A Molecular Basis for Estrogen-Induced Cryptorchidism

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Male sexual differentiation relies upon testicular secretion of the hormones testosterone, Mullerian inhibiting substance, and insulin-3 (Insl3). Insl3 is responsible for testicular descent through virilization and outgrowth of the embryonic gubernaculum. In mouse, prenatal exposure to 17β-estradiol and the nonsteroidal synthetic estrogen diethylstilbestrol (DES) disturbs the endocrine balance, causing demasculinizing and feminizing effects in the male embryo, including impaired testicular descent (cryptorchidism). In the current study, we show that maternal exposure to estrogens, including 17α- and β-estradiol, as well as DES, specifically down regulates *Insl3* expression in embryonic Leydig cells, thereby providing a mechanism for cryptorchidism. These experiments may have implications for the widespread use of estrogenic substances in agriculture and the environment. © 2000 Academic Press
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INTRODUCTION

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 3% of newborn males. Cryptorchidism can result in infertility and is associated with an increased risk for development of testicular cancer (Hutson *et al.,* 1994). In mice, while the ovaries form and remain positioned just below the metanephros, the testes undergo relocation in the scrotum. This descent occurs in two distinct, hormonally regulated phases (Hutson, 1985; Hutson *et al.,* 1994; Satokata *et al.,* 1995). During the transabdominal phase (embryonic day 15.5 (E15.5) to E17.5) the testes relocate from the urogenital ridge to the inguinal region. The gubernaculum, a mesenchymal tissue that connects the urogenital ridge to the inguinal abdominal wall, mediates this transabdominal descent. Contraction of the gubernacular cord and outgrowth of the gubernacular bulb, concomitant with elongation of the embryonic rostrocaudal axis, place the testes above the inguinal canal at birth. In mice, the inguinoscrotal phase (testicular migration into the scrotum) occurs in the second or third postnatal week.

Different hormones control the two phases of testicular descent. Inguinoscrotal descent is mediated by androgens since this phase fails to occur in some humans or animals with mutations in the androgen receptor that cause androgen resistance (Hutson, 1986) or with defects in androgen synthesis (Grocock *et al.,* 1988). In rats, impaired inguinoscrotal descent can be elicited by prenatal blockade with anti-androgens (Husmann and McPhaul, 1991; Spencer *et al.,* 1991). The first phase of testicular descent (transabdominal phase), however, does not require androgens as it is normal in eutherian mammals with androgen deficiency or resistance (Griffin and Wilson, 1980; Hutson, 1986). The factor promoting transabdominal testicular descent via contraction and outgrowth of the gubernaculum remained unknown for decades. This hormone, which received names such as "factor X" (Habenicht and Neumann, 1983), "third hormone" (van der Schoot *et al.,* 1995), or even "descendin" (Fentener *et al.,* 1988), was thought to be expressed by the testes and to promote gubernaculum outgrowth and contraction. This factor was recently identified as insulin-3 (*Insl3*) based on studies in gene knockout mice (Nef and Parada, 1999; Zimmermann *et al.,* 1999).

The *insulin-3* gene product, also known as Leydig insulin-like hormone or relaxin-like factor, is part of the insulin family which includes insulin, relaxin, insulin-like growth factors I and II, insulin-4 (or EPIL) (Chassin *et al.,* 1995), insulin-5 (Conklin *et al.,* 1999) or RIF-2 (Hsu,

1999), and insulin-6 (Kasik *et al.,* 2000) or RIF-1 (Hsu, 1999). The mature Insl3 peptide, like insulin, is composed of A and B chains linked by disulfide bonds (Adham *et al.,* 1993). *Insl3* expression is sexually dimorphic (Zimmermann *et al.,* 1997). In male mice, *Insl3* transcripts are first detected at E13.5 in Leydig cells, and levels remain constant until postnatal sexual maturation and initiation of spermatogenesis, at which time a second increase in transcript levels is observed. In females, *Insl3* transcripts are first detected at postnatal day 6 (P6) in theca and luteal cells of the ovary (Balvers *et al.,* 1998). Mice homozygous for an *Insl3* gene deletion are viable but exhibit bilateral cryptorchidism, abnormal gubernacular development, and spermatogenesis defects and are infertile. Steroidogenic factor 1 (SF-1) appears to be involved in regulation of *Insl3* transcription (Zimmermann *et al.,* 1998). SF-1 is an orphan nuclear receptor that regulates the expression of several genes in testes, ovaries, adrenals, and pituitaries (Wong *et al.,* 1997).

Abnormal estrogen action has been implicated as a possible cause for sporadic cryptorchidism in humans. Several observations have been posited to support this theory. First, treatment of pregnant women with diethylstilbestrol (DES), a nonsteroidal estrogenic substance, is associated with undescended testis in male offspring (Gill *et al.,* 1979). In addition, nausea in early pregnancy, which is thought to result from high estrogen levels, is associated with an increased incidence of impaired testicular descent (Henderson *et al.,* 1979). Moreover, one clinical study showed that mothers of cryptorchid children had higher levels of free estradiol during the first trimester versus mothers whose offspring had normally descended testes (Bernstein *et al.,* 1988). Finally, Hadziselimovic and colleagues (1999) demonstrated an increased expression of estradiol in placenta of boys with cryptorchid testes.

Animal studies support the human correlations. In mice, *in utero* exposure to estradiol induces cryptorchidism. Indeed, the main experimental model for intra-abdominal cryptorchidism involves exposure of pregnant rodents to exogenous estrogens (Grocock *et al.,* 1988; Hadziselimovic and Girard, 1977; Hutson, 1992; Khan *et al.,* 1998; Perez *et al.,* 1996; van der Schoot, 1992). The effects of estradiol include a reduction of gubernacular outgrowth, the induction of estrogen receptors within the Wolffian ducts, and the stabilization of the Mullerian ducts. Finally, male mice lacking estrogen receptors have overdeveloped cremaster muscles of the gubernaculum (Donaldson *et al.,* 1996). This outcome is consistent with the notion that estrogen normally inhibits the development of the cremasteric muscles and therefore may impede testicular descent.

Since both the absence of *Insl3* and *in utero* exposure to exogenous estrogen induce bilateral cryptorchidism in newborns, we investigated the possible relationship between these hormones using the estrogen-induced cryptorchidism model. Our data provide the first molecular link concerning estrogen-induced cryptorchidism and support the hypothesis that excess estrogen may be a causal factor in improper testicular descent.

MATERIALS AND METHODS

Steroid hormone-treated mice. Five- to six-week CD1 females were mated and presence of vaginal plug was counted as day E0.5. E13.5 pregnant females received a subcutaneous injection of 0.2 ml dimethyl sulfoxide (DMSO) alone or containing 6 mg of one of the following steroids: 17α-estradiol, 17β-estradiol, estriol, progesterone, 17α-hydroxyprogesterone, hydrocortisone. In the case of DES, 20 μg in 0.2 ml DMSO was injected at E11.5, E13.5, and E15.5 of gestation. The time of hormonal treatment was selected because E13.5 corresponds to the beginning of sexual development and also from previous similar studies (Grocock *et al.,* 1988; Hadziselimovic and Girard, 1977). 17β-Estradiol-treated females failed to deliver normally; cesarean sections were performed on E19 and pups were fostered to surrogate females. Male offspring were killed at E17.5, P0, P7, P20, and P42. For the dose–response experiment, pregnant females at 13.5 dpc received a single injection of 0.2 ml of DMSO with decreasing amounts of 17β-estradiol (6 mg, 1 mg, 500 μg, 100 μg, and 20 μg).

Scanning electronic microscopy. CD1 treated and untreated E17.5 embryos and P0 pups were decapitated and fixed overnight in 2.5% glutaraldehyde, 4% paraformaldehyde in a phosphate-buffered solution. After fixation, the specimens were dissected to expose the urogenital organs and the gubernaculum. The samples were then processed for SEM by rinsing twice in 0.1 M phosphate buffer, fixing in 0.1% osmium tetroxide for 1.5 h, and dehydrating through a gradient of EtOH. The tissues were dried using a Denton DCP-1 critical point drying apparatus, carbon-coated in the sputter coater, and viewed in the scanning electron microscope (JEOL JSM 840A SEM).

Histology and in situ hybridization. Pregnant mice were allowed to deliver or offspring were delivered by cesarean section on day 19 of gestation for mice treated with 17α-estradiol, 17β-estradiol, or DES. The abdominal cavity of neonates was opened, the positions of testes were recorded, and the whole embryo was or isolated tissues were fixed in Bouin's solution overnight. After fixation, the samples were dehydrated and embedded in paraffin. Seven-micrometer sections mounted on coated slides were stained with hematoxylin and eosin as described (Liebl *et al.,* 1997) or processed for *in situ* hybridization as described (Nef and Nef, 1997). ^{35}S-labeled radioactive antisense cRNA was produced by *in vitro* transcription with T3, T7, or Sp6 RNA polymerases. The mouse antisense *Insl3* cRNA probe is a 369-bp fragment comprising the full coding region of the Insl3 cDNA. The mouse antisense SF-1 probe was produced from a 502-bp cDNA fragment corresponding to the first 502 bp of the *SF-1* cDNA (gift from K. L. Parker), and the mouse antisense probe for the cholesterol side-chain cleavage cytochrome P450 (P450scc) was produced from a 700-bp fragment corresponding to the first 700 bp of the *P450scc* cDNA. The sections were exposed 7 days in Kodak emulsion NTB2 and then developed, fixed, stained with toluidine blue, and mounted.

Northern blots. Total RNA was extracted using Trizol solution (Gibco BRL) and analyzed by Northern blot for *Insl3* and *glyceraldehyde-3-phosphate dehydrogenase* (G3PDH) transcripts. Ten micrograms of denatured total RNA from tissues or cell lines was electrophoresed on a 1.5% formaldehyde/agarose gel and transferred to nylon membrane (Hybond N+). Hybridization was performed with the NorthernMax prehybridization/hybridization

buffer (Ambion) using the manufacturer's protocol. Probes were generated by random primer labeling using dCTP. Autoradiography was performed with an intensifying screen overnight at $-70°C$. Blots were then stripped of the remaining radioactivity by rinsing the membranes twice with a boiling 0.5% SDS (v/w) solution, rehybridized with a G3PDH-specific probe to determine the quantity of RNA loaded, and exposed overnight at $-70°C$.

RESULTS

Mice Exposed in Utero to 17β-Estradiol Have Cryptorchid Testes

Pregnant mice received a subcutaneous injection of 6 mg of 17β-estradiol on E13, corresponding to the beginning of male sexual differentiation in the gonad (Grocock *et al.,* 1988; Hadziselimovic and Girard, 1977). SEM analysis of untreated E17.5 male embryos (Fig. 1A) showed a well-developed gubernacular bulb and a contracted gubernacular cord, whereas the gubernaculum of 17β-estradiol-treated embryos (Fig. 1B) displayed a small bulb and an elongated cord. The testes of untreated neonate male mice (Fig. 1C) are localized beneath the bladder neck and proximal to the inguinal region. The once swollen gubernacular bulbs have regressed and appear embedded in the peritoneal hollow (see arrowheads), where they will ultimately draw the testes through the inguinal canal after birth. In contrast (Fig. 1D), neonates previously exposed to 17β-estradiol show the testes located above the bladder neck or, in some cases, just below the kidney in a position similar to that of the ovaries. These are classical examples of impaired testicular descent (cryptorchidism). As previously described, excess estrogen also causes retention of the Mullerian ducts and feminized or undeveloped gubernaculae, which likely explains the intra-abdominal localization of the testes. Except for the stabilization of the Mullerian structures, the urogenital anatomy of mice treated with 17β-estradiol shares striking features with *Insl3* $-/-$ mice (Nef and Parada, 1999; Zimmermann *et al.,* 1999). Both displayed intra-abdominal testes and feminized gubernaculae.

17β-Estradiol Regulates Insl3 Transcription in Embryonic Leydig Cells

The similar phenotypes (undifferentiated gubernaculum and intra-abdominal cryptorchidism) of *Insl3* $-/-$ and 17β-estradiol-exposed male mice prompted us to examine the possibility that fetal estrogen exposure may interfere with *Insl3* action. We therefore performed *in situ* mRNA hybridization on testis from normal and 17β-estradiol-treated neonatal (P0) mice using an *Insl3*-specific probe. In untreated testes, *Insl3* transcripts were abundant in Leydig cells (Nef and Parada, 1999). In contrast, we were unable to detect *Insl3* transcripts in the testes of males exposed to 17β-estradiol *in utero* (compare Figs. 2C and 2D). Histological examination of the estradiol-treated testes revealed normal Leydig cell numbers and morphology (Figs. 2A and 2B). To examine gene expression in Leydig cells we per-
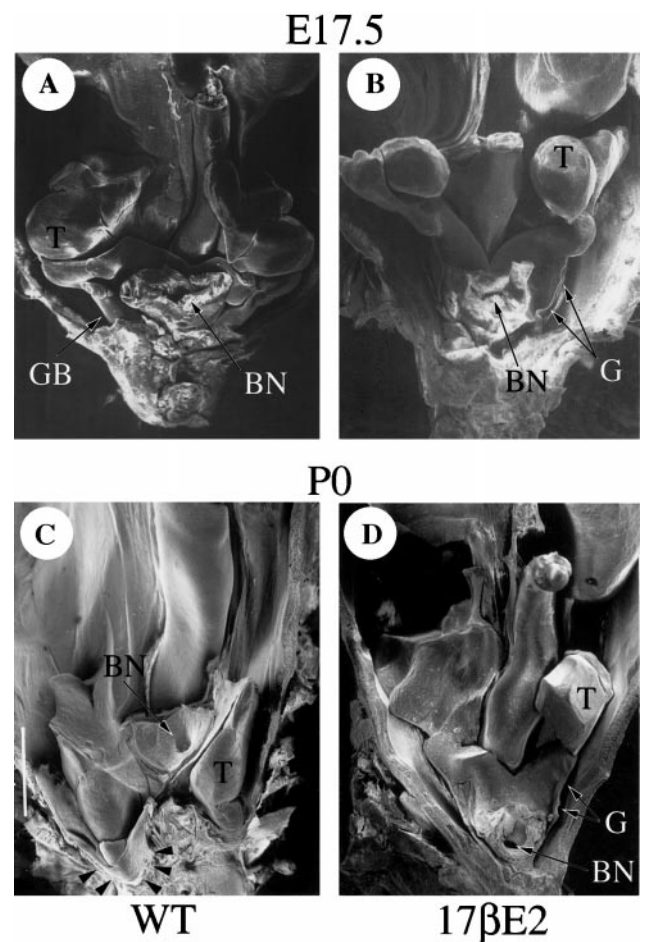

FIG. 1. SEM of the urogenital anatomy of E17.5 and neonate male pups treated with 17β-estradiol. Pictures of (A and C) untreated males compared to (B and D) 17β-estradiol-treated males at E17.5 (A and B) and P0 (C and D). (A) At E17.5 the male gubernaculum is swollen and contracted and the testes are located slightly above the bladder neck. (B) In contrast, in the 17β-estradiol-treated embryos the gubernaculum is completely feminized with a very thin and elongated cord similar to female gubernaculum. (C) At P0, the testes of untreated neonate pups have migrated below the bladder neck, and the gubernaculum, which has regressed and inverted, is embedded within the peritoneal hollow (arrowheads). (D) The testes of 17β-estradiol-treated males are clearly cryptorchid and located above the bladder neck. The gubernaculum is thin and elongated, similar to female gubernaculum. There is no peritoneal hollow. The bladder has been surgically removed to ensure better accessibility. Abbreviations: BN, bladder neck; G, gubernaculum; GB, gubernacular bulb; T, testes.

formed *in situ* mRNA hybridization for the cholesterol side-chain cleavage cytochrome P450 mRNA, a specific marker for Leydig cells. Expression of *P450scc* was the same in Leydig cells from both treated and untreated testes (Figs. 2E and 2F). The expression of *Desert hedgehog,* a specific marker for Sertoli cells, is normal in estradiol-exposed
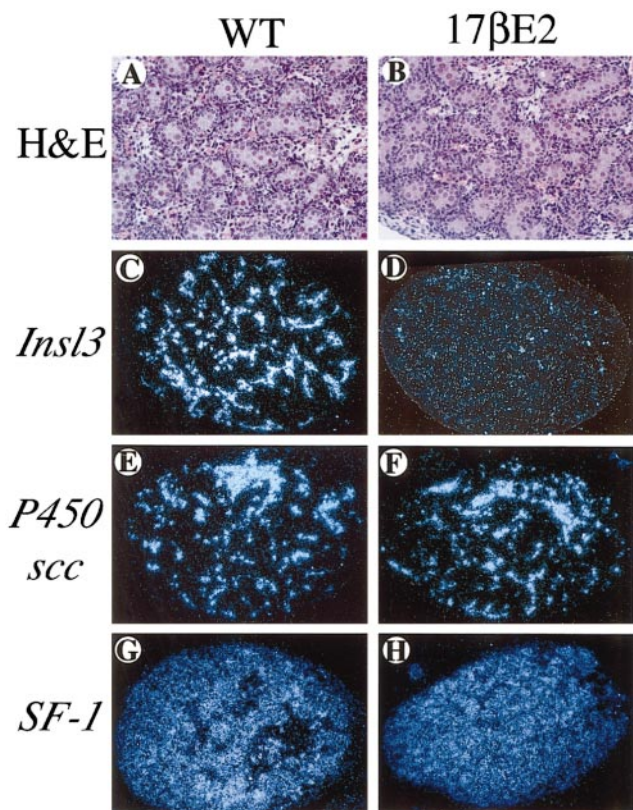
FIG. 2. *In situ* hybridization of untreated and 17β-estradiol-treated testes. (A, C, E, and G) Untreated and (B, D, F, H) 17β-estradiol-treated testes sections were stained with (A and B) hematoxylin and eosin or hybridized with an antisense probe for *Insl3* (C and D) or *P450scc* (E and F) or *SF-1* (G and H). Note the absence of *Insl3* transcripts in 17β-estradiol-treated males but the presence of *P450scc* transcripts, which indicates that the *Insl3* gene is specifically blocked by the 17β-estradiol treatment.

testes (data not shown). These data indicate that estrogen-exposed testes differentiated normally and that general gene transcription in Leydig and Sertoli cells was normal, while *Insl3* transcription was severely altered.

The *SF-1* gene has been reported to control transcription of *Insl3* (Zimmermann *et al.,* 1998). We observed normal levels of SF-1 transcripts in estradiol-exposed Leydig cells despite the fact that *Insl3* expression was abolished (Figs. 2G and 2H). These data indicate that *Insl3* transcriptional blockade was specific and not mediated by SF-1. Thus, exposure of male embryos to unusually high levels of estradiol resulted in specific blockade of Leydig cell *Insl3* gene expression.

Developmental Insl3 Expression

Insl3 transcript levels were affected throughout development by exposure to estradiol (Fig. 3A). Postnatally, how-

ever, *Insl3* expression was normal in prenatally estradiol-exposed males (Fig. 3A). The reemergence of *Insl3* transcripts between P0 and P7 coincided with the replacement of fetal-type Leydig cells by a new adult-type Leydig cell population (Balvers *et al.,* 1998; Huhtaniemi and Pelliniemi, 1992). Thus, *Insl3* expression appeared to be permanently and specifically blocked in fetal-type Leydig cells by exposure to a single bolus of exogenous estradiol. *Insl3* transcripts reappeared only in the new adult-type Leydig cell population that was exposed to 17β-estradiol in the differentiated state.
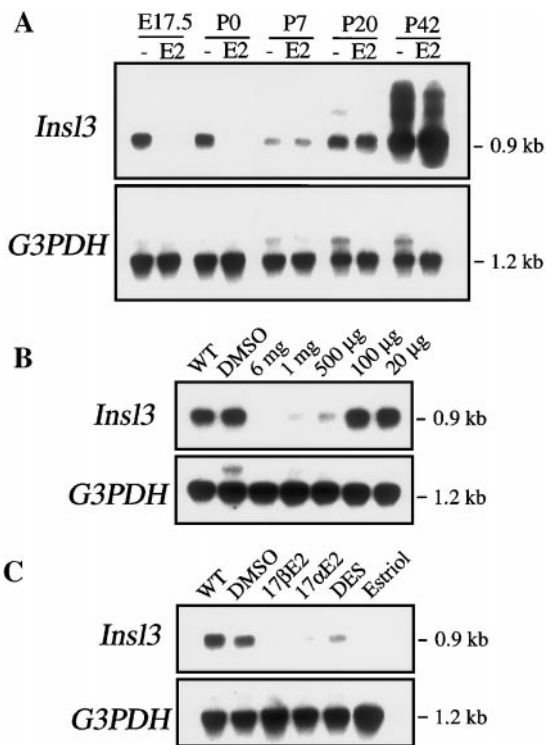

FIG. 3. (A) Time course of *Insl3* expression in 17β-estradiol-treated testes. 10 μg of total RNA from testes untreated (−) or 17β-estradiol-treated (E2) at different stages were hybridized against a specific probe for the *Insl3* gene. At E17.5 and P0 the *Insl3* expression is completely blocked but at P7, P20, and P42 the expression of the *Insl3* gene is similar in treated and untreated testes. (B) Dose–response analysis of *Insl3* gene expression in mice treated with decreasing amounts of 17β-estradiol. 10 μg of total RNA from P0 testes untreated (WT) or treated with the vehicle DMSO or decreasing amounts of 17β-estradiol were hybridized against a specific probe for the *Insl3* gene. The minimum quantity to block *Insl3* gene transcription is 500 μg (15 μg 17β-estradiol per gram of tissue). (C) Northern blot analysis of *Insl3* gene expression in 17β-estradiol, 17α-estradiol, DES, or estriol-treated testes. 10 μg of total RNA from P0 testes untreated (WT) or treated with the vehicle DMSO, 17β-estradiol, 17α-estradiol, or DES was hybridized against a specific probe for the *Insl3* gene. Note the blockade of *Insl3* expression in 17β-estradiol-, 17α-estradiol-, estriol-, and DES-treated testes.

Typically, experimental cryptorchidism is produced by intraperitoneal injection of 1–6 mg estradiol into a pregnant female (40–240 μg/g of tissue). These artificially high concentrations ensure saturation and reproducibility under experimental conditions, but have limited implications for mechanisms of sporadic cryptorchidism. We therefore performed a dose–response experiment to determine the minimum quantity of 17β-estradiol necessary and sufficient to block *Insl3* gene transcription when applied as a single dose at E13.5. Northern blot analysis of total RNA from testes treated with decreasing amounts of estradiol show that *Insl3* transcripts were absent or markedly reduced after treatment with 6 mg, 1 mg, and 500 μg but were present at 100 μg of 17β-estradiol (Fig. 3B). Therefore, 17β-estradiol doses in the range of 20 μg per gram of tissue (500 μg of 17β-estradiol/30 g pregnant female) were sufficient to reproducibly block *Insl3* gene transcription after an acute exposure. In each case, the reduction in *Insl3* transcripts correlated with the intra-abdominal localization of the testes (data not shown), indicating that the presence of *Insl3* hormone was necessary for normal testicular descent and that exposure to estrogen can impede its action. The consequences of chronic exposure to estrogens at lower concentrations may further reduce the level required for biological effect.

Estrogen-Related Molecules and Nonsteroidal Estrogens Induce Cryptorchidism via Insl3 Regulation

It has been hypothesized that estrogen-related molecules, including so-called environmental or xenoestrogens, may be responsible for the increasing incidence of sporadic cryptorchidism. To obtain experimental evidence in support of this idea, we next tested whether the estrogen stereoisomer 17α-estradiol and the related estriol, two molecules known to have reduced estrogenic activity, have effects on *Insl3* gene transcription and the development of the gubernaculum and testicular descent. In addition, the nonsteroidal synthetic estrogen DES was tested in this assay. Northern blot analyses performed with total RNA from untreated or 17α-estradiol-, 17β-estradiol-, estriol-, and DES-treated testes at P0 indicated that *Insl3* transcripts were absent in 17β-estradiol- and estriol-treated testes or significantly reduced in 17α-estradiol and DES-treated testes (Fig. 3C). SEM analysis of neonatal male embryos treated with estrogenic substances displayed intra-abdominal testes and thin, elongated gubernaculae (see Fig. 4; compare the gubernaculae of treated and untreated neonate pups). The peritoneal hollows were also absent in treated pups. Again, the feminized gubernaculae were similar to those observed in *Insl3* −/− neonatal mice (Nef and Parada, 1999; Zimmermann *et al.,* 1999) and explained the intra-abdominal localization of the testes. *Insl3* transcripts levels were unaffected in vehicle (DMSO)-treated testes. Thus, estrogen-related compounds that have been reported to
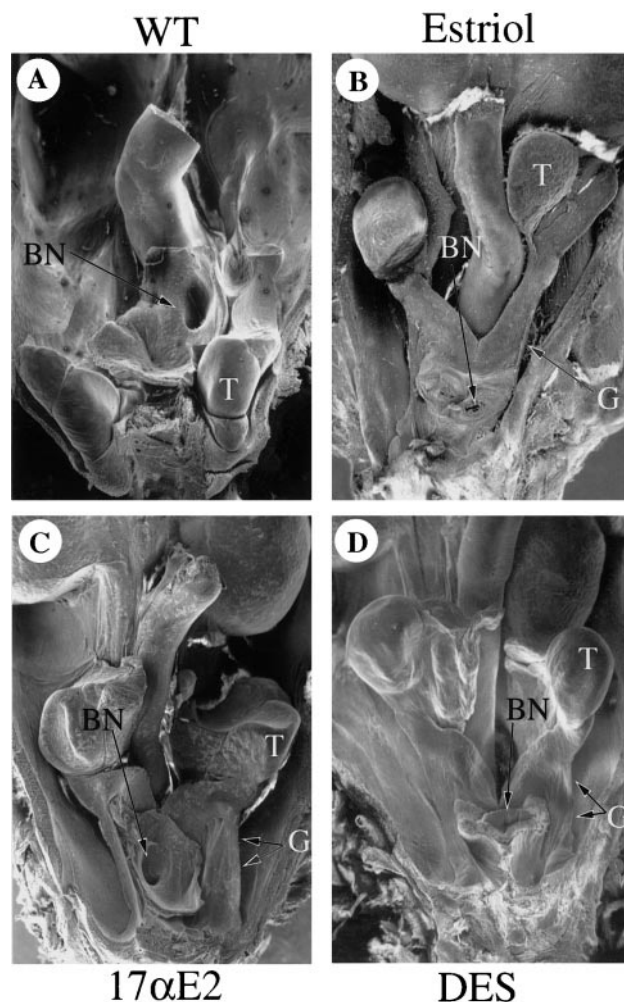

FIG. 4. SEM of the urogenital anatomy of neonates (A) untreated or treated with (B) estriol, (C) 17α-estradiol, or (D) DES. In each case, note the feminization of the gubernaculum, the intra-abdominal localization of the testes, and the absence of peritoneal hollow. The bladder has been surgically removed to ensure better accessibility. Abbreviations: BN, bladder neck; G, gubernaculum; T, testes.

have reduced estrogen activity in other assays can promote cryptorchidism in mice through down regulation of the *Insl3* gene. In addition, partial *Insl3* blockade was sufficient to retard gubernacular development.

To determine whether the effect on *Insl3* expression was a general feature of steroid hormones, pregnant females were exposed to progesterone, 17α-hydroxyprogesterone, and hydrocortisone under the same conditions. Neither steroid had any effect on intra-abdominal testicular descent (data not shown). Thus, *Insl3* blockade appears specific to estrogen-like substances that likely act through estrogen receptors α or β or some novel estrogen receptor and not through a nonspecific effect.

DISCUSSION

The purpose of this study was to determine the relationship between estrogens, *Insl3* expression, and testicular descent. The data unambiguously show that *in utero* exposure to 17α-estradiol, 17β-estradiol, estriol, or DES negatively regulates *Insl3* gene expression. Histological examination of the cryptorchid Leydig cells, as well as normal expression of additional genes, suggests that *Insl3* blockade is not caused by general metabolic depression in these cells. Estrogens are produced mainly in gonads and some peripheral tissues. These hormones are cell-membrane permeable, but are retained with high affinity and specificity in target cells expressing the estrogen receptor (ER). The estrogen–ER complex undergoes a conformational change allowing the receptor to bind with high affinity to chromatin and modulate the expression of target genes. Analysis of the *Insl3* proximal promoter (Zimmermann *et al.,* 1998) did not reveal sequences obviously related to estrogen-response elements. Thus, either the entirety of *in vivo Insl3 cis*-regulatory sequences has not yet been identified or the 17β-estradiol effect is not mediated by direct inhibiting interaction by ERα. Inhibition of *Insl3* transcription may be mediated via indirect ER transcriptional control of *Insl3* regulatory genes other than SF-1. The reported *Insl3* promoter possesses four consensus binding sites for SF-1, and mutations in any of these sites abolish *Insl3* transcription in a mouse tumor cell line (MA-10), indicating that in these cells, the *Insl3* gene is under direct control of SF-1 (Zimmermann *et al.,* 1998). However, our data indicate that the effects of estrogens on *Insl3* expression in fetal Leydig cells are not related to alteration in *SF-1* expression. High levels of estrogen result in the abolition of *Insl3* expression without affecting *SF-1* expression or its function on the *P450scc* gene (Clemens *et al.,* 1994).

In utero exposure to exogenous estradiol causes several effects on paratesticular structures. In addition to bilateral intra-abdominal cryptorchidism, there is stabilization of the Mullerian ducts. Persistence of Mullerian structures is unrelated to *Insl3* transcription since *Insl3* −/− male mice have normal vas deferens and no evidence of a uterus (Nef and Parada, 1999; Zimmermann *et al.,* 1999). A likely explanation for the persistence of Mullerian structures in these mice is that, independent of their action on *Insl3,* estrogens also block *Wnt7a* gene transcription. Previous work has shown that DES is able to block *Wnt7a* gene transcription (Miller *et al.,* 1998). Wnt7a is required for Mullerian inhibiting substance receptor expression, and its absence blocks receptor expression and subsequent regression of the Mullerian structures (Parr and McMahon, 1998).

The phenotype of estrogen-treated mice is strikingly similar to that of *Insl3* mutant mice. Despite the fact that estradiol is known to inhibit androgen production and that androgens have been implicated in testicular descent, the intra-abdominal position of the testes in estrogen-treated mice is due to the absence of *Insl3* hormone but not of androgens. Estrogens block the first phase of testicular descent (transabdominal descent), which is controlled hormonally by *Insl3* (Nef and Parada, 1999; Zimmermann *et al.,* 1999), whereas androgens control only the second phase (inguinoscrotal descent; Hutson *et al.,* 1994). In fact, estrogen exposure does not block outgrowth of the gubernaculum by acting as an anti-androgen since neonatal male mice treated with the anti-androgen flutamide display normal gubernacular development with complete transabdominal descent (Shono *et al.,* 1996).

Estrogens inhibit androgen production either by limiting the development and growth of Leydig cells or by directly inhibiting the activities of several steroidogenic enzymes involved in testosterone synthesis (for review see Abney, 1999). Estradiol is produced not only by the mother but also in significant amounts by Sertoli cells (Dorrington and Armstrong, 1975; Pomerantz, 1980). In addition, testes concentrate estradiol as much as 10- to 50-fold higher than in peripheral blood (Kelch *et al.,* 1972). Normal buffering mechanisms control the concentration of free estradiol in Leydig cells. First, estradiol and estrone bind with high affinity to α-fetoprotein, a protein present in high quantities in fetal serum (Keel and Abney, 1984; Nunez *et al.,* 1974). In addition, the presence of an estrogen sulfotransferase in Leydig cells has been reported (Hobkirk and Glasier, 1992; Song *et al.,* 1995), which may facilitate the catabolism of estrogen. Evidently, abnormally high levels of free estradiol or related estrogenic molecules can overcome the buffering mechanism leading to reduce *Insl3* transcription.

Blockade of *Insl3* gene transcription by *in utero* exposure to estrogens occurs during neonatal development. The reappearance of *Insl3* transcripts coincides with the replacement of fetal Leydig cells by an adult population (Balvers *et al.,* 1998; Huhtaniemi and Pelliniemi, 1992). Adult Leydig cells do not down regulate *Insl3* gene transcription upon estrogen exposure (data not shown). Therefore, the differentiation switch associated with replacement of the fetal with the adult-type cell population between P5 and P15 also corresponds to a change in *Insl3* gene regulation. Fetal Leydig cells express *Insl3* in a gonadotropin-independent fashion and are estrogen-sensitive, whereas *Insl3* expression is estrogen independent but gonadotropin dependent in the adult population (Balvers *et al.,* 1998).

The main argument against estrogens as potential factors in impaired testicular descent is the lack of persistent Mullerian structures in affected humans (Heyns and Hutson, 1995). In rodents, estrogen-induced cryptorchidism is obtained by injecting pregnant females with 1–6 mg of estradiol at 13.5 dpc (Grocock *et al.,* 1988; Hadziselimovic and Girard, 1977). This represents supraphysiologic amounts of estrogen, which in no case represent a normal *in vivo* state. A likely scenario for the effectiveness of this experimental strategy is the saturation of any potential relevant target. If estrogens underlie sporadic cryptorchidism, then it is likely that these effects are mediated by smaller doses localized to the correct target tissue at the precise time, thus achieving maximal effect. It may be possible that a small excess of free estradiol at the right

developmental stage (i.e., during the first trimester in human) may have a strong impact on testicular descent. It is well known that testes concentrate estradiol and it is generally agreed that estrogens have a dramatic, usually inhibitory, influence on cell function (Abney, 1999). Therefore, it seems reasonable to postulate that subtle differences in estrogen levels may alter *Insl3* transcription and impact testicular descent.

External factors, such as natural or synthetic molecules with estrogenic activities (xenoestrogens), may disrupt the normal endocrine balance and cause demasculinization and feminization effects in the developing male embryo. It has been widely speculated that the increased presence in the environment of xenoestrogens in the past 50 years may be the cause for declining male reproductive health such as the increased incidence of testicular cancer, cryptorchidism, hypospadias, and a lowered sperm count (Jensen *et al.,* 1995; Toppari, 1998). A classic example of the effects of a xenoestrogen is DES, which was prescribed during the late 1940s to early 1970s to pregnant women to prevent abortion, preeclampsia, and other complications of pregnancy. Daughters of women treated with the drug during pregnancy have a high risk of developing clear cell adenocarcinoma of the vagina, while male offspring are at risk of developing multiple reproductive defects including cryptorchidism, epididymal cysts, low sperm counts, and testicular cancers (Gill *et al.,* 1979; Herbst *et al.,* 1971, 1979; Wilcox *et al.,* 1995). Here we show that DES is able to induce intra-abdominal cryptorchidism and block *Insl3* gene transcription. These data likely explain the higher proportion of cryptorchidism in boys whose mothers were treated with DES during pregnancy and suggest that the *Insl3* gene may be a potential target for endocrine disrupters with estrogenic activities. We hope that these results will trigger a reexamination of estrogens as potential mediators of cryptorchidism. While this article was in completion, Emmen *et al.* (2000) reported DES-mediated regulation of *Insl3* transcripts. Their report is in agreement with ours and we extend the findings to include additional estrogens and to demonstrate the specificity of the blockade. Further studies to elucidate the mechanism by which estrogens block *Insl3* transcription are clearly warranted.

ACKNOWLEDGMENTS

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