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Research Article

Mice lacking membrane estrogen receptor 1 are protected from reproductive pathologies resulting from developmental estrogen exposure†

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Abstract

Both membrane and nuclear fractions of estrogen receptor 1 (ESR1) mediate 17β-estradiol (E2) actions. Mice expressing nuclear (n)ESR1 but lacking membrane (m)ESR1 (nuclear-only estrogen receptor 1 [NOER] mice) show reduced E2 responsivity and reproductive abnormalities culminating in adult male and female infertility. Using this model, we investigated whether reproductive pathologies caused by the synthetic estrogen diethylstilbestrol (DES) are mitigated by mESR1 ablation. Homozygous and heterozygous wild-type (WT and HET, respectively) and NOER male and female mice were subcutaneously injected with DES (1 mg/kg body weight [BW]) or vehicle daily from postnatal day (PND) 1–5. Uterine histology was assessed in select DES-treated females at PND 5, whereas others were ovariectomized at PND 60 and treated with E2 (10 μg/kg BW) or vehicle 2 weeks later. Neonatal DES exposure resulted in ovary-independent epithelial proliferation in the vagina and uterus of WT but not NOER females. Neonatal DES treatment also induced ovary-independent adult expression of classical E2-induced transcripts (e.g., lactoferrin [*Ltf*] and enhancer of zeste homolog 2 [*Ezh2*]) in WT but not NOER mice. At PND 90, DES-treated WT and HET males showed smaller testes and a high incidence of bacterial pyogranulomatous inflammation

encompassing the testes, epididymis and occasionally the ductus deferens with spread to lumbar lymph nodes; such changes were largely absent in NOER males. Results indicate that male and female NOER mice are protected from deleterious effects of neonatal DES, and thus mESR1 signaling is required for adult manifestation of DES-induced reproductive pathologies in both sexes.

Summary Sentence

Current results reveal that male and female NOER mice are protected from harmful effects of DES, providing strong evidence that membrane ESR1 signaling is required for adult manifestation of DES-induced reproductive pathologies in both sexes.

Key words: xenoestrogens, steroid receptors, uterus, vagina, estrogen, testis, epididymis.

Introduction

Estrogenic actions in mammals are primarily mediated by signaling through estrogen receptor 1 (ESR1; also known as ERα), which is widely distributed throughout male and female reproductive and non-reproductive organs. In addition, ESR2 (also known as ERβ) plays a role in some facets of reproduction [1–4]. Most ESR1 in target tissues/cells is located in the nucleus and to a lesser extent in the cytoplasm, where it functions as a ligand-activated transcription factor and signals through classic genomic pathways. However, in addition to nuclear ESR1 (nESR1), 5%–10% of cellular ESR1 is palmitoylated after protein synthesis, and this biochemical modification directs these receptors to the cell membrane, giving rise to membrane ESR1 (mESR1) [5, 6]. Estrogen binding to mESR1 induces a variety of rapid effects, including activation of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathways and increasing intracellular cyclic adenosine monophosphate (cAMP) and Ca^{+2} concentrations, resulting in pronounced and possibly longstanding downstream cellular changes [7–10].

The recent development of transgenic mice expressing nESR1 but with reduced or undetectable mESR1 [11–13] provides a novel animal model for understanding the role of mESR1 in estrogen signaling. Female nuclear-only ESR1 (NOER) mice with a complete lack of mESR1 [11] have reduced uterine estrogen responsiveness, and adult females from both lines of NOER mice created by separate laboratories are reported to be infertile [11, 12]. In addition, male NOER mice [13] have reproductive pathologies similar to *Esr1* knockout (*Esr1*KO) males that totally lack ESR1, suggesting that loss of mESR1 alone is sufficient to impair estrogen responses. NOER males show transient fertility immediately after puberty but become progressively infertile [13].

Early exposure of rodents to the potent synthetic estrogen diethylstilbestrol (DES) has been used for many decades as a model of developmental estrogen effects, but this work also has relevance to human health. Pregnant women were treated with DES from the 1940s to early 1970s under the mistaken notion that it reduced miscarriages. It subsequently became clear that many children exposed to DES in utero developed reproductive problems in adulthood, including vaginal clear cell carcinoma and other male and female reproductive abnormalities [14–16]. Moreover, it is now becoming apparent that daughters and even grand-daughters of women exposed to DES in utero have similar reproductive pathologies as their mothers, which is suggestive of a maternally transmitted transgenerational effect [17, 18]. Comparable results occur in rodent models of DES exposure [17, 19]. In addition, DES continues to be used as a growth promoter in cattle and other food animals [20, 21] and thus remains an important human health concern.

Early DES exposure has dual effects on target organs such as the uterus, where it produces short-term changes in uterine epithelial proliferation, secretory protein production, and other pleiotropic effects [22]. In addition, early exposure to DES and other xenoestrogens causes epigenetic changes that can permanently and even heritably change gene function without altering the DNA sequence itself. There has been intense interest in the role of epigenetic changes induced by DES and other xenoestrogens in causing reproductive pathologies since this phenomenon was first described 20 years ago [23]. These epigenetic effects include methylation changes of estrogen-target genes such as lactoferrin (*Ltf*) [23, 24], as well as histone modifications that can also alter gene transcription [9, 25]. The latter at least appear to be mediated through mESR1 [9, 25], as estrogen treatment stimulates the PI3K/AKT pathway by acting through mESR1 to cause methylation changes in histone sites such as H3K27me3, which are associated with inhibition of transcription. Long-term disruptions in the transcriptome and proteome resulting from epigenetic changes are likely associated with adult health consequences, a classic example of adverse developmental origins of health and disease effects [26, 27].

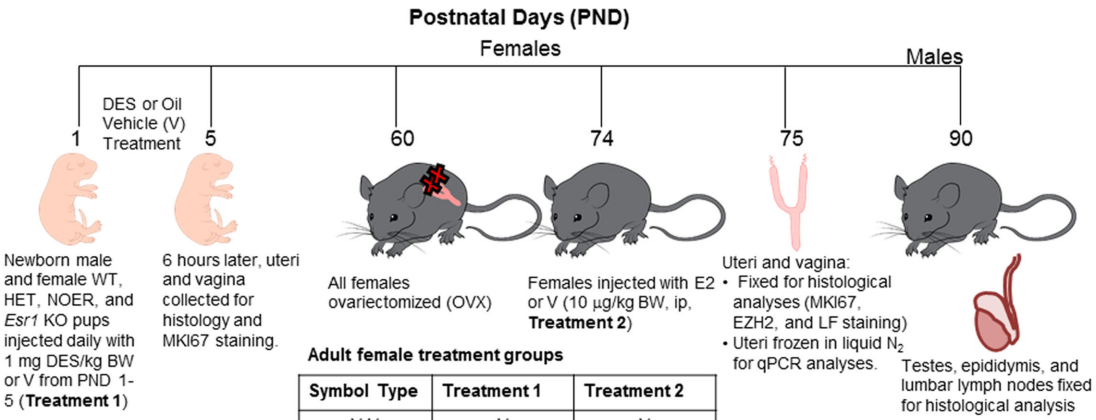
If DES-induced reproductive pathologies and epigenetic changes depend upon mESR1, such responses should presumably be abolished or blunted in NOER mice. We therefore hypothesized that male and female NOER mice lacking mESR1 would be at least partially protected from effects of early DES exposure. To test this, wild-type (WT), heterozygous (HET), and NOER mice deficient in mESR1 were administered DES neonatally and then immediate effects on the uterus and long-term effects on the male and female reproductive effects were examined.

Materials and methods

Animals and treatments

Wild-type, HET, NOER, and *Esr1*KO mice on a mixed C57BL/6 and 129SvEv background were generated from our colony at the University of Florida, as detailed previously [13]. Heterozygous male and female NOER and *Esr1*KO mice were bred to obtain homozygous NOER and *Esr1*KO males, respectively. This breeding scheme also yielded WT and HET (one WT allele and one *Esr1*KO allele) males and females that were used in these studies. Genotyping of NOER [13] and *Esr1*KO [28] mice was performed by multiplex PCR on genomic DNA, as described previously.

Mice were housed in standard mouse cages at 22°C, with 12h:12h light/dark cycles and ad libitum access to water and Teklad 2918 rodent diet (Envigo, Madison, WI). This diet is formulated with reduced soybean meal, contains 18% protein, and is irradiated. All procedures were approved by the University of Florida IACUC and



Adult female treatment groups

Symbol Type	Treatment 1	Treatment 2
V V	V	V
V E2	V	E2
DES V	DES	V
DES E2	DES	E2

Figure 1. Schematic of experimental procedure for treating WT, HET, NOER, and *Esr1*KO mice with neonatal DES, and then subsequent collection of male and female reproductive organs for histological, immunohistochemical, or qPCR analysis. Adult females included four treatment groups, as shown in the table below the timeline. In contrast, males were only treated with neonatal DES or V.

conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Neonatal female and male pups were weighed each day and given daily subcutaneous injections of 10 μl/g of DES in corn oil such that their final DES dose was 1 mg/kg body weight (BW), or an equivalent volume of corn oil vehicle (V) alone from the day of birth (PND1) to PND 5 (Figure 1). Assignment to these groups was randomized. Pups were injected at approximately the same time of the day during this treatment period. All pups in a litter were subjected to the same treatment to prevent cross-contamination. Six hours after the final injection, uteri from some WT, NOER, and *Esr1*KO female pups were collected for histology and immunostaining, as described below (Figure 1). Mice were genotyped following weaning. At 60 days of age, all WT, HET, and NOER females were ovariectomized. Two weeks later, they were randomly allocated to treatment groups detailed below. Mice were treated with a single intraperitoneal (ip) injection of 17β-estradiol (E2, 10 μg/kg BW) in 0.1 ml of corn oil, or V alone. Twenty-four hours later, uteri and vagina were collected and frozen for qPCR or fixed and processed for histology or immunohistochemistry (Figure 1). At 90 days of age, male reproductive organs were collected from WT and NOER mice given either DES or V neonatally, weighed, and processed for histology. Multiple males from one litter were sometimes used for the analysis of neonatal DES effects, and thus, litter effects were taken into account for the statistical analysis, as detailed below.

Histological analysis and immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin (NBF) until embedded. Samples were paraffin embedded, and 5–6 μm sections were then cut from each block. Samples were either stained with hematoxylin and eosin or left unstained and then used for MKI67 (proliferation marker) or enhancer of zeste homolog 2 (EZH2) immunohistochemistry. Immunohistochemistry for MKI67 (Supplementary Table 1) was performed as described previously [13, 28]. Immunohistochemistry for EZH2 was performed with these same procedures, except that the primary antibody was a rabbit monoclonal IgG for EZH2 (catalog #5246; Cell Signaling Technology, Danvers, MA) that was used at a 1:1000 dilution (Supplementary Table 1). In both cases, the chromogen used to detect positively stained cells was 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA), resulting in a brown precipitate in these cells.

Initial histological assessments of testes, epididymis, and surrounding lymph nodes from the male mice showing lesions suggested severe and acute inflammation, which were further evaluated as detailed below. Thus, these sections were stained at the IDEXX Laboratory in Columbia, MO, with Gram stain by the Brown and Hopps method [29] to examine for potential bacteria and, if present, to be able to classify whether the microorganisms were Gram negative or positive and their morphology, such as cocci or rod shaped.

Morphometric analysis of MKI67 staining in adult uterus

To quantify MKI67 staining in adult uterine sections from different treatment groups, images of sections were acquired under a 25X objective using an Olympus BH-2 microscope. In Adobe Photoshop CS2 Version 9.0 (Adobe, San Jose, CA), a box was constructed that measured 160 by 80 pixels. The box was then placed over 10 random luminal epithelial areas for the uterine sections. The number of MKI67$^+$ and total epithelial cells within each boxed region was determined by two independent investigators who were blinded to the mouse genotype and treatments for each section examined.

Uterine gene expression studies

Total RNA extracted from frozen uterine tissue was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Catalogue #205310, Qiagen, Germantown, MD). The qPCR procedure was performed on the Applied Biosystems 7500 Real-Time PCR System (Carlsbad, CA) by using the QuantiTect SYBR Green PCR Kit (Catalogue #204143; Qiagen). Primer sequences for the genes examined are listed in Supplementary Table 2, and primers were purchased from IDT (Coralville, IA). The qPCR conditions employed were as follows: (1) 15 min at 95°C for polymerase activation, (2)

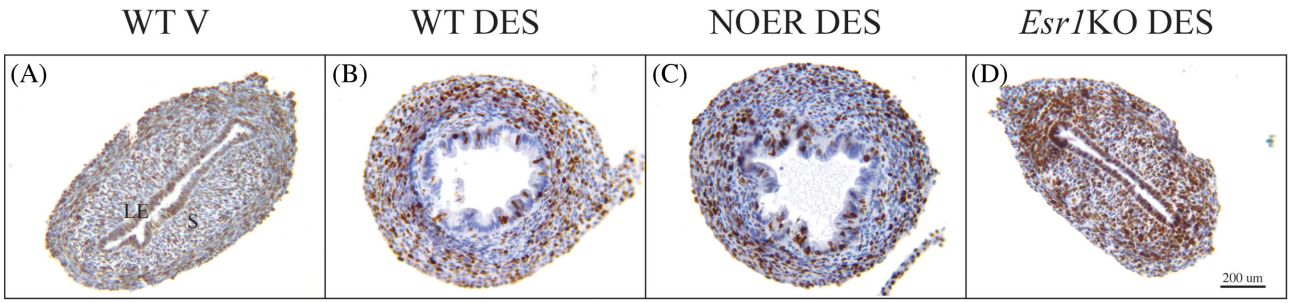


Figure 2. Neonatal DES treatment induced changes in uterine structure and epithelial proliferation in uteri of neonatal NOER and WT, but not *Esr1*KO, mice. Uteri were collected 6 h after the last injection of DES or oil vehicle on PND 5 and immunostained for MKI67. Uteri from DES-treated WT (B) and NOER (C) pups showed enlarged lumens and reduced MKI67 staining, pseudo-stratification, and corrugation in their luminal epithelium (LE) compared to uteri from oil-treated WT (A) or DES-treated *Esr1*KO (D). All images were photographed at the same magnification, and cells that are positive for the proliferation marker MKI67 are stained brown. The N = 3–6 for all groups. S = Stroma, LE = luminal epithelium

40 cycles of denaturation 40 s at 94°C, annealing 40 s at 55°C, and extension 72°C for 1.50 min, (3) dissociation melt curve analysis from 60°C to 90°C. The internal housekeeping gene was ribosomal protein L7 (*Rpl7*) and test estrogen-responsive genes included: *Esr1*, *Esr2*, *Ezh2*, DNA methyltransferase 1 and 3a (*Dnmt1* and *Dnmt3a,* respectively), histone acetyl transferase 1 (*Hat1*), histone deacetylase 2 and 4 (*Hdac2* and *Hdac4,* respectively), lactotransferrin (*Ltf*), and sine oculis homeobox 1 (*Six1*).

These candidate genes were selected based on their known regulation by estrogen binding to ESR1 and/or acting as epigenetic modifiers [31–33]. EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2) complex that provides methyltransferase activity, and thus PRC2 activity is regulated primarily by EZH2 expression. Estrogen may also regulate EZH2 expression transcriptionally in mouse uteri [34]. DNMT1 and DNMT3a are part of a family of DNA methyltransferases that add methyl groups to CpG structures in DNA. Both HDAC2 and HDAC4 regulate histone deacetylation and thus can regulate eukaryotic gene expression. HAT1 catalyzes the addition of acetylate groups to lysine residues within histone proteins, especially H4 histone molecules, which is generally associated with increased gene expression [35]. LTF is considered a classic-estrogen induced gene within the uterus and is associated with regulation of cell growth, differentiation, and cancer development [36].

Statistics

Uterine gene expression and uterine MKI67 staining were analyzed with ANOVA in SAS 9.4 (Cary, NC). For uterine gene expression, Grubb's outlier test function in GraphPad Software (https://www.graphpad.com/quickcalcs/Grubbs1.cfm, La Jolla, CA) was used to identify potential outliers. Uterine gene expression differences were determined based on ΔCt value. Data for dependent variables of uterine gene expression and uterine MKI67-positive epithelial cells as a percentage of total epithelial cell number within each defined region were analyzed using the MIXED procedure of Statistical Analysis Systems (SAS). Sources of variation considered were genotype, DES treatment, E2 treatment, and all possible combinations. Replicates for each individual were averaged beforehand, and the individual mouse served as the experimental unit. As two independent observers calculated the number of MKI67$^+$ cells and total cells within each defined region, a Pearson correlation coefficient was performed with Microsoft Excel to determine the inter-observer agreement (R^2 value).

To determine the $2^{-\Delta\Delta Ct}$ values for the uterine gene expression, the WT V V group was considered the reference control group and the average relative expression for this group set at 1. All data are presented as the mean ± standard error of the mean. Using principal component analysis (PCA) in the mixOmics R package (v 6.61) [37], the correlations among the genes were determined and correlation circle plots were generated by plotting of these variables. In these analyses, the variables are projected inside a circle with radius 1 centered at the origin or correlation circle. Strongly correlated variables project in the same direction from the circle origin. The more variables extend from the central origin point indicate that they are strongly associated. Results were confirmed with Pearson correlation coefficient, as determined by SAS v 9.4.

As in some cases, males of the same genotype and who received the same treatment from the same litter were tested, a split plot in space analysis was used [38]. This analysis controls for potential litters effects as the dam becomes the statistical unit. While we sought to control for potential litter effects in analyzing the incidence of testicular lesions (fibrous adhesions and orchitis) in the various male groups, this was not possible. The reason is that statistical methods that control for potential litter effects with non-parametric data (yes/no), in particular calculating an odds ratio with PROC GLIMMIX, require at least one incidence for all test groups as the log of 0 cannot be determined. Consequently, in collaboration with a biostatistician, these results were instead analyzed with chi-square analysis using Graph Pad Prism version 6.0.

Results

Neonatal DES treatment induces similar immediate uterine changes in WT and NOER, but not Esr1KO, mice

Mice were treated from PND 1 to 5 with DES or oil vehicle and then uteri were examined 6 h after the final treatment. Uteri from oil-treated WT and NOER mice had a flattened, oblong shape (Figure 2). The lumen had a slit-like morphology typical of neonatal uterus, with a simple columnar epithelium. Extensive cell proliferation was evident in both the stroma and epithelium, and the beginnings of myometrial differentiation was noted in the peripheral mesenchyme. In sharp contrast, uteri from WT or NOER mice treated PND 1–5 with DES had a rounded, distended shape, and there was a pronounced enlargement of the uterine lumen in both groups compared to WT vehicle controls. Uterine epithelium in both DES-treated WT and

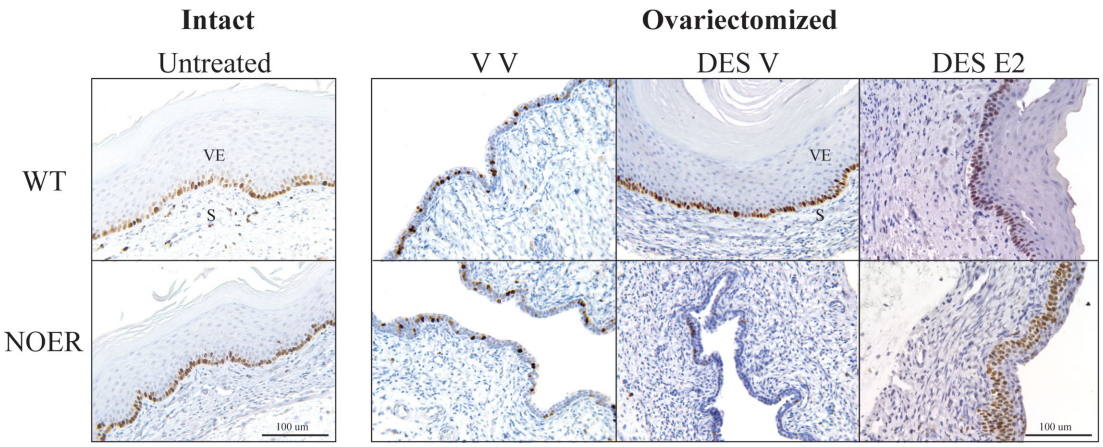


Figure 3. Ovary-independent persistent vaginal epithelial proliferation and cornification is induced in adult WT, but not NOER mice, by neonatal DES treatment. In intact WT and NOER vaginas during estrus, proliferation of the basal epithelium (VE) was high, and the epithelium was stratified and keratinized. Some stromal (S) MKI67 labeling was also seen. In WT V V and NOER V V mice, vaginal epithelium was 2–3 cell layers thick and atrophic, with low MKI67 labeling. In WT DES V mice, basal epithelium showed high proliferation, and the epithelium was stratified and highly keratinized. In sharp contrast, vaginal epithelium from NOER DES V mice was totally atrophic and resembled that of the WT V V or NOER V V mice. Vaginal epithelium in WT DES E2 mice resembled that in WT DES V mice. In NOER DES E2 mice, the basal epithelium was highly proliferative and the epithelium was somewhat stratified, but this epithelium had not had the chance to develop the full stratification and keratinization because in this case E2 was administered only 24h before sacrifice. All photos are at the same magnification. The N = 3–8 for all WT and NOER groups, respectively.

NOER females was pseudostratified with a scalloped appearance, and the initial appearance of the myometrial layer seen at this stage in vehicle-treated WT controls was not apparent. Uterine epithelial proliferation was dramatically reduced and stromal proliferation also appeared to be less extensive in DES-treated WT or NOER mice compared to oil-treated WT mice. In contrast to WT and NOER, the morphology of DES-treated *Esr1*KO uteri that lacked both the membrane and nuclear forms of ESR1 was essentially unchanged by DES treatment.

Neonatal DES induces ovary-independent persistent vaginal cornification and proliferation in adult WT but not NOER mice

Vaginal histology and cell proliferation were comparable in intact WT and NOER mice during estrus (Figure 3). In WT or NOER mice that were given V neonatally and then treated with E2 following adult ovariectomy (WT V E2 and NOER V E2, respectively), vaginal epithelial proliferation was robust and comparable in intact WT or NOER vaginas (not shown). In WT or NOER mice that were given V both neonatally and following adult ovariectomy (WT V V and NOER V V, respectively), vaginal epithelium was atrophic with minimal proliferation (Figure 3). Conversely, vaginal epithelial proliferation in WT or NOER mice that were given V neonatally and then treated with E2 following adult ovariectomy (WT V E2 and NOER V E2, respectively) was robust and comparable to the intact WT or NOER vaginas (not shown). The vaginas of WT V E2 and NOER V E2 mice showed an increase in epithelial height compared to vaginas from WT V V and NOER V V mice, but because these tissues were sampled only 24 h after E2 treatment, they did not show full stratification, cornification, or keratinization.

Unlike the vaginal epithelial atrophy in WT V V mice, vaginas of DES-treated ovariectomized WT mice had persistent vaginal epithelial proliferation and were cornified irrespective of whether they were given E2 (WT DES E2) or oil (WT DES V) 24 h prior to collection. In sharp contrast, vaginal epithelium from NOER DES V females did not exhibit persistent vaginal cornification, and was indistinguishable from WT V V vaginas, with minimal basal epithelial proliferation resulting in an atrophic epithelium that was only 2–4 cell layers thick. Vaginas of NOER mice given DES neonatally and then E2 in adulthood (NOER DES E2) showed a strong epithelial proliferative response to E2.

Neonatal DES induces ovary-independent uterine proliferation in adult WT but not NOER mice

Uterine epithelial proliferation was low and comparable in WT and NOER mice treated with V neonatally and following adult ovariectomy (WT V V and NOER V V, respectively), as shown by MKI67 immunohistochemistry (Figure 4A) and quantitation of MKI67 labeling (Figure 4B) by two independent observers with an inter-observer agreement of $R^2 = 0.97$ and who were each blinded to the mouse genotype and treatments received for each section examined. In contrast, in WT mice given oil neonatally and then E2 following adult ovariectomy (WT V E2), almost the entire epithelial lining stained with MKI67 ($P \leq 0.01$ vs. control WT V V). Epithelial cell MKI67 staining was not statistically different in NOER V E2 mice compared to NOER V V mice, but epithelial MKI67 labeling was clearly lower in NOER than WT mice. In WT DES V mice, the epithelium showed an ovary-independent proliferation ($P = 0.04$ relative to WT V V). Treatment with E2 (WT DES E2) did not produce a significant further increase in uterine epithelial MKI67 staining relative to relative to WT DES V. In contrast, in NOER DES V mice, epithelial MKI67 staining was relatively low and comparable to NOER V V, and E2 treatment (NOER DES E2) resulted in a strong trend toward an increase in epithelial labeling ($P = 0.06$), although this did not reach significance, likely because of limited sample size.

Uterine mRNA and protein expression in adult WT and NOER mice

Expression of *Ezh2* mRNA in uteri of WT and NOER mice following various neonatal and adult treatments is shown in Figure 5. Expression of *Ezh2* mRNA expression was elevated in uteri of WT

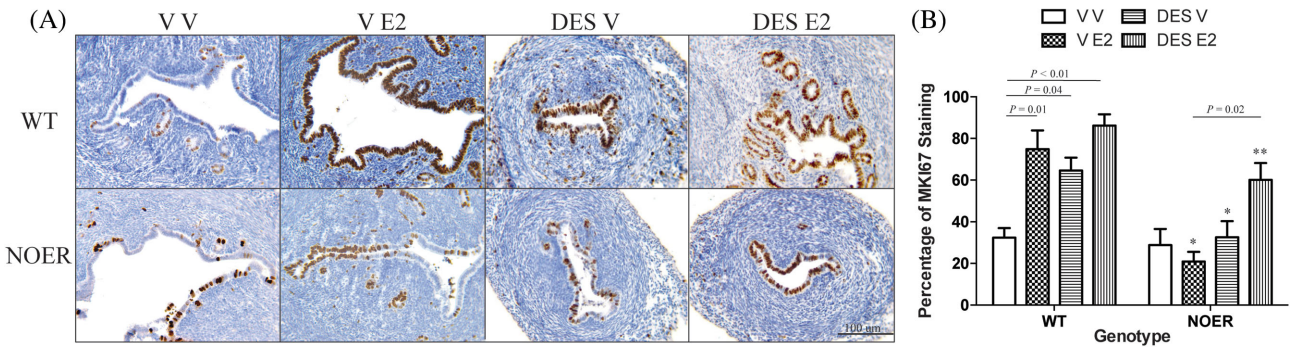


Figure 4. Neonatal DES induces ovary-independent uterine proliferation in WT, but not NOER, mice. (A) In WT V V and NOER V V mice, epithelial proliferation was low. Treatment with E2 following adult ovariectomy (WT V E2) stimulated epithelial proliferation, but this response was diminished in NOER V E2 mice. The WT DES V mice showed an ovary-independent epithelial proliferation that was not seen in NOER DES V mice, and epithelial proliferation was robust in both WT DES E2 and NOER DES E2 mice. (B) Quantitation of uterine epithelial proliferation in the various treatment groups also indicated that WT DES V mice, but not NOER DES V mice, show ovary-independent epithelial proliferation. The N = 4–7 for all WT and NOER groups, respectively. $^{*}P < 0.05$ vs. WT group given the same treatment; $^{**}P = 0.07$ vs. WT DES E2 group.

DES V mice compared to WT V V mice (Figure 5A; $P = 0.008$), and in WT V E2 mice relative to WT V V mice ($P = 0.05$). Conversely, *Ezh2* mRNA expression was decreased in WT DES E2 vs. DES V ($P = 0.04$). Significant differences were not detected among any of the treatment groups of NOER mice.

Expression of the classic estrogen-induced gene *Ltf* was increased in WT V E2, WT DES V, and WT DES E2 mice compared to WT controls (WT V V, Figure 5B; $P < 0.005$). For unexplained reasons, NOER V V mice demonstrated greater basal *Ltf* expression than WT V V ($P = 0.04$). Within NOER groups, only NOER DES E2 was significantly greater than NOER DES V. Expression of *Hat1* mRNA was more pronounced in WT DES V than WT V V and WT DES E2 (Figure 5C; $P = 0.02$ and 0.03, respectively). Comparison across genotypes revealed that expression of this gene was greater in WT DES V than NOER DES V ($P = 0.004$). Similarly, *Esr2* mRNA was greater in WT DES V than WT V V or WT DES E2 (Figure 5D; $P = 0.002$ and 0.02, respectively). WT DES V expressed greater amounts of *Esr2* than NOER DES V ($P = 0.007$). Circle correlation plotting based on PCA and correlation analyses revealed strong positive association between *Ezh2* and *Hat1* expression ($r = 0.89$, $P < 0.0001$), suggesting neonatal DES exposure can simultaneously alter several histone marks (Figure 5E). This figure and Pearson correlation coefficient revealed *Hat1* and *Ezh2* also positively correlated with *Esr2* ($r = 0.40$, $P = 0.01$ and $r = 0.50$, $P = 0.001$, respectively). None of the other genes showed significant differences based on genotype and DES/E2 treatment interactions. These other results are shown in Supplementary Table 3.

Most of the EZH2 qPCR results were confirmed with immunohistochemistry (Figure 6). Immunohistochemistry for EZH2 revealed that expression of EZH2 was low and similar in both WT V V and NOER V V mice (Figure 6). In WT V E2, EZH2 protein expression was increased relative to WT V V, but in NOER mice this E2 response was muted. The epithelium of WT DES V mice strongly expressed EZH2 despite the lack of adult uterine stimulation by ovarian E2 in these animals, but a similar response was not detected in NOER DES V mice. Treatment with E2 produced some further increases in epithelial EZH2 protein expression in both WT DES E2 and NOER DES E2 mice, which is in contrast to the results obtained with qPCR. Potential reasons for the differences in mRNA vs. protein expression in the WT DES E2 group are detailed in section Discussion.

Male reproductive pathologies in adult WT and NOER mice in response to DES

An analysis comparing male reproductive tract changes in NOER and WT mice has been previously published by our group [13]. Thus, the current work focuses on male reproductive changes due to DES treatment in WT, HET, and NOER mice. In DES-treated WT and HET males, neonatal DES treatment resulted in a high incidence of fibrous adhesions between the testes, epididymis, epididymal fat pad, and sometimes involving the ductus deferens. These organs formed one large connected mass in many WT and HET males, making it difficult to obtain clear pictures (Figure 7A), with 52% of the WT (12/23) and 78% of the HET (14/18) males showing lesions. In sharp contrast, only 1/16 (6%) of DES-treated NOER males ($P \leq 0.001$ vs. DES-treated WT males) demonstrated this overt gross pathological change, and in the one occurrence observed, there were relatively limited adhesions between the testis and epididymis. No lesions of this type were observed in oil-treated WT (0/14), HET (0/3), or NOER (0/17) males (Figure 7B). As WT and HET mice showed similar incidences of testicular lesions, as well as similar changes in testes and seminal vesicle weights in response to DES treatment, they were combined for statistical and graphing purposes.

In WT/HET mice, DES treatment reduced paired testes weights (collected from mice not showing any lesions) by 33%, respectively, relative to those exposed to vehicle alone ($P \leq 0.0002$ vs. oil-treated controls for both genotypes; Figure 7C). Neonatal DES treatment reduced paired testes weights in NOER males by 12% ($P \leq 0.05$ vs. oil-treated NOER controls), but these changes did not reach significance ($P = 0.16$).

Similar results were seen for weights of the seminal vesicle-coagulating gland complex (Figure 7D). In WT and HET mice, DES treatment reduced the weights of the seminal vesicle/coagulating gland complex by 64%, respectively, relative to counterparts exposed to vehicle alone ($P \leq 0.001$ vs. oil-treated controls for both genotypes). On the other hand, neonatal DES treatment reduced the weights of the seminal vesicle/coagulating gland complex in NOER males by 38% ($P \leq 0.0002$) vs. oil-treated NOER controls.

Histological examination of male reproductive tracts from DES-treated WT and HET males that showed fibrous adhesions revealed extensive pyogranulomatous orchitis with central areas of necrosis and cellular debris, suggestive of acute and necrotizing inflammation

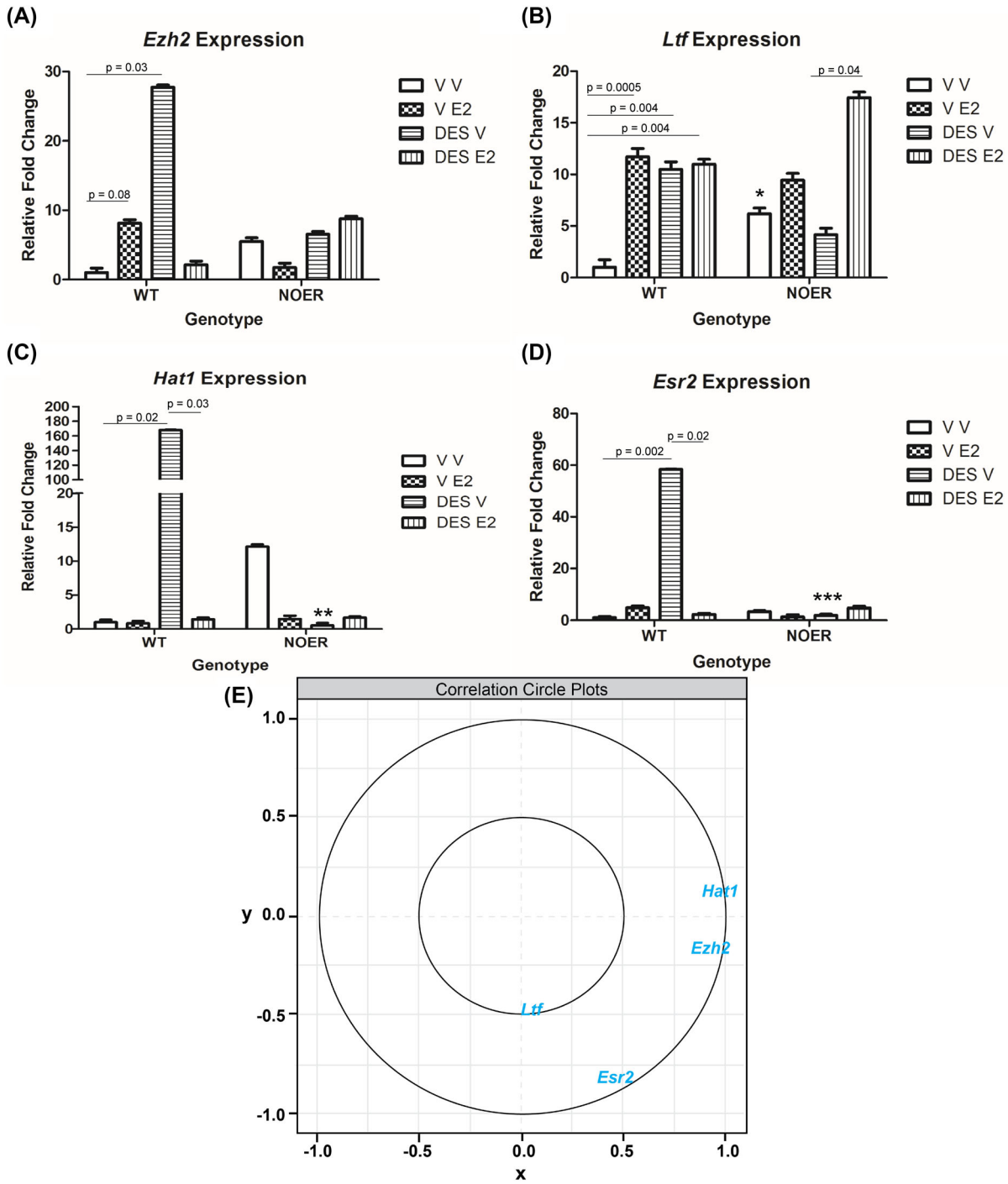


Figure 5. Uterine mRNA expression in adult WT and NOER mice given various neonatal and adult treatments. Expression of *Ezh2* mRNA was elevated in uteri of WT DES V mice compared to WT V V mice. A trend toward an increase in *Ezh2* mRNA was detected in WT V E2 mice relative to WT V V mice. In contrast, increased *Ezh2* mRNA expression was not detected in NOER DES V mice. Expression of *Ltf, Hat1, and Esr2* mRNA (B, C, and D) in uteri of WT and NOER mice given various neonatal and adult treatments was similar to the results observed for *Ezh2*, in that in all cases neonatal DES treatment induced a high expression of the gene in WT mice that was seen even following ovariectomy and oil treatment, but a similar phenomenon was not seen in NOER mice where expression of all of these transcripts was low following neonatal DES treatment and oil administration after adult ovariectomy. The analyses of *Ezh2* mRNA expression was based on 6–10 samples each from the various WT and NOER groups. Analysis of mRNA expression for *Hat1, Esr2,* and the other genes listed in Supplementary Tables 2 and 3 was based on N = 4–9 and N = 6–8 mice for the various WT and NOER groups, respectively. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ vs. WT group given the same treatment. (E) To examine the inter-relationships between uterine gene expression patterns, correlation circle plots based on PCA was performed with mixOmics R package (v 6.61) [37]. As *Hat1* and *Ezh2* extend the furthest distance in the same direction from the central origin point, this indicates a strong association. This association was also reflected by the Pearson correlation coefficient, which revealed the association to be r = 0.89, $P < 0.0001$. The figure further reveals that *Hat1* and *Ezh2* positively correlated with *Esr2*, whose expression pattern extended a similar distance from the central origin point as these other two gene expression patterns. The Pearson correlation coefficient revealed *Hat1* and *Ezh2* associations with *Esr2* were r = 0.40, $P = 0.01$ and r = 0.50, $P = 0.001$, respectively.

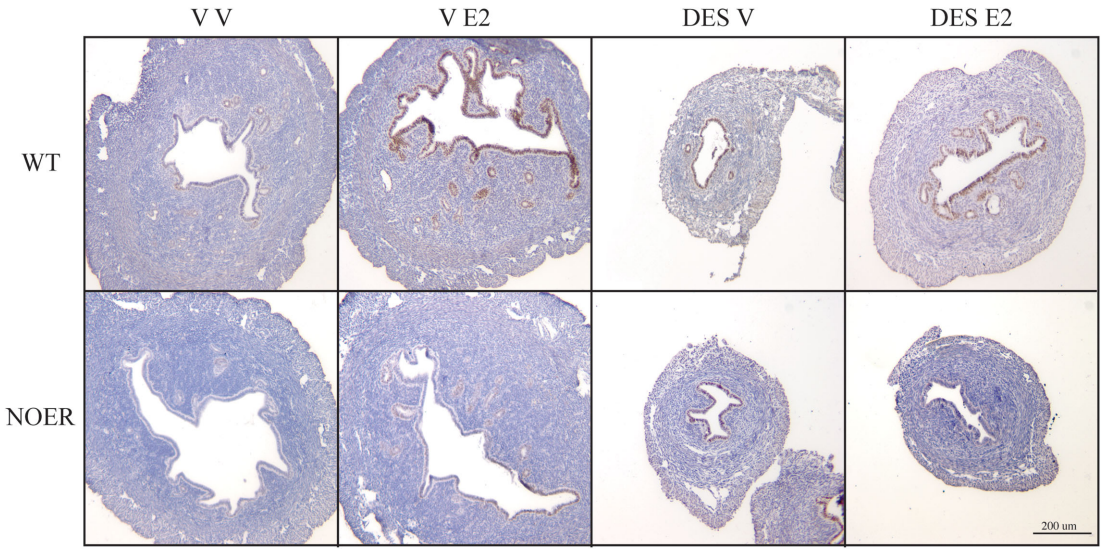


Figure 6. Neonatal DES induces ovary-independent EZH2 expression in uterine epithelium of WT, but not NOER, mice. Epithelial EZH2 expression was minimal in WT V V and NOER V V, and increased in WT V E2 and NOER V E2 mice, although the response was more robust in WT mice. Glandular and luminal epithelium of WT DES V mice expressed EZH2, but this was absent in NOER DES V uteri. Note also the small atrophic appearance of both the WT and NOER uteri after neonatal DES treatment. Expression of EZH2 was increased by E2 in both WT DES E2 and NOER DES E2 mice. The N = 4–5 for all WT and NOER groups, respectively.

(Figure 8). Similar abnormalities were evident in the epididymis (data not shown).

To determine whether the orchitis/epididymitis lesions in WT/HET DES-treated mice could be due to bacterial infection, sections of testes, epididymis, and surrounding lumbar lymph nodes that were enlarged were analyzed with Gram stain. In all three organs, bacterial colonies were evident and were surrounded by a shell of inflammation admixed with necrosis. Visualization of these bacteria under higher magnification revealed that these bacteria appeared to be Gram-positive cocci (Figure 9).

Discussion

An extensive literature extending back over 50 years documents that perinatal administration of DES or other estrogens produces pronounced and long-term alterations in adult reproductive organs of both male and female rodents [19, 39, 40]. These effects include estrogen-independent persistent adult vaginal epithelial proliferation and cornification, alterations in uterine and oviductal structure and cell proliferation, testicular, epididymal and seminal vesicle abnormalities, and an increased susceptibility to neoplastic changes in the effected organs [10, 19, 25, 39–43]. Analogous pathological changes also occur in humans exposed to DES prenatally [14–16] and even transgenerationally [17, 19]. Reproductive effects of DES in both males and females are mediated predominately by ESR1 [44–48]. Recent results reveal that mESR1 is critical for mediating normal estrogen effects in males and females [11–13]. In this study, we sought to determine the role of mESR1 in mediating the deleterious effects of neonatal DES treatment. We compared the effects of neonatal DES treatment at a dose similar to previous studies [19, 40] in WT and NOER mice to see if the lack of mESR1 in NOER mice altered the immediate neonatal or final adult responses to DES administration.

Analysis of uteri from WT, NOER, and *Esr1*KO mice shortly after the neonatal treatments indicated that DES caused dramatic changes in histology and epithelial proliferation in WT and NOER, but not *Esr1*KO, mice. The DES-treated WT and NOER mice showed a corrugated epithelial appearance, uterine lumen enlargement and dilation, and uterine epithelial proliferation was inhibited compared to vehicle-treated WT or DES-treated *Esr1*KO uteri; all of these changes are identical to those previously reported in WT mice immediately after neonatal DES treatment [22]. Thus, WT and NOER uteri show comparable short-term responses to neonatal DES, indicating that membrane ESR1 is not required for this response, whereas lack of both nuclear and membrane ESR1 in *Esr1*KO mice abolishes DES-induced uterine pathological changes.

Despite similar neonatal responses, adult responses of WT and NOER females to neonatal DES treatment dramatically diverged. Estrogen administration to female mouse pups in early neonatal life results in a cornified and keratinized vaginal epithelial morphology and ovary-independent epithelial proliferation of basal vaginal epithelium in adulthood [39]. This ovary-independent adult proliferation can also be obtained through prenatal DES administration, as shown by McLachlan and co-workers [41]. Perinatal estrogen effects are mediated through ESR1 [48] and associated with altered *Wnt* expression [49]. The mechanism of these effects has been suggested to involve estrogen effects on PI3K/AKT and MAP kinase pathways, and potentially changes in DNA methylation and histone modifications [24].

Since all of these responses are thought to involve mESR1 signaling, we hypothesized that adult effects of early estrogen exposure would be absent or diminished in NOER mice lacking mESR1. Indeed, despite comparable responses to neonatal DES immediately post-treatment, long-term responses of WT and NOER females to neonatal DES treatment dramatically diverged. The ovary-independent vaginal epithelial proliferation and keratinization that are hallmarks of developmental estrogen exposure were absent in DES-treated NOER mice but not their WT siblings. Both ovariectomized adult NOER and WT mice showed increased vaginal epithelial proliferation and cornification in response to post-ovariectomy E2 treatment, indicating that this tissue remained responsive to estrogen in NOER mice. These results emphasize the critical role of

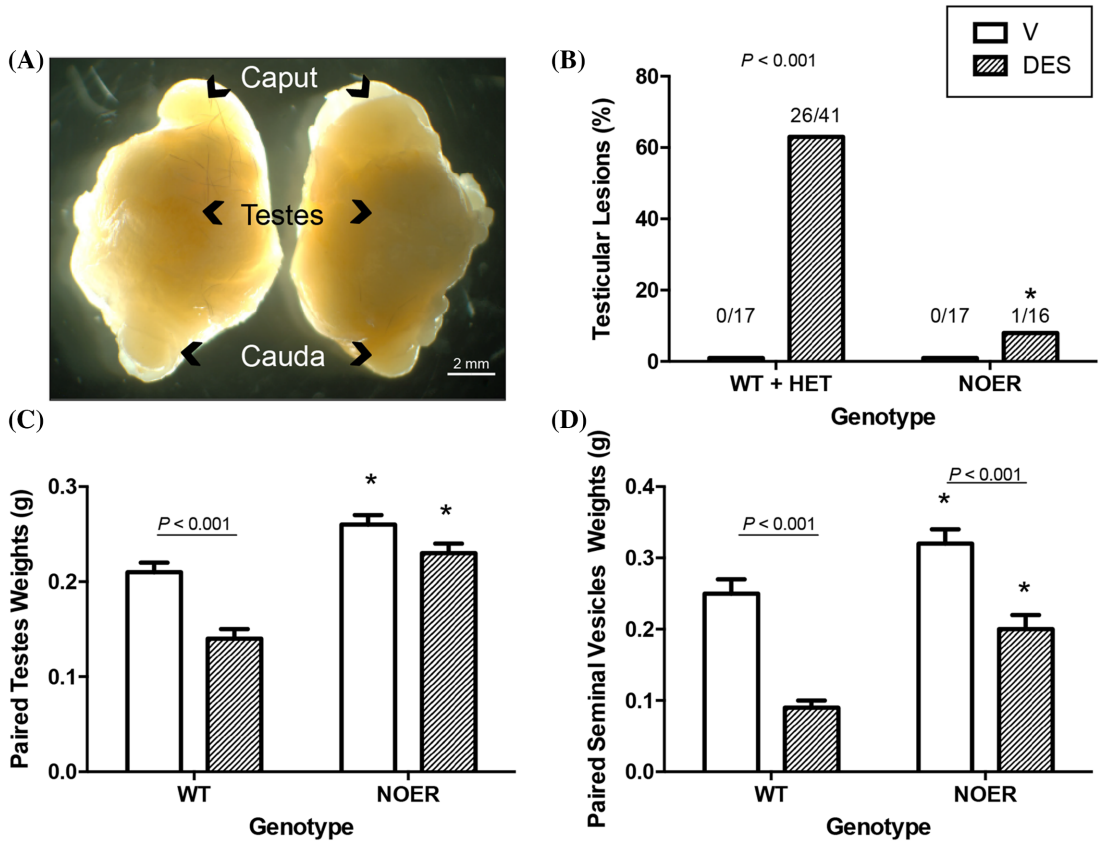


Figure 7. Effects of neonatal DES treatment on WT/HET and NOER mice. (A) Male reproductive tract of a WT mouse that was treated with DES from PND 1 to 5 and fixed in Bouins at 90 days of age and photographed with transmitted light. Treatment with DES produced substantial gross lesions in WT/HET mice consisting of a tightly adhered aggregate of the testis, the caput and cauda epididymis, the epididymal fat pad, and sometimes the ductus deferens. With the extensive adhesions, it was difficult to photograph these organs in a single planar view. For this reason, labels have been added to delineate the caput and cauda epididymides and testes. These gross pathological changes resemble those shown in Figure 1A in Miyaso et al. [55]. (B) Despite a highly significant increase in lesions in DES-treated vs. oil-treated WT/HET mice ($P \leq 0.001$), the incidence of these lesions was only 1/16 in DES-treated NOER mice and was not different than the oil-treated NOER group ($P = 0.5$), although it was significantly different than the DES-treated WT group ($P \leq 0.001$; denoted by *). The N values for the groups are shown above the bars. Testis (C) and seminal vesicle (D) weights in WT/HET and NOER mice treated neonatally with DES. The N = 15–17 for the various groups shown in panel C and N = 12–22 for panel D. When testicular lesions were extensive or severe, testes could not be completely dissected from the lesion and accurately weighed, so sample numbers for DES-treated groups in C are less than in B. Neonatal DES treatment decreased ($P \leq 0.001$) testes weights in WT but not in NOER mice, and testes weights were greater in both groups of NOER mice compared to WT. Neonatal DES treatment significantly ($P \leq 0.001$) decreased seminal vesicle weights in both WT and NOER mice, and seminal vesicle weights were different in both groups of NOER mice vs. WT. For panels C and D, * denotes a NOER group that differed at $P < 0.03$ relative to its corresponding WT/HET group.

mESR1 in harmful effects of developmental DES exposure and characteristic lesions associated with it. Moreover, they suggest that genetic ablation or potential pharmacological inhibition of mESR1 signaling might confer at least partial resistance to developmental effects of DES and possibly other xenoestrogens.

It is abundantly clear that DES, E2 or environmental estrogens administered to rodents during perinatal development can alter uterine architecture and induce proliferative abnormalities that manifest at adulthood. Importantly, such pre-neoplastic lesions have a high incidence of progressing to endometrial cancer [41, 50]. Neonatal DES treatment also results in ovary-independent proliferation of uterine epithelium in adulthood, as shown by constitutive high uterine epithelial proliferation in neonatally DES-treated mice following neonatal, pubertal or adult ovariectomy [42]. The ovary-independent uterine epithelial proliferation is similar to that seen in the vagina after neonatal DES treatment, and may occur by similar mechanisms.

Our present immunohistochemical and cell proliferation data indicate that WT mice given DES neonatally show ovary-independent epithelial proliferation after adult ovariectomy and subsequent V treatment. This effect only occurs in WT mice treated with DES, and is not observed in WT V V mice, as expected. Critically, this DES effect was not seen in NOER mice, where epithelial proliferation in NOER DES V mice was similar to NOER V V mice. Thus, the characteristic induction of ovary-independent uterine epithelial proliferation by neonatal DES treatment is mitigated in NOER mice, consistent with our vaginal data where lack of mESR1 also precludes ovary-independent epithelial proliferation following neonatal DES treatment. These results again emphasize the essential role of mESR1 in modulating the typical adult sequelae of neonatal (and presumably prenatal) DES exposure.

Neonatal DES treatment induces epigenetic effects in organs such as uterus, and these epigenetic changes can result in the adult uterus from neonatally DES-treated WT mice expressing high levels of *Ltf* and other estrogen-dependent proteins even after adult ovariectomy. These effects may involve demethylation of the promoter of estrogen-target genes as well as histone modifications that lead to more active

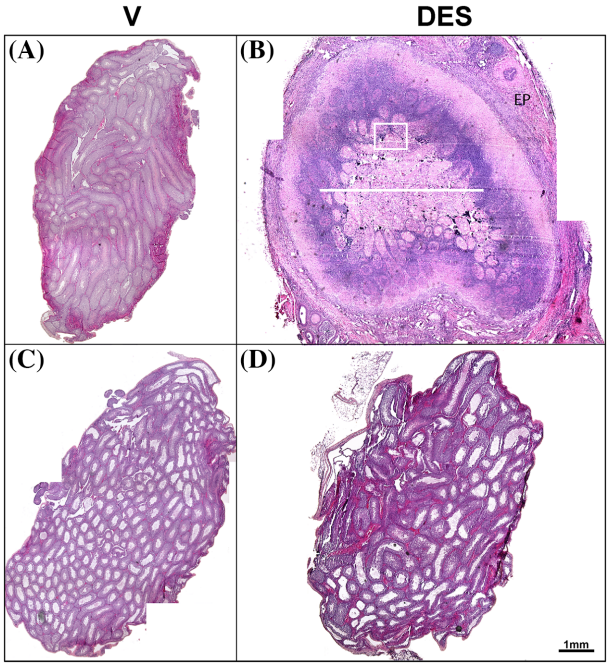


Figure 8. Histological sections of testes from adult (90-d) WT (A, B) and NOER (C, D) males that were treated from PND 1 to 5 with vehicle (A, C) or DES (B, D). Images of testes captured with a microscope and a color digital camera and then stitched together by computer to show complete cross sections. In WT and NOER vehicle-treated males (A, C), no evidence of inflammation or necrosis is evident. However, in WT DES-treated males (B), overall testis shape was changed, extensive pyogranulomatous and necrotizing inflammation was present, and the seminiferous tubules were occluded and abnormal. The necrotizing inflammation in the testes and epididymis is similar to that shown in Figure 3D in [55]. The necrotic area in this testis is indicated by a solid white line. Note that the caput epididymis (EP) is tightly adhered to the superior aspect of the testis; a white box in the testes indicates an area that was imaged at higher power to show the extensive inflammation and presence of bacteria in Figure 9. Such pathological changes were for the most part absent in DES-treated NOER males. Representative images are provided from the N = 12–22 mice/group that were examined. These same WT/HET DES mice also showed extensive fibrous adhesions of the testes, epididymis, and sometimes encompassing the ductus deferens with 52% of the WT (12/23) and 78% of the HET (14/18) males showing fibrous adhesions and these histopathological changes. In sharp contrast, only 1/16 (6%) of DES-treated NOER males ($P \leq 0.001$ vs. DES-treated WT males) showed fibrous adhesions and these above histopathological changes. Note the distended lumens in the seminiferous tubules of the V and DES-treated testes; this is not detected in WT V testis, and is indicative of changes unique to NOER mice, as we reported previously [13].

transcription [10, 17, 21, 24, 30, 39–42]. Our results show that neonatal DES treatment of WT mice induces high levels of ovary-independent expression of genes for normally estrogen-dependent proteins such as *Ezh2*, *Ltf*, *Hat1*, and *Esr2* even when the animals are ovariectomized during adulthood and subsequently treated with only V. In contrast, these effects are essentially eliminated in NOER mice lacking mESR1, again illustrating the critical role of mESR1 in neonatal effects of DES in general, and in epigenetic effects of neonatal DES in particular. Informatics analyses also revealed strong and positive association of *Ezh2* and *Hat1* expression, suggesting that neonatal DES exposure might simultaneously alter several uterine histone protein marks.

The qPCR and immunohistochemistry for EZH2 in WT DES V and NOER DES V mice were generally confirmatory. However,

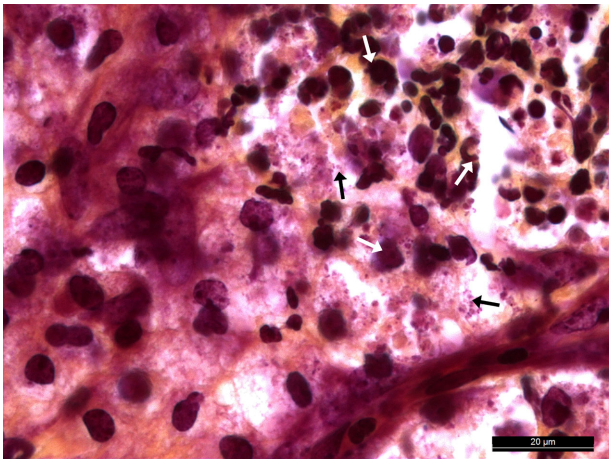


Figure 9. Gram stain of testes from DES-treated WT mouse. The parenchyma of the testes contained extensive necrotizing pyogranulomatous inflammation (white arrows denote inflammatory cells) with bacteria admixed in these areas that were predominantly Gram-positive cocci (black arrows). Similar bacterial colonies and inflammatory responses were evident in the epididymis and lumbar lymph nodes of DES-treated WT/HET males.

there were differences in relation to E2 treatment, with qPCR results suggesting that this gene was decreased in WT DES E2, but a comparable decrease in expression for the protein was not evident with immunohistochemistry. Instead, this group seemed to exhibit even greater EZH2 protein expression than those administered DES V. Previous studies suggest that the correlation between mRNA and protein expression is only ~40% [51, 52]. Moreover, in some instances xenoestrogens increase EZH2 mRNA or protein expression, whereas in other cases, these same chemicals decrease this histone modifier [31–33].

By examining the reproductive system in both males and females, it is increasingly apparent that both sexes of NOER mice are refractory to the full effects of neonatal exposure to DES, providing further evidence that DES pathogenic effects require the presence of mESR1. Consistent with previous literature showing deleterious effects of developmental DES treatment of males [26, 27, 53–55], in WT males exposed to DES neonatally, pyogranulomatous and necrotizing inflammation was evident in the testes, epididymis, and surrounding lumbar lymph nodes. A similar incidence of these lesions was seen in HET males, indicating that one copy of ESR1 is sufficient to mediate the full range of pathological DES effects. Gram staining revealed bacterial infection as the likely underlying cause of such changes in the testes and epididymides in these DES-treated groups; this has been observed the first time. Such changes were almost entirely absent in NOER males, suggesting they are resistant to these particular DES-induced pathological lesions. Lymphatic fluid from the testes and epididymis drains to the lumbar lymph nodes (Supplementary Figure 1), which is a possible route for spread of infection to these organs. Two DES-treated males also had bladder abnormalities, one with a large bladder stone and the other with cellular debris in the bladder, suggesting that the infection in the lumbar lymph nodes could also originate in the bladder or from impairments in the urinary system.

It is unclear why WT/HET mice neonatally exposed to DES develop adult bacterial pyogranulomatous lesions in the testes and epididymis. Previous studies in rodents and humans indicated that developmental DES exposure can result in epididymal cysts [15, 16, 53–58]. Miyaso et al. [55] postulated that neonatal DES exposure of

mice results first in epididymal inflammation followed by orchitis. However, the underlying cause leading to the initial inflammation is uncertain. Notably, the gross and microscopic anatomical changes reported in this previous work [55] are nearly identical to what we observed in WT mice neonatally exposed to DES. Neonatal treatment of mice with 17-cypionate β-estradiol resulted in later inflammation within the efferent ductules, epididymis, and vas deferens but not the testis, although obstructive azoospermia eventually results [59]. A study with rats suggested that neonatal treatment with 10 μg, but not 0.1 μg, of DES caused an infiltration of neutrophils (elastase positive) and CD45-positive inflammatory cells across the epididymal and vas deferens epithelial lining [60]. Multi-layering of basal cells with increased cell proliferation was coincident with these inflammatory changes. Stromal overgrowth has also been reported in epididymides of DES-treated mice [61]. Conceivably, DES or other estrogen exposure may impair the barrier function of the epithelium, resulting in chemotaxis of neutrophils, macrophages, and other inflammatory cells into these sites.

Current results show that bacteria are the likely causative agents of DES-induced inflammatory lesions in the testes and epididymis. Under normal conditions, such bacteria may be commensal residents within the male reproductive system [62], but disruption of the epithelial basement membrane permits penetration into the organ parenchyma with ensuing inflammation and tissue necrosis. Notably, present results suggest that NOER mice are partially to fully protected against these damaging effects of DES.

Neonatal DES treatment produced more pronounced decreases in testicular weights in WT than in NOER males, consistent with female and other male data showing that responses to neonatal DES treatment are sharply ameliorated in the absence of mESR1. The small increases in testicular weights in NOER mice at 3 months of age compared to WT controls are consistent with our previous observations in 4-month-old NOER mice [13], and presumably reflect the distended seminiferous tubules in NOER mice, which were apparent in this study and that we have reported previously [13]. Testicular changes in NOER mice are due to impaired efferent ductule function.

Seminal vesicle weights are increased in untreated NOER mice compared to untreated WT controls, and neonatal DES treatment produced more pronounced decreases in seminal vesicle weights in WT than NOER males. We and others have shown that seminal vesicles express high amounts of estrogen receptor during development and that deleterious effects of early DES treatment are mediated through ESR1 [45, 47, 63]. Seminal vesicle weights of *Esr1*KO mice are increased compared to WT controls [64]. This may result from a normal inhibitory effect of signaling through ESR1 on seminal vesicle growth, which, when removed, results in increased seminal vesicle weight. It likely also reflects endocrine alterations in *Esr1*KO mice. The loss of mESR1 in NOER males, and the partial reduction in ESR1 signaling, likely results in increased growth and ultimately adult weight in seminal vesicles of NOER males, although these increases are less pronounced than in *Esr1*KO mice which totally lack ESR1 signaling. The *Esr1*KO mice are completely refractory to the effects of neonatal DES administration on adult seminal vesicle weights [64], and these findings are consistent with our present observation that NOER mice lacking mESR1 are partially refractory to neonatal DES, as evidenced by these mice showing attenuated overall responses to estrogens [11, 13].

Many endocrine disrupting chemicals (EDCs) can bind and activate ESR1 and ESR2, sometimes at low concentrations. Notably, some of these compounds appear to have a greater ability to bind and activate mESR1 than nESR1 [65–68]. These data, in conjunction with the present findings that the full effects of the xenoestrogen DES require mESR1, imply that effects of EDCs mediated through mESR1 must be taken into account when evaluating the consequences of EDC exposure.

In summary, our findings emphasize the critical role of signaling through mESR1 in the overall epigenetic changes and other effects resulting from developmental DES exposure in the vagina, uterus, and male reproductive tract and provide a valuable model system for understanding effects of early DES treatment.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table 1. Antibodies utilized.
Supplementary Table 2. Primer sequences for genes tested.
Supplementary Table 3. qPCR results for those genes that did not show uterine expression differences based on genotype or DES treatment.
Supplementary Figure 1. Lymphatic fluid drainage of testes and epididymis. Lymphatic fluid from the testes and epididymis drains to the lumbar lymph node. Thus, infections beginning in the testes and epididymis will first spread to these lymphatic organs.

Acknowledgments

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