

## Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol

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**Prenatal exposure to diethylstilbestrol (DES) is associated with reproductive tract abnormalities, subfertility and neoplasia in experimental animals and humans. Studies using experimental animals suggest that the carcinogenic effects of DES may be transmitted to succeeding generations. To further evaluate this possibility and to determine if there is a sensitive window of exposure, outbred CD-1 mice were treated with DES during three developmental stages: group I was treated on days 9–16 of gestation (2.5, 5 or 10 µg/kg maternal body weight) during major organogenesis; group II was treated once on day 18 of gestation (1000 µg/kg maternal body weight) just prior to birth; and group III was treated on days 1–5 of neonatal life (0.002 µg/pup/day). DES-exposed female mice (F<sub>1</sub>) were raised to maturity and bred to control males to generate DES-lineage (F<sub>2</sub>) descendants. The F<sub>2</sub> males obtained from these matings are the subjects of this report; results in F<sub>2</sub> females have been reported previously [Newbold *et al.* (1998) *Carcinogenesis*, 19, 1655–1663]. Reproductive performance of F<sub>2</sub> males when bred to control females was not different from control males. However, in DES F<sub>2</sub> males killed at 17–24 months, an increased incidence of proliferative lesions of the rete testis and tumors of the reproductive tract was observed. Since these increases were seen in all DES treatment groups, all exposure periods were considered susceptible to perturbation by DES. These data suggest that, while fertility of the DES F<sub>2</sub> mice appeared unaltered, increased susceptibility for tumors is transmitted from the DES ‘grandmothers’ to subsequent generations.**

### Introduction

For many years, research in our laboratory has centered on studying the effects of diethylstilbestrol (DES) and other estrogens on differentiating reproductive tract tissues. Using the CD-1 outbred mouse, we have shown that benign and

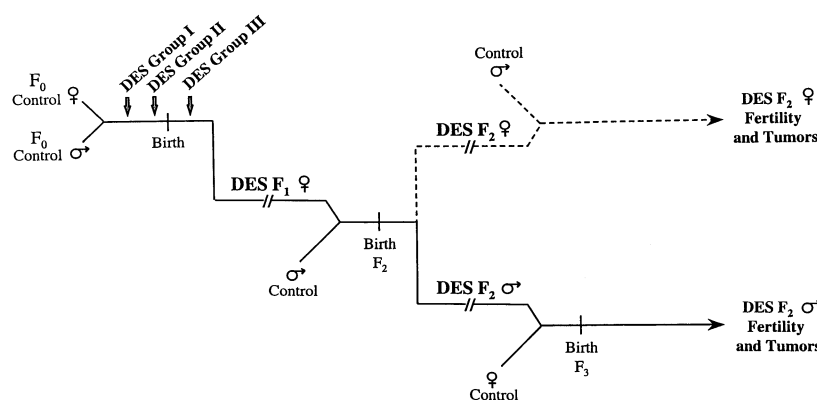
malignant changes in the developmentally DES-exposed murine genital tract closely parallel those reported in humans (1–12). In fact, this DES-exposed animal model has both replicated and predicted lesions observed in similarly exposed humans (13–15); however, the etiology of these various DES-induced abnormalities has remained unclear. While many of the effects are considered teratogenic (6,10–12) and may be associated with abnormal gene expression during development (16), the pathogenesis of some of the neoplastic lesions is more difficult to discern (7,9,10,17). Although DES and other estrogens are known carcinogens in humans and rodents (18), the cellular and molecular mechanisms by which these hormones induce neoplasia have not been fully elucidated.

Stimulation of cell proliferation and gene expression by binding to the estrogen receptor have been suggested to be important mechanisms in hormonal carcinogenesis (19). The significance of these mechanisms is supported by our recent study showing increased DES-induced tumor prevalence and reduced time to tumor formation in the uteri of transgenic mice that overexpress the estrogen receptor (20). These findings are consistent with other studies that suggest estrogens can be epigenetic carcinogens, acting via a promoting effect related to cellular proliferation, mediated through the estrogen receptor (21–23). However, binding to the estrogen receptor and estrogenicity alone are not altogether sufficient to explain the carcinogenic activity of estrogens because some estrogens are not carcinogenic (24).

Other mechanisms may be related to estrogen-induced carcinogenesis (for review, see ref. 25). While estrogens are not mutagenic in many assays, they do exhibit specific types of genotoxic activity under certain conditions. In cell culture, DES, 17β-estradiol and their metabolites have been reported to induce morphological and neoplastic transformation of Syrian hamster embryo (SHE) cells; SHE cells express no measurable levels of estrogen receptor, and estrogen treatment is not mitogenic to the cells (26). Thus, estrogenic activity apparently does not play a role in the transformation of these cells. SHE cell transformation rates do, however, correlate with aneuploidy induction and DNA damage caused by DNA adducts (25). Further evidence of genetic and epigenetic effects associated with estrogen treatment has been described in our studies of developmentally DES-exposed mice (27,28) and humans (29), and in studies from other laboratories (30–32). These data raise the possibility that the neoplastic changes seen following developmental exposure to DES may be related to epigenetic and/or genetic changes imprinted at the molecular level. Recent reviews lend support to this hypothesis (33,34).

Whether these DES-induced changes persist and are transferred to subsequent generations is not known, but growing evidence in experimental animals suggests that this is indeed a possibility; increased incidence of second generation tumors has been reported (35–38). Although one study reported no adverse second generation effects of DES in a group of 8–12-

**Abbreviations:** DES, diethylstilbestrol; H&E, hematoxylin and eosin.



**Fig. 1.** Generation of DES-lineage mice. Details are described in Materials and methods. Group I, prenatal DES treatment at a dose of 2.5, 5 or 10  $\mu\text{g}/\text{kg}$  of maternal body weight was administered as s.c. injections on days 9–16 of gestation. Group II, prenatal DES treatment at a dose of 1000  $\mu\text{g}/\text{kg}$  of maternal body weight was injected s.c. on day 18 of gestation. Group III, neonatal DES treatment at a dose of 0.002  $\mu\text{g}/\text{pup}/\text{day}$  was administered as s.c. injections on days 1–5. Developmentally exposed DES female mice ( $F_1$ ) obtained from these treatment groups were mated at sexual maturity to control male mice of the same strain to obtain DES-lineage mice ( $F_2$ ). DES  $F_2$  female mice represented by the dotted line have been reported in a separate publication (40). DES-lineage males ( $F_2$ ) are the subjects of this study.

week-old  $F_2$  female mice, long-term abnormalities including cancer were not examined (39). Adding support to the idea of a DES transgenerational effect, a recent study from our laboratory described the increased prevalence of uterine adenocarcinoma in DES-lineage female mice who themselves were never directly exposed to DES (40).

The current study was designed to determine if either benign or malignant abnormalities could be transmitted along the maternal germ line to DES-lineage males, as shown previously for DES-lineage females (40). As described in the DES-lineage female study, three windows of developmental exposure were included to identify whether a particularly critical stage of differentiation for the DES-exposed mouse ( $F_1$ ) was essential in transmitting adverse effects: (i) DES exposure on days 9–16 of gestation, the period of major organogenesis in the mouse and a time we have shown to be sensitive to DES adverse effects (2,3,41); (ii) DES exposure on day 18 only, the day preceding birth, an exposure time that was reported by Walker (35) to be associated with multigenerational effects; and (iii) DES exposure on days 1–5 of neonatal life, which we have previously reported to result in an increased incidence of uterine adenocarcinoma in the  $F_1$  generation (9,10), although the tumorigenic dose used previously was 1000 times higher than that used in the current study. In the current study, we report that the increased susceptibility for reproductive tract tumors in developmentally DES-exposed female mice ( $F_1$ ) is passed on to their male descendants ( $F_2$ ) as reported for the female descendants (40). The implications that DES and other estrogenic chemical carcinogens may be associated with genetic/epigenetic changes that can be transmitted to subsequent generations is discussed.

## Materials and methods

### $F_0$ generation

As described previously (3), adult CD-1 [CrI: CD-1 (ICR) BR] mice were obtained from Charles River Breeding Laboratories (Raleigh, NC) and bred to male mice of the same line in the breeding facility at the National Institute of Environmental Health Sciences (NIEHS; Research Triangle Park, NC). Vaginal plug detection was considered day 0 of pregnancy. On day 9 of gestation, pregnant female mice were individually housed in cages with hardwood chip bedding and a cotton fiber nesting block. Pregnant mice were housed under controlled lighting (12 h light and 12 h dark) and controlled temperature (21–22°C) conditions. NIH-31 lab mouse chow and fresh water

were supplied *ad libitum*. All animal procedures complied with an approved NIEHS/NIH animal care protocol.

### $F_1$ generation

**Group I.** DES (Sigma Chemical Co., St Louis, MO) dissolved in corn oil, or corn oil alone (control), was administered as an s.c. injection to the pregnant dam on days 9–16 of gestation at a daily dose of 2.5, 5 or 10  $\mu\text{g}/\text{kg}$  of maternal body weight (prenatal DES-2.5, prenatal DES-5 and prenatal DES-10, respectively) as described previously (3). These doses administered under this particular dosing scheme were previously reported to cause subfertility but not infertility (4) and to result in reproductive tract lesions later in life (3). Pregnant mice delivered their young and litters were standardized to eight female pups each.

**Group II.** DES dissolved in corn oil, or corn oil alone (control), was administered as a single s.c. injection to the pregnant dam on day 18 of gestation at a dose of 1000  $\mu\text{g}/\text{kg}$  maternal body weight (prenatal DES-day 18) as described (42). This dose and treatment scheme were chosen because a previous study using this protocol reported multigenerational effects (35). Pregnant mice delivered their young and litters were standardized to eight female pups.

**Group III.** Untreated pregnant mice delivered their young and litters were standardized to eight female pups. Pups were injected s.c. once daily on days 1–5 of life with DES (neonatal DES) dissolved in corn oil (0.002  $\mu\text{g}/\text{pup}/\text{day}$ ; weight of pups ranged from 1 g on day 1 to 3.5 g on day 5), or corn oil alone (control), as described (9,10). From a pilot study which determined the fertility of female mice exposed neonatally to DES (unpublished data), the dose of 0.002  $\mu\text{g}/\text{pup}/\text{day}$  was chosen to generate a second generation for this study, since the dose of 2  $\mu\text{g}/\text{pup}/\text{day}$  used in our previous studies (9,10) was not compatible with fertility.

All mice were weaned at 3 weeks of age and housed five per cage until further study. These mice are referred to as the  $F_1$  generation. A schematic diagram of the experimental design for the generation of DES-lineage mice is shown in Figure 1.

### $F_2$ generation

According to a previously described protocol (4), 8–12-week-old  $F_1$  female mice [group I (42 prenatal DES-2.5, 42 prenatal DES-5, 39 prenatal DES-10 and 25 control); group II (99 prenatal DES-day 18 and 25 control); and group III (42 neonatal DES and 25 control)] were bred to proven untreated male mice of the same line (four females per male). Because the controls for all three groups were similar, they were averaged together and the data are presented as a single set. (In the course of the study, a larger number of females was determined to be necessary in group II, prenatal DES-day 18, so that sufficient numbers of  $F_2$  animals could be generated.) Females observed to be pregnant were removed and housed individually until delivery. When  $F_1$  female mice delivered their young, pups were counted and litters were standardized to eight pups per litter whenever possible. The offspring of the  $F_1$  mice are referred to as second generation ( $F_2$ ) or DES-lineage mice. All  $F_2$  mice were weaned at 3 weeks of age and held four per cage for further study. The  $F_2$  female littermates of the males described in this study have been reported separately (40).

**Table I.** Body and reproductive tissue weights among DES-lineage (F<sub>2</sub>) male mice<sup>a</sup>

F <sub>1</sub> treatment	n	Body wt (g)	Right testis weight (g)		Left testis weight (g)		Epididymis weight (g)		Seminal vesicle weight (g)	
			Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
Control	10	56.0 ± 1.8	0.130 ± 0.005	0.234 ± 0.011	0.122 ± 0.004	0.218 ± 0.008	0.067 ± 0.002	0.121 ± 0.005	0.632 ± 0.062	1.134 ± 0.107
Group I										
Prenatal DES-2.5	4	49.3 ± 5.8	0.117 ± 0.010	0.243 ± 0.019	0.108 ± 0.010	0.225 ± 0.019	0.062 ± 0.008	0.126 ± 0.009	0.511 ± 0.186	0.950 ± 0.317
Prenatal DES-5	5	55.1 ± 2.9	0.126 ± 0.007	0.233 ± 0.023	0.084 ± 0.023	0.146 ± 0.038	0.071 ± 0.005	0.130 ± 0.007	0.441 ± 0.060*	0.788 ± 0.083*
Prenatal DES-10	7	47.8 ± 2.1*	0.126 ± 0.005	0.265 ± 0.015	0.124 ± 0.004	0.262 ± 0.014*	0.078 ± 0.002*	0.164 ± 0.004*	0.441 ± 0.038*	0.949 ± 0.107
Group II										
Prenatal DES-day 18	8	58.6 ± 2.2	0.130 ± 0.003	0.223 ± 0.010	0.119 ± 0.003	0.203 ± 0.008	0.066 ± 0.002	0.113 ± 0.005	0.650 ± 0.075	1.059 ± 0.126
Group III										
Neonatal DES	3	53.2 ± 6.3	0.121 ± 0.021	0.232 ± 0.040	0.111 ± 0.019	0.213 ± 0.035	0.061 ± 0.003	0.118 ± 0.011	0.555 ± 0.117	1.019 ± 0.105

Values shown are means ± SE.

<sup>a</sup>F<sub>1</sub> DES-exposed female mice were mated with untreated control male mice. The male offspring (F<sub>2</sub>) from these matings are referred to as DES-lineage male mice. Treatment of F<sub>1</sub> female mice is described in Materials and methods. These F<sub>2</sub> males were killed at 14–15 months of age following termination of the breeding study.

\*P < 0.05 versus controls (Mann–Whitney U-test).

### F<sub>2</sub> breeding

The fertility of a subset of F<sub>2</sub> males was determined at 11–12 months of age. DES-lineage (F<sub>2</sub>) male mice (10 control; group I, four DES-2.5, five DES-5, seven DES-10; group II, eight DES-day 18; and group III, three neonatal DES) were bred to proven untreated control females, two control females per DES-lineage male. When a female mouse appeared pregnant, she was removed from the breeding cage, weighed and individually housed. At delivery, pups (F<sub>3</sub>) were counted, weighed and examined for gross abnormalities. At the end of 12 weeks, breeding was discontinued and F<sub>2</sub> DES-lineage male mice were killed. Body weights were determined, serum samples collected and reproductive tract tissues were examined and weighed.

### Estradiol and testosterone levels

Serum samples were analyzed for total estradiol and testosterone levels as described previously (4).

### F<sub>2</sub> tumor incidence

For tumor studies, F<sub>2</sub> male mice were killed at 17–19 or 22–24 months of age. At necropsy, body weights were recorded and animals were observed for any gross abnormalities. Reproductive tract tissues were quickly removed and fixed in 10% neutral buffered formalin. Other tissues including liver, lung, kidneys, adrenal glands and heart were also removed and similarly fixed. All tissues were processed, embedded in paraffin and sectioned at 6 μm. A standardized sectioning method was used for testes since the rete testis had previously been identified to be a target for DES-adverse effects (7,14). A mid-sagittal cut along the long axis of the testis through the hilus was made and both cut surfaces embedded. Ten serial sections usually yielded sections through the tubulus rectus and the intratesticular rete testis. If the rete was not observed, an additional 10 sections were made. If the rete was not observed in the recuts, no additional sections were cut. All tissue sections were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. Additional serial sections were made on some lesions to include the entire area of pathological change. Data from the two age groups were evaluated separately and then combined; findings are presented as a single set (17–24 months). Survival rates were similar between the two age groups.

### Statistical analyses

Mann–Whitney U-tests (43) were used to compare body weights, reproductive tissue weights and serum hormone levels in DES and control groups. The incidences of proliferative lesions (hyperplasia and tumors) were compared by Fisher's exact test (43).

## Results

When 11–12-month-old DES-lineage (F<sub>2</sub>) males were bred to control females, few differences in fertility were observed between the control and DES-treated groups. One male from group I: prenatal DES-2.5, did not impregnate any females over the 12 week breeding period, even though housed with multiple proven partners; all other F<sub>2</sub> DES males in the study were fertile. The average litter size (mean ± SE) for the pregnant females was 11.4 ± 0.4 control; group I, 10.3 ± 0.5 prenatal DES-2.5, 10.0 ± 0.7 prenatal DES-5,

10.3 ± 0.6 prenatal DES-10; group II, 11.1 ± 1.2 prenatal DES-day 18; and group III, 10.3 ± 0.5 neonatal DES. No malformed neonates (F<sub>3</sub>) were noted in any group. Thus, no biologically significant difference in fertility between control and DES-lineage males was readily apparent.

At the end of the breeding period, F<sub>2</sub> males were killed; body weights and reproductive tissue weights are shown in Table I. Body weights varied, but no biologically significant differences between DES and control groups were seen except in group I, prenatal DES-10, which showed a statistically significant decrease in body weight when compared with controls (47.8 ± 2.1 g versus 56.0 ± 1.8 g).

Testicular weights were generally similar in DES and control groups. After correction for body weight differences, the mean left testis weight (actual and relative to body weight) was decreased somewhat in group I, prenatal DES-5 as compared with controls; this was reflective of one F<sub>2</sub> DES animal with unilateral testicular atrophy (testis weight = 0.01 g).

Conversely, the left testis/body weight ratio was significantly (P < 0.05) elevated in the prenatal DES-10 group. However, this was due entirely to the reduced body weight, since actual mean testis weights were virtually identical in DES-10 and control groups. Epididymal weights were not statistically different except in group I, prenatal DES-10, which was statistically larger than controls. Seminal vesicle weights were decreased in group I, prenatal DES-5 and prenatal DES-10 when compared with controls.

Serum testosterone and estrogen levels from the animals in the breeding study are plotted in Figure 2. Although there is a trend for testosterone levels to be lower in the DES F<sub>2</sub> males, none of the reductions was statistically significant, and any difference in these levels did not apparently affect fertility. Serum estrogen levels were significantly (P < 0.05) reduced in the prenatal DES-2.5 group relative to controls (Figure 2) but the biological importance is uncertain. Whether additional animals or younger animals show a similar trend needs further examination.

In contrast to the lack of a demonstrable affect on fertility, DES-lineage (F<sub>2</sub>) males clearly showed an increased incidence in proliferative lesions of the rete testis (hyperplasia and tumors) and reproductive tract tumors as compared with control mice. Abnormalities observed in DES-lineage mice at 17–24 months of age are summarized in Table II.

A normal rete testis from a control mouse is illustrated in

Figure 3. The irregular tubules of the rete are located at the mediastinum of the testis. The channels of the rete are lined by cuboidal or flat epithelium. Rete testis hyperplasia (Figure 4) was seen in all F<sub>2</sub> groups but the incidence and degree of severity was more pronounced in the DES groups as compared with controls. Furthermore, two tumors of the rete testis were seen in DES F<sub>2</sub> treated groups. In group I, prenatal DES-5, the rete tumor was composed of neoplastic cells that had focally penetrated through the basement membrane. The rete was cystic, but the lining epithelial cells were not flattened (Figure 5A and B). The other rete testis tumor was in a group II, prenatal DES-day 18 animal (Figure 6A); the lesion had both solid and papillary components composed of cells which

demonstrate loss of polarity and nuclear atypia (Figure 6B). The combined incidence of proliferative lesions of the rete testis (rete testis hyperplasia and tumors) summarized in Table III, suggests that the rete testis is a target for the transgenerational effects of DES. In this study, hyperplasia and tumors of the rete were summarized together because these lesions represented stages in the progression of rete disease based on prior information from DES-exposed male mice (7,14). Occurrence of other rare lesions in this area was

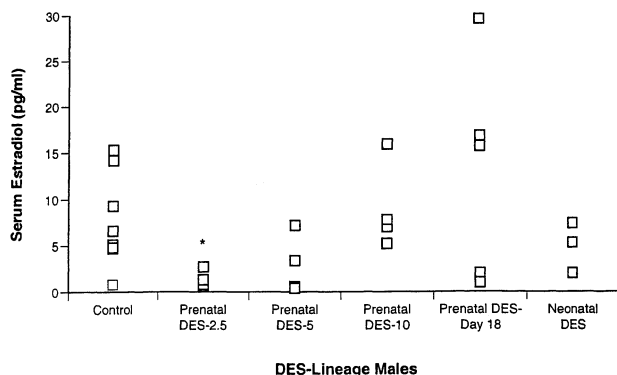
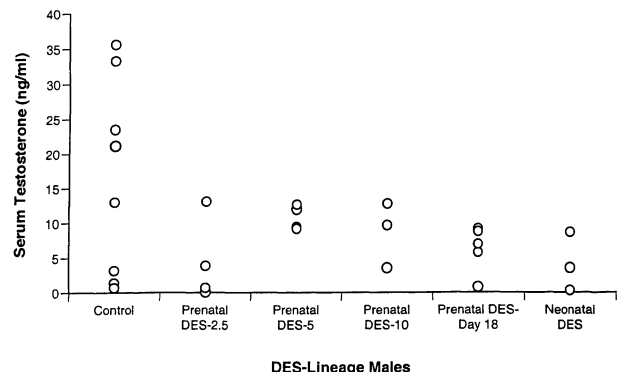


Fig. 2. Total testosterone (ng/ml) and total estradiol (pg/ml) levels measured in serum from DES-lineage (F<sub>2</sub>) male mice killed at the end of the breeding study. \*P < 0.05 versus controls (Mann-Whitney U-test).

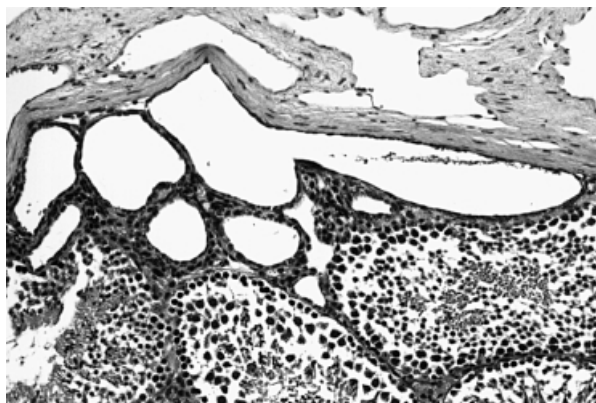


Fig. 3. Normal rete testis in a control male mouse. Irregular tubules of the rete are located at the mediastinum of the testis. The channels of the rete are lined by cuboidal or flat epithelium. (H&E, ×25.)

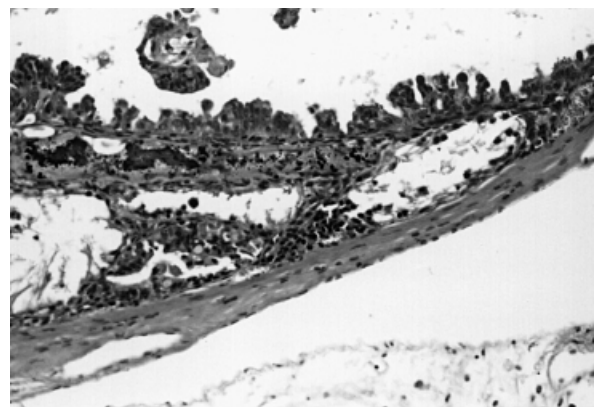


Fig. 4. Rete testis hyperplasia in a DES-lineage (F<sub>2</sub>) male mouse, group I, prenatal DES-5. Most of the dilated rete is lined by flattened or cuboidal cells except near the tunica, where there are pleomorphic papillary projections of cells with highly variable nuclei. Focal hobnail cell change and loss of polarity can be seen. (H&E, ×50.)

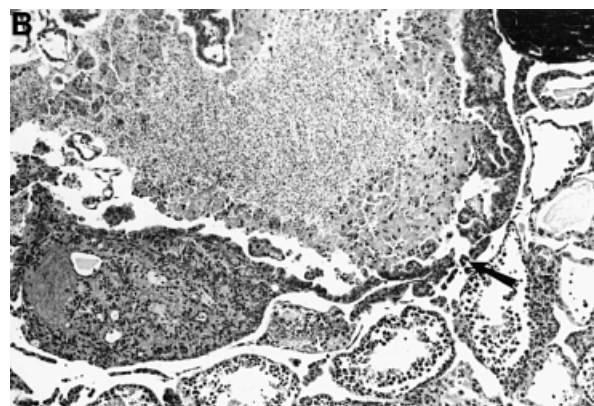
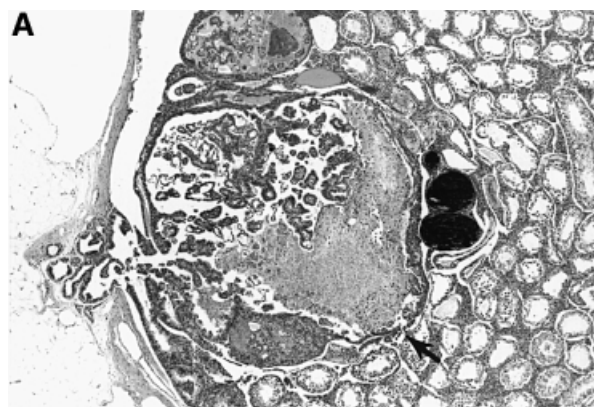


Fig. 5. (A) Rete testis tumor in a DES-lineage (F<sub>2</sub>) male mouse, group I, prenatal DES-5. There is a papillary growth in the rete with a cystic component that has focally extended through the basement membrane. (Arrow, H&E, ×5.) (B) Enlargement of (A). Tumor is composed of neoplastic cells that have focally penetrated through the basement membrane. (Arrow, H&E, X25.)

**Table II.** Abnormalities in male DES-lineage (F<sub>2</sub>) mice

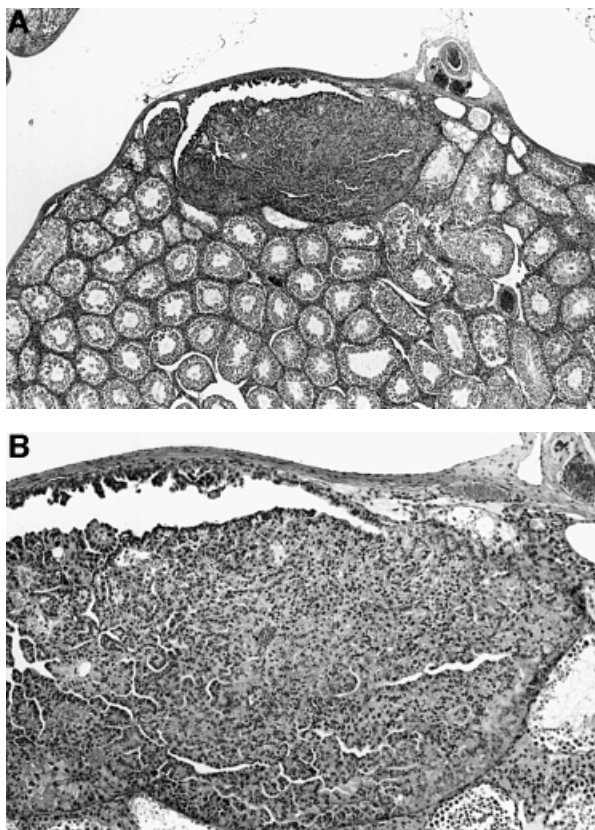
Developmental dose regime	F <sub>1</sub> DES treatment <sup>a</sup>	Testis <sup>b,c</sup>	Reproductive tract
Control	Corn oil	Interstitial cell tumor 1/68 (2) <sup>b</sup> Rete hyperplasia 3/53 (6) <sup>c</sup>	Prostatic hyperplasia 2/68 (3)
Group I	Prenatal DES-2.5	Interstitial cell hyperplasia 4/89 (4) Tubuli recti hyperplasia 1/73 (1) Rete hyperplasia 15/73 (21)	Prostatic hyperplasia 4/89 (4)
	Prenatal DES-5	Interstitial cell hyperplasia 2/100 (2) Interstitial cell tumor 2/100 (2) Rete hyperplasia 26/83 (31) <b>Rete tumor</b> 1/83 (1)	Prostatic hyperplasia 9/100 (9) SV hyperplasia 2/100 (2) <b>SV papilloma</b> 1/100 (1) <b>SV carcinosarcoma</b> 2/100 (2)
	Prenatal DES-10	Interstitial cell hyperplasia 2/62 (3) Interstitial cell tumor 2/62 (3) Hemangioma 1/62 (2) Rete hyperplasia 17/49 (35)	Prostatic hyperplasia 3/62 (5) Ampullary gland hyperplasia 1/62 (2)
Group II	Prenatal DES-day 18	Interstitial cell hyperplasia 1/65 (2) Interstitial cell tumor 1/65 (2) Lipoid cell hyperplasia of rete 1/52 (2) Rete hyperplasia 4/52 (8) <b>Rete tumor</b> 1/52 (2)	Prostatic hyperplasia 4/65 (6)
Group III	Neonatal DES	Interstitial cell hyperplasia 2/29 (7) Rete hyperplasia 7/23 (30)	Prostatic hyperplasia 1/29 (3) <b>Prostatic neoplasia</b> 1/29 (3) SV hyperplasia 1/29 (3) <b>SV sarcoma</b> 1/29 (3) CG hyperplasia 1/29 (3)

SV, seminal vesicle; CG, coagulating gland.

<sup>a</sup>As described in Materials and methods; F<sub>1</sub> DES-exposed female mice were mated with untreated control male mice. The male offspring from these matings are referred to as DES-lineage (F<sub>2</sub>) male mice. The F<sub>2</sub> males were killed at 17–24 months of age.

<sup>b</sup>Percentages are shown in parentheses.

<sup>c</sup>The denominator in rete lesions (hyperplasia and tumors) is lower than other lesions because tissue sections were not always available for evaluation through the rete.



**Fig. 6.** (A) Rete testis tumor in a DES-lineage (F<sub>2</sub>) male mouse, group II, prenatal DES-day 18. This tumor has both solid and papillary components. (H&E, ×5.) (B) Enlargement of (A). Loss of cellular polarity and nuclear atypia can be seen. (H&E, ×50.)

**Table III.** Summary of rete testis proliferative lesions in DES-lineage (F<sub>2</sub>) male mice<sup>a</sup>

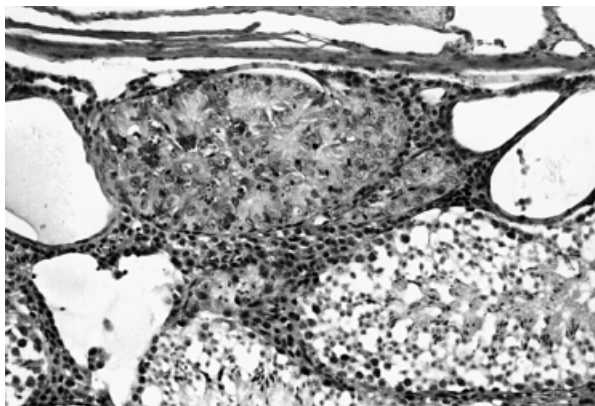
Developmental dose regime for F <sub>1</sub>	Incidence of lesions	Statistical significance <sup>b</sup>
Control	3/53 (6)	–
Group I		
Prenatal DES-2.5	15/73 (21)	<i>P</i> < 0.05
Prenatal DES-5	27/83 (33) <sup>c</sup>	<i>P</i> < 0.01
Prenatal DES-10	17/49 (35)	<i>P</i> < 0.01
Group II		
Prenatal DES-day 18	5/52 (10) <sup>c</sup>	NS
Group III		
Neonatal DES	7/23 (30)	<i>P</i> < 0.01

<sup>a</sup>F<sub>1</sub> female mice were exposed prenatally or neonatally to DES as described in Materials and methods. These DES F<sub>1</sub> females were mated with untreated control male mice to obtain the DES-lineage (F<sub>2</sub>) males. The incidence of rete abnormalities was observed in the F<sub>2</sub> DES-lineage males (Figure 1).

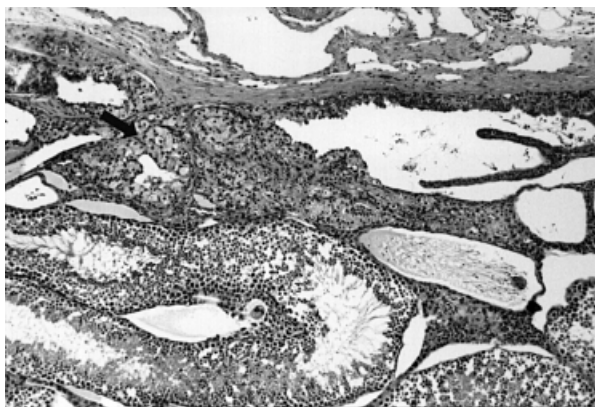
<sup>b</sup>Relative to control (Fisher's exact test); NS, not significant.

<sup>c</sup>One of these lesions was a rete testis tumor.

also observed. Tubuli recti hyperplasia in the testis (Figure 7) was seen in group I, prenatal DES-2.5. In this lesion, the terminal end of the seminiferous tubules was distended with an increased number of cells resembling those normally seen in this area. However, nuclear size variation and hyperchromatic nuclei were seen. Another rare lesion, found in the rete testis of a group II, prenatal DES-day 18 animal, was lipoid cell hyperplasia (Figure 8). Cells with foamy cytoplasm were interspersed with the rete epithelial cells. There was extension of the foamy cells for a short distance into the efferent ducts. The foamy cells were larger and more vacuolated than interstitial cells in the same area. Elsewhere in the rete, there were focal hobnail changes and loss of polarity (Figure 8). Interstitial cell hyperplasia and interstitial tumors were seen



**Fig. 7.** Tubuli recti hyperplasia in a DES-lineage ( $F_2$ ) male mouse, group I, prenatal DES-2.5. The terminal end of the seminiferous tubule is distended with an increased number of cells that resemble those normally found in this area. However, nuclear size variation and hyperchromatic nuclei can be seen. (H&E,  $\times 25$ .)



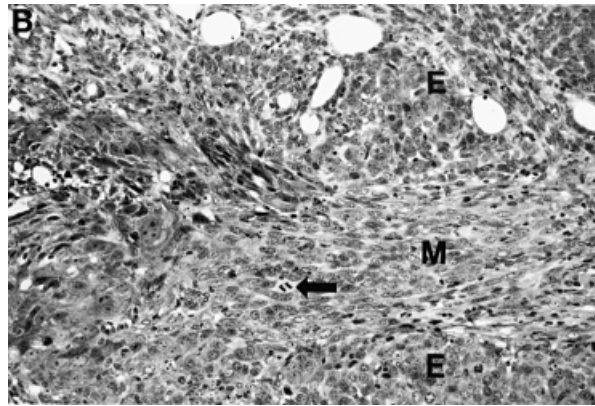
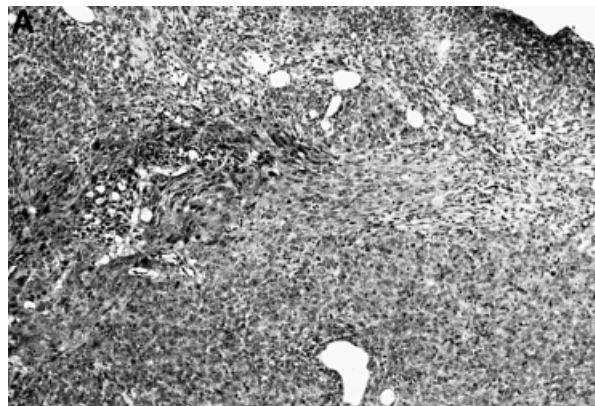
**Fig. 8.** Lipoid cell hyperplasia in a DES-lineage ( $F_2$ ) male mouse, group II, prenatal DES-day 18. Cells with foamy cytoplasm are interspersed with the rete epithelial cells. (Arrow, H&E,  $\times 50$ .)

in controls and all DES treatment groups; this finding is consistent with reports of these spontaneous occurring tumors in this mouse strain (44,45).

Other rare tumors in reproductive tract tissues were observed in the DES-lineage ( $F_2$ ) males. Of particular interest was a seminal vesicle papilloma [1/100 (1%)] and two seminal vesicle carcinosarcomas [2/100 (2%), Figure 9A and B] in group I, prenatal DES-5; prostatic neoplasia [1/29 (3%), Figure 10A and B] and seminal vesicle sarcoma [1/29 (3%)] in group III, neonatal DES. These lesions are summarized in Table II. Sperm granulomas and inflammation in the epididymis were observed in all DES-lineage ( $F_2$ ) groups.

The mammary gland was not routinely screened, but two animals in group II, prenatal DES-day 18 had lesions identified on gross examination; microscopic evaluation of these tumors showed pathological changes consistent with fibrosarcomas of the milk line.

Other tissues were also screened for abnormalities. The incidence of hepatocellular neoplasms was not different between control and DES-lineage mice, but pulmonary neoplasms occurred at approximately twice the rate [group I, prenatal DES-2.5 (37%), prenatal DES-5 (34%), prenatal DES-10 (21%); group II, prenatal DES-day 18 (38%); group III, neonatal DES (38%)] in DES-lineage ( $F_2$ ) males as compared with controls (12%). This finding is of uncertain



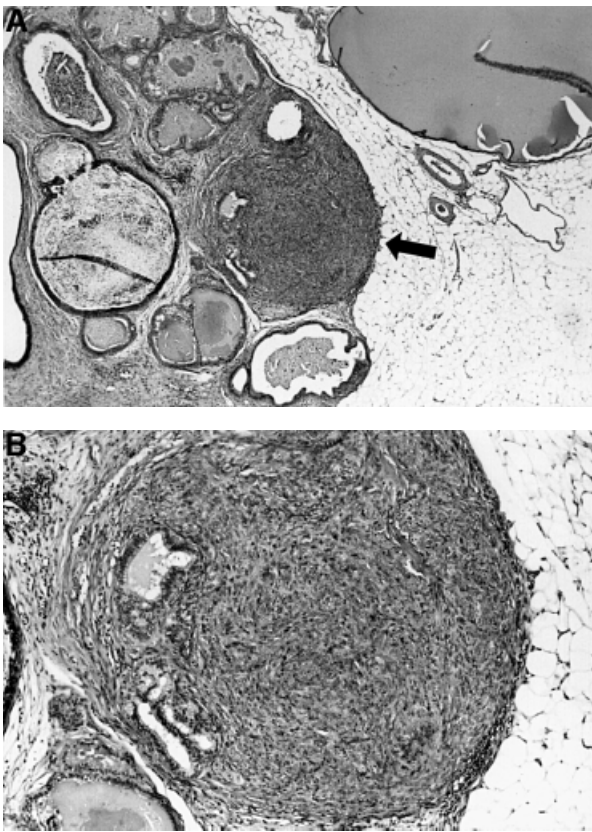
**Fig. 9.** (A) Seminal vesicle carcinosarcoma in a DES-lineage ( $F_2$ ) male mouse, group I, prenatal DES-5. (H&E,  $\times 5$ .) (B) Enlargement of (A). There are epithelial cells (E) on both sides of a mesenchymal component (M). Numerous mitotic figures and atypical nuclei can be seen. (Arrow, H&E,  $\times 50$ .)

biological significance especially since female DES descendants showed no corresponding increase (40). All other organs examined in this study showed no significant differences in incidence of tumors.

**Discussion**

While the main focus of this study was to determine the incidence of proliferative lesions in DES-lineage male mice, the fertility of a subset of these  $F_2$  DES males was also examined at 11–12 months of age. Although one DES-lineage male from group I, prenatal DES 2.5 was not able to impregnate any females over the course of study, this is not an unusual finding in rodent breeding colonies. Of the fertile males in this study, no significant difference in reproductive outcome between control and DES-lineage males was observed. Litter sizes were similar between control and all DES-lineage groups. Sex ratios of the litters were not determined. Since a recent report described altered sex ratios in  $F_2$  litters resulting from the prenatal hormone environment of the mother (46), this endpoint warrants further examination. Although variations in body weights, reproductive tract tissue weights, and hormone levels were observed across groups, the differences did not result in apparent altered reproductive outcomes. Additional DES-lineage animals need to be studied, however, to determine if subtle effects on fertility exist, since infertility in laboratory animals may not be a sensitive endpoint in detecting an adverse response to reproductive toxicants (47).

In contrast to the apparent lack of effects on fertility,



**Fig. 10.** (A) Prostatic neoplasia in a DES-lineage ( $F_2$ ) male mouse, group III, neonatal DES. The circumscribed lesion is located in the dorsolateral prostate. (Arrow, H&E,  $\times 5$ ). (B) Enlargement of (A). There is a complex mixture of polyhedral and spindle-shaped cells. The lesion is mostly a solid tumor but it has some tubular structures. (H&E,  $\times 25$ .)

histological abnormalities in the genital tracts of the DES-lineage mice ( $F_2$ ) evaluated at 17–24 months of age suggested an increased incidence of tumors in the rete testis and reproductive tract tissues. While there is ample evidence that exposure of pregnant females to DES and other chemicals results in tumors in their  $F_1$  offspring (18,48,49), the data in this report add increasing support to the idea that exposure to some chemical carcinogens may result in increased incidences of tumors in more than one generation of ‘untreated’ descendants (50).

The transgenerational effects reported in this study are very interesting. As we have previously proposed for  $F_1$  DES-exposed males (7), the rete testis is a target for DES adverse effects; this observation has further support from a recent study from another laboratory demonstrating adverse effects on the rete testis following exposure to a number of environmental estrogenic compounds (51). In the current study, the highest rate of proliferative lesions in the rete testis was in group I, prenatal DES-10 with 17/49 (35%) of the  $F_2$  males having lesions at 17–24 months of age. Of particular significance, was the occurrence of two rete tumors, one in group I, prenatal DES-5 [1/83 (1%)] and another in group II, prenatal DES-day 18 [1/52 (2%)]. Rete testis adenocarcinoma was reported previously by this laboratory in 5% of prenatally DES-exposed  $F_1$  males (100  $\mu\text{g}/\text{kg}$  maternal body weight) (7,14). Rete testis tumors are rare and were not observed in any control males in this study or in any other historical controls from this lab. Other lesions in the rete testis observed in this study included tubuli recti hyperplasia in a group I, prenatal DES-2.5 mouse

and lipid cell hyperplasia in group II, prenatal DES-day 18 mouse. Considering the changes in the intra-testicular duct system of the testis, reported in this study, this area appears to be a target for the transgenerational effects of DES with the distal portion of the ductuli recti and rete being particularly affected. Together, these DES-induced changes could have retrograde effects on the rest of the seminiferous tubule and further alter sperm transport resulting in sperm granulomas, which were also observed in DES-lineage males. A recent review summarized the toxic effects of several compounds including DES on the developing excurrent duct system (52); as pointed out, the rete testis and efferent ductules have received little attention in male reproductive toxicology in the past; therefore, it is difficult to know if these tissues are insensitive to toxicants or if they have been just overlooked. The transgenerational effects of DES described in this paper along with previous reports from this lab describing adverse effects including neoplasia of the rete testis following prenatal exposure to DES (7), suggest these tissues warrant additional study.

Other interesting findings in this study were the occurrence of rare tumors such as a seminal vesicle papilloma, two seminal vesicle carcinosarcomas, a seminal vesicle sarcoma and a prostatic neoplasm in DES-lineage mice.

The mechanisms involved in these transgenerational events are unknown. However, considering all the genetic/epigenetic effects (25,27–32,53) that have been associated with DES treatment, the possibility of germ cell alterations are feasible. In fact, one explanation for the transgenerational DES-effects (36,37,40,54) is that the effect could be transmitted by abnormally imprinting DNA methylation patterns. Interestingly, a recent report from our laboratory describes imprinting of abnormal methylation patterns in estrogen-responsive genes in  $F_1$  females following developmental DES exposure (28); whether this is related to DES-lineage carcinogenicity remains to be determined. However, changes in DNA methylation patterns are receiving renewed interest (55–57).

In summary, the data described in this report suggest that irreversible changes exist in developmentally DES-exposed females that can be transmitted to their ‘grandsons’. This concept is further strengthened by similar results in the  $F_2$  female siblings in which, like the males ( $F_2$ ) described in this study, reproduction is not apparently altered but cancers are observed (40). The results obtained using this experimental animal model indicate that the cascade of events that lead to the appearance of a tumor may well begin before birth and perhaps before conception. The data described in this report are further significant because this animal model can be used to study both genetic and epigenetic changes associated with developmental exposure to DES. Using this animal model, we can now systematically analyze and detect the changes caused by DES, which will enable us to compare similarities and differences between mice and humans. The ability to detect these genetic/epigenetic changes represents an important advancement in future cancer therapy and prevention. Furthermore, this animal model permits us to reach across species and learn more about mechanisms involved in cancer, in particular, the factors underlying the genetic predisposition to cancer.

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