B) in association with a severe downregulation of *Insl3* transcription.^[19,24] Similarly, gestational exposure to phthalate esters, an endocrine disruptor with weak estrogenic activities, also impairs *Insl3* gene expression and the transabdominal phase of testicular descent in rat embryos.^[25]

Transcriptome Analysis of Testes from Embryos Exposed to E2 or DES

In order to identify the molecular mechanisms implicated in xenoestrogen-dependant cryptorchidism, we have characterized the transcriptional changes in the fetal testis after exposure to E2 (6 mg, single I.P. injection) and DES (10 µg/day, osmotic pump delivery). Our expression profiling analysis indicated that 6 and 31 genes are respectively down-regulated and up-regulated (>4 fold change) by estrogen exposure *in utero*.^[26] Amongst the genes whose transcription is inhibited by both E2 and DES, we found four which are specifically expressed in Leydig cells (*i.e.* *Insl3*, Steroidogenic acute regulatory protein (*Star*), Cytochrome p450

17 α -hydroxylase/17-20-lyase (*Cyp17a1*) and *Renin*). Genes expressed specifically in Sertoli cells (*i.e.* Müllerian Inhibiting Substance (*MIS*)) and in the germ cell lineage (*e.g.* *Oct4*) are not affected by xenoestrogen exposure *in utero*. *In vitro* studies have shown that *Insl3* transcription is regulated by *Sf-1* and its cofactor *Dax*,^[27,28] a negative regulator of transcription. *Sf-1* and *Dax-1* transcription were not affected by E2 or DES, excluding possible effects on the transcription of these genes as mediator of estrogen action on *Insl3* transcription. On the other hand, *Star* and *Cyp17a1* were already known to be targets of estrogens after fetal exposure.^[24,29] Both genes are crucial for the synthesis of testosterone. *Star* is the rate limiting enzyme for steroidogenesis by regulating cholesterol transport into the inner membrane of the mitochondria. Mice lacking the *Star* gene display male to female phenotypic sex reversal and have impaired inguinoscrotal descent of the testis due to the absence of androgen production.^[30] *Cyp17a1* is an enzyme that is part of the steroidogenic cascade but the proof of its essential function in testis steroid synthesis has never been shown since mice lacking *Cyp17a1* die at E7.5, before gastrulation.^[31] Thus disruption of *Star* and *Cyp17a1* expression is consistent with lower androgen production. Amongst the probe sets that are upregulated, we found genes implicated in cancer, metabolism, bone formation and cell architecture, but no gene with testicular function has been found.

Interestingly, we found that *Star* expression is not down-regulated by E2 or DES in the fetal adrenal gland. In this organ, the levels of *ER α* and *ER β* transcripts were low in comparison with the fetal testis, suggesting that ERs may play a role. Since *ER β* inacti-

vation has no consequence on the endocrine functions of the fetal Leydig cells,^[32,33] these results suggested that *ER α* was responsible for the estrogen-dependant downregulation of endocrine genes in the testis.

ER α Mediates DES-induced Cryptorchidism and Testis Dysgenesis

This hypothesis has been tested by exposing *ER α* KO embryos to E2 and DES and analyzing the transcriptome profile of the fetal testis. As depicted on the scatterplot (Fig. 2), in *ER α* KO testis, very few genes are affected by estrogen exposure, suggesting that *ER α* plays a major role during fetal exposure to E2 or DES. We found that all genes down-regulated when exposed to estradiol or DES have their expression restored in absence of *ER α* (Fig. 3). Concerning the genes that are upregulated, two-thirds were overexpressed in absence of *ER α* because of either (i) an *ER α* -independent mechanism, (ii) the absence of *ER α* itself, or (iii) E2 toxicity.

In contrast to *ER α* mutant mice, testicular descent in both wild type and *ER β* mutant mice was impaired by *in utero* exposure to E2 or DES. This indicates that *ER α* , but not *ER β* , mediates estrogen-dependent inhibition of the endocrine functions of fetal Leydig cells. In *ER α* mutant mice, testicular descent appeared normal, the gubernaculum was contracted and developed even in the presence of very high levels of exogenous estrogens (Fig. 4). In addition, *Insl3* transcription is fully restored in absence of *ER α* , but not in absence of *ER β* even in presence of saturating levels of exogenous estrogens, indicating that *ER α* mediates *Insl3* down-

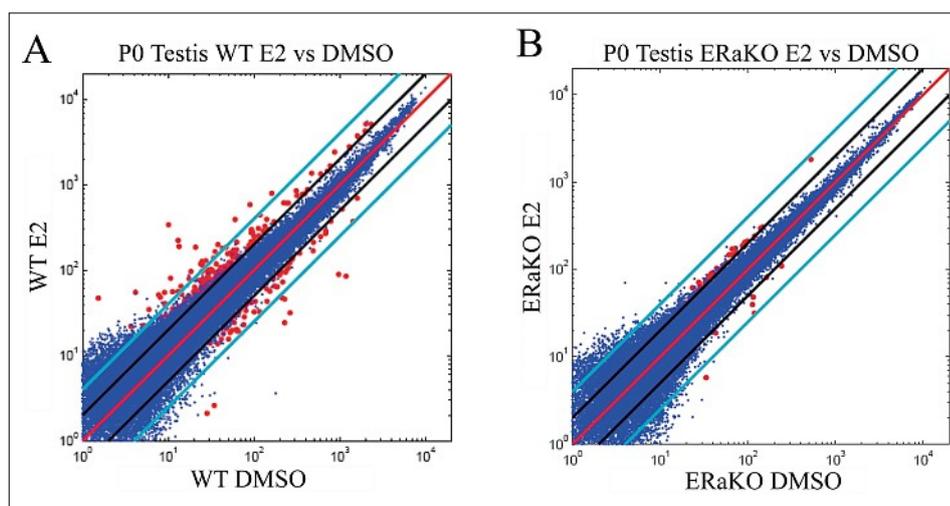


Fig. 2. Global expression analysis of the transcriptomes of WT or *ER α* mutant testes exposed to 17 β estradiol. Scatter plot analysis representing the systematic comparison of the log₂-scaled expression signals from WT (A) or *ER α* mutant (B) testes exposed *in utero* to E2 or DMSO. Blue dots represent genes with expression levels statistically similar in both conditions, while red dots represent genes whose expression profiles were found to be affected by E2 exposure (≥ 2 - and ≥ 4 -fold change, black and blue diagonals respectively). Reprinted with the permission of Endocrinology see Cederroth *et al.*^[26]

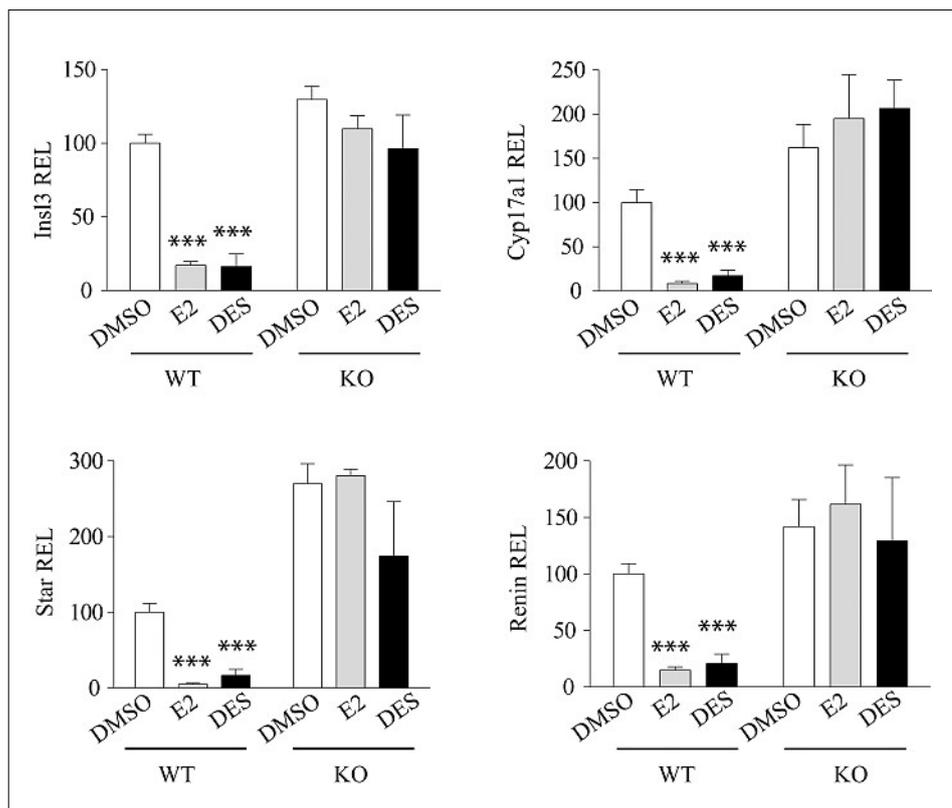


Fig. 3. Quantitative RT-PCR using WT and ER α mutant testes as template. Messenger RNA expression levels of representative genes modulated by *in utero* exposure to E2 and DES in WT and ER α mutant testes. White bars represent control (DMSO-treated) animals while gray and black bars symbolize E2 and DES exposed animals. Results are mean \pm SEM (n = 3/group), *p < 0.05, **p < 0.01, ***p < 0.001 versus DMSO. Reprinted with the permission of Endocrinology see Cederroth *et al.*^[26]

regulation and subsequent cryptorchidism upon exposure to xenoestrogens *in utero*.

Mechanisms of Estrogen Action

Previous studies support a direct effect of xenoestrogens on fetal Leydig cells.^[32,34] Our own studies demonstrate a critical role for ER α in this effect. However, the mechanism regulating ER α -dependent down-regulation of Ins13 and steroidogenic genes remains unclear. It is possible that estrogen-activated ER α binds directly to estrogen regulatory elements (ERE) or other non-ERE binding sites (*e.g.* SF-1 response element, SFRE) in target genes *via* a classical mode of action and induces changes in gene expression.^[27] So far, no experimental data indicate that ER α may act directly, through ERE or SFRE, on the promoter of Ins13. Analysis of the 190 bp Ins13 proximal promoter required for Leydig cell-specific transcription did not reveal any putative EREs^[27,28] and electromobility shift assays (EMSA) performed with *in vitro* translated ER α or ER β failed to show that these receptors interact with the Ins13 190 bp promoter.^[26] Alternatively, DES-dependent cryptorchidism may be mediated through a non-genomic pathway involving a crosstalk between ER α and unknown cellular signaling pathways. It has been reported that E2 induces rapid responses through pathways that involve ER α , c-Src tyrosine kinases and mitogen activated protein kinases (MAPK).^[35] These rapid pathways are activated not only by estrogen but also by endocrine disruptors such as BPA.^[36] It has been recently shown that estrogen actions on breast cancer cells require the insulin like growth factor-1 receptor (IGF-1R)/MAPK pathway. In cholinergic neurons, inhibition of MAPK blocks estrogen-dependant cAMP element-binding protein (CREB) phosphorylation^[37] and in cortical neurons, PI3K inhibitors inhibit estrogen activation of Akt and ERK.^[38] The crosstalk between estrogen and insulin pathways may be more important than expected and future studies should investigate the implications of the insulin pathways in mediating fetal actions of xenoestrogens.

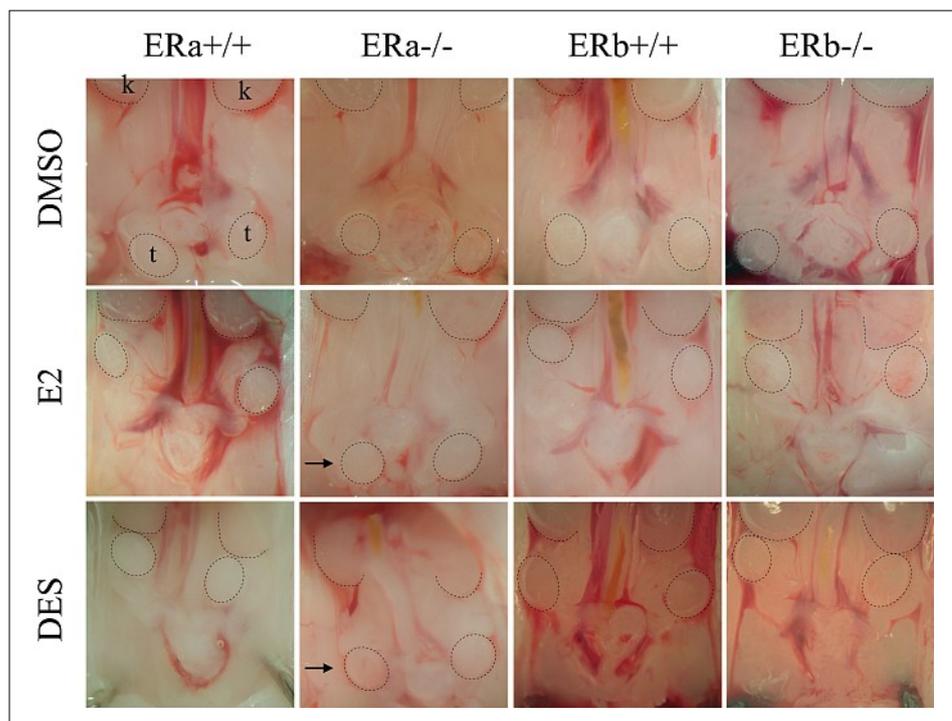


Fig. 4. Normal testicular descent in mice lacking ER α but not ER β exposed to E2 or DES. Photomicrographs of the urogenital system of E18.5 embryos exposed to E2, DES or vehicle (DMSO) and that are mutant for ER α or ER β . Note that the transabdominal descent is completed only in ER α mutant animals despite exposure to E2 or DES (arrows). The bladder has been surgically removed to ensure better accessibility and the position of the testes and kidneys have been encircled with dashed lines for better visibility. Legend: kidney (k), testis (t). Reprinted with the permission of Endocrinology see Cederroth *et al.*^[26]

Discussion

Most probably no single compound with endocrine-disrupting activities is the culprit of cryptorchidism, but additive effects of a mixture of EDs *in utero* may contribute to testicular maldescent. In addition, variation in the severity of xenoestrogen-induced cryptorchidism may be related to animal models, genetic influences, dose-dependant effects and/or epigenetic changes. For instance, rats exposed *in utero* the phthalate esters exhibit cryptorchidism and reduced Ins13 gene expression,^[25] whereas, no effects on cryp-

torchidism and *Ins13* transcription could be observed in mice at similar doses of phthalates (personal data). Even between different strains of mice, the genetic background could influence the susceptibility to EDs: for example it has been shown that CD-1 outbred mice are 16 times more sensitive to the disruptive effects of estradiol on spermatogenesis than inbred strains such as the C57/B6J.^[39] Fetal exposure to endocrine disruptors such as BPA or DES at parts-per-billion (ppb) (equivalent to 20 ng/kg/day) has irreversible effects on the reproductive organs.^[40] The fact that very small doses may have severe effects in rodents causes a lot of attention among the scientific community and is crucial for risk assessment.^[41] To our knowledge, effects of fetal exposure to such low doses of DES on cryptorchidism are lacking. We found that exposure to DES doses in the range of 8 µg/kg/day during gestation was not sufficient to adversely affect testicular descent and *Ins13* transcription although we do not exclude a potential effect at ppb doses. Finally, the increased frequency in reproductive disorders may include transgenerational effects. Gestational exposure to EDs has been reported to induce epigenetic changes^[42] that can permanently affect male fertility through several generations.^[43] While data demonstrating the adverse effects of EDs on human reproductive function are sparse, the homology in reproductive system and endocrine regulations between animal models and humans indicates a potential for detrimental effects on testicular endocrine function and cryptorchidism.

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