

Developmental Toxicant Exposure Is Associated with Transgenerational Adenomyosis in a Murine Model¹

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ABSTRACT

The common environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or, commonly, dioxin) is a known endocrine disruptor that has been linked to the development of endometriosis in experimental models. Using a murine model, we previously demonstrated that in utero TCDD exposure promotes the transgenerational development of an “endometriosis-like” uterine phenotype consisting of reduced responsiveness to progesterone, as well as subfertility and an increased risk of preterm birth. Because adenomyosis is frequently observed as a comorbidity in women with endometriosis, herein we sought to determine the incidence of adenomyosis in nonpregnant mice with a history of direct or indirect TCDD exposure. Using histologic assessment and immunohistochemical staining, we analyzed murine uteri for adenomyosis, microvessel density, and expression of estrogen receptors alpha and beta (ESR1 and ESR2). Our studies revealed that unexposed control mice did not exhibit adenomyosis, whereas this disease was frequently observed in mice with a history of early-life TCDD exposure. A transgenerational impact of developmental TCDD exposure was demonstrated, because a subset of mice with only an indirect exposure (F3) also exhibited adenomyosis. Microvessel density within the uterus was significantly higher in all groups of TCDD-exposed mice compared with control animals, with density correlated to the severity of disease. Both ESR1 and ESR2 proteins exhibited alterations in expression in experimental mice compared with controls. Similar to women with endometriosis, we observed a significant reduction in the ratio of *Esr1:Esr2* mRNA in all F1 mice compared with controls. Although this retrospective study was not designed to specifi-

cally address mechanisms associated with the development of adenomyosis, our data suggest that developmental TCDD exposure permanently alters adult steroid responses, which may contribute to the transgenerational development of adenomyosis.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), adenomyosis, endometriosis, transgenerational

INTRODUCTION

Commonly occurring as comorbidities, endometriosis and adenomyosis remain poorly understood gynecologic disorders affecting millions of women worldwide [1–3]. Endometriosis is characterized by the presence of endometrial glands and stroma at extrauterine sites, whereas adenomyosis is defined as the presence of endometrial glands and stroma embedded within the uterine muscle [4, 5]. Although the etiology of each disease remains uncertain, the symptomatology of both diseases is similar: reduced fertility, pelvic pain, heavy menstrual bleeding, and dysmenorrhea [5, 6]. Additionally, human and animal studies have implicated a role of inflammatory processes and epigenetic events in the development of each of these diseases [7–11], suggesting that endometriosis and adenomyosis share a common or overlapping natural history. For example, both diseases are considered to be estrogen dependent [12, 13], and, not surprisingly, exposure to environmental toxicants with endocrine-disrupting effects has been suggested to play a role in their pathogenesis [14]. Numerous endocrine-disrupting compounds have been identified, many of which can act as estrogens and are capable of using both estrogen receptor (ESR)-dependent and ESR-independent signaling pathways [15].

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is a ubiquitous environmental toxicant known to disrupt steroid synthesis and action, affecting both estrogen and progesterone signaling processes [16]. A potential role for TCDD in the pathogenesis of endometriosis was first reported by Rier et al. [17] following the development of severe, life-threatening disease in a primate colony exposed to this toxicant. However, human epidemiology studies have not consistently found a link between TCDD body burden and the presence of endometriosis [18, 19]. Because humans and other animals are known to be more susceptible to environmental exposures during development, our laboratory previously examined the impact of developmental TCDD exposure on subsequent adult reproductive function. Our initial studies revealed that in utero exposure of female mice to TCDD led to an adult uterine phenotype consistent with that of women with endometriosis. Specifically, adult female mice with a history of TCDD exposure in utero

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(F1 generation) exhibited reduced uterine progesterone receptor (PGR) protein and mRNA expression, as well as subfertility, and, when fertile, showed an increased risk of preterm birth [20, 21]. Surprisingly, the endometriosis-like uterine phenotype persisted in three subsequent generations (F2–F4) in the absence of additional toxicant exposure [20, 21]. Because endometriosis and adenomyosis may share common etiologies, herein we conducted a retrospective examination of archived uteri from control mice and mice with a direct (F1–F2) or indirect (F3) history of TCDD exposure. Prior to examining murine tissues, we examined archived uteri from women with endometriosis for the presence of adenomyosis in order to develop a histological scoring system for this disease. In addition to the histologic assessment of adenomyosis, we examined archived murine tissues with regard to microvessel density (MVD) and expression of estrogen receptors α and β proteins (ESR1/ESR2). Finally, we identified an additional cohort of frozen murine uteri from control and F1 mice and examined expression of *Esr1* and *Esr2*.

Our current understanding of the mechanisms associated with the pathogenesis of adenomyosis is limited, although several theories (most involving altered estrogen action) have been proffered [22]. Treatment options for women with this disease are limited, and hysterectomy is common [5]. Thus, understanding the potential role of estrogen-mimicking or estrogen-disrupting compounds in the pathogenesis of adenomyosis may enable the development of novel preventive and therapeutic options that can also preserve fertility.

MATERIALS AND METHODS

Acquisition of Human Samples

For the current study, we used archived, fixed samples of uteri obtained from women (N = 10) who had previously had a hysterectomy for endometriosis at Vanderbilt University Medical Center. Prior to surgical removal of any organ, all patients consented for the use of discarded tissue for medical research. Tissues were not collected specifically for this project; however, Vanderbilt's Institutional Review Board approved our use of archived human tissues for retrospective analysis.

Animals

Herein, we describe the retrospective analysis of archived tissues, culled from a number of previous studies. For all studies, virgin female and male C57BL/6 mice were purchased from Harlan Sprague-Dawley (now Envigo) and housed in Vanderbilt University Medical Center's Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals. All animals received food and water ad libitum. Animal rooms were maintained at a temperature of 22°C–24°C and a relative humidity of 40%–50% on a 12L:12D schedule. All original studies involving mice were approved by the Vanderbilt University Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act.

Chemicals

TCDD in nonane solution was obtained from Cambridge Isotope Laboratories. As a class II/III carcinogen, it is handled only by trained personnel and according to EPA guidelines, including the use of personal protection and a dedicated workspace with an externally vented hood. Within our laboratory, TCDD is kept in a locked biosafety cabinet when not in use. Our use of TCDD has been approved by Vanderbilt University's Biosafety office. All other chemicals were obtained from Sigma Chemical Co. unless otherwise noted.

In Utero TCDD Exposure

Virgin C57BL/6 females (N = 25), aged 10–12 wk, were mated with intact males of similar age. Upon observation of a vaginal plug, females were separated and denoted as Day 0.5 of pregnancy (E0.5). Mice were monitored

for weight gain and nipple prominence, which are indicative of pregnancy. Pregnant mice (F0) were exposed to TCDD (10 $\mu\text{g}/\text{kg}$) in corn oil or vehicle alone by gavage on E15.5 (when organogenesis is complete). To ensure consistency across all studies, exposure of pregnant mice is routinely performed at 1100 h local time. This in utero plus lactational exposure paradigm results in direct exposure of the fetuses (F1 mice) as well as direct exposure of the fetal germ cells, which have the potential to become the F2 generation. This dose of TCDD reflects the more rapid clearance of this toxicant in mice compared with humans and is well below the LD50 for adult mice of this strain (230 $\mu\text{g}/\text{kg}$) [23]. TCDD given at this time and dose is not overtly teratogenic, and gestation length was not affected in the F0 animals; pups (F1 mice) were typically born on E20.

Generation of F2/F3 Females

A single control, proven breeder male was placed with a single F1 female and monitored for the presence of a vaginal plug (E0.5) each morning. Following the identification of a plug, the male was removed. Females were weighed prior to mating and again on E16.5, when they were examined for signs of pregnancy (weight gain, nipple prominence). An animal was considered infertile after four positive vaginal plugs with no subsequent pregnancy, because, in our hands, control mice typically require only one or two matings to achieve pregnancy, but never more than three. Second-generation (F2) animals were weaned at 4 wk of age, at which time male pups were removed. At 10–12 wk, F2 females were mated to control males with proven fertility of similar age and were monitored as above. As previously described, like their siblings, F1 and F2 males exhibited subfertility, with $\geq 50\%$ of F1/F2 animals being completely infertile [8, 20, 24, 25]. Perhaps as a consequence of reduced fertility in directly exposed animals, to date, mating of nonsibling F1 male and female mice or nonsibling F2 male and female mice has not resulted in offspring [8]; thus, toxicant-exposed mice are necessarily mated to control partners.

Euthanasia

Adult female mice were euthanized at 6–7 mo of age by cervical dislocation after anesthetic overdose (isoflurane). Studies included mated animals (fertile and infertile) as well as virgin females of the same age. Animals were typically euthanized during the estrus phase, as indicated by microscopic examination of the vaginal smear.

Immunohistochemistry

Immunohistochemical staining of murine and human tissues was performed by standard methodology for formalin-fixed, paraffin-embedded tissues. Briefly, tissues were deparaffinized with xylene and rehydrated in serial dilutions of ethanol. After heat-activated antigen retrieval, primary antibody was applied (rabbit anti-human von Willebrand factor [vWF] at 1:3000 dilution [original concentration 200 $\mu\text{g}/\text{ml}$; DakoCytomation], rabbit anti-smooth muscle actin [anti-SMA] at 1:500 dilution [original concentration 200 $\mu\text{g}/\text{ml}$; Thermo-Scientific], or rabbit anti-human ESR1 [ESR1 catalogue no. ab32063; Abcam] and rabbit anti-human ESR2 [PA1-310B; Thermo-Scientific]; both of the last two antibodies were used at 1:150 dilution with original concentration of 1 mg/ml). Slides were incubated for 1 h at room temperature, washed in PBS and vWF, and visualized using the Dako Envision+ HRP/DAB System (DakoCytomation), followed by counterstaining with Mayer hematoxylin. Smooth muscle actin was visualized using the Bond Polymer Refine detection system (Leica Biosystems) and was counterstained with Mayer hematoxylin. ESR1/ESR2 were visualized using the Dako Envision+ HRP/DAB System (DakoCytomation) and were not counterstained. After staining, all slides were dehydrated, cleared, and coverslipped for morphological analysis. For the negative control, prior to incubating tissues with primary antibody, the ESR1 and ESR2 antibodies were neutralized by 1-h incubation with an excess of the immunizing peptide. Histopathological assessments were performed using an Olympus BX51 microscope system, and images were captured using an Olympus DP71 digital camera.

Assessment of Staining Intensity of ESR1 and ESR2

Staining intensity was assessed based on the Histologic score (H-score) method originally developed by Budwit-Novotny and colleagues [26]. For calculation of H-score, 10 fields were selected at random per group and photographed at 400 \times magnification. Staining intensity within endometrial glands and stroma was independently assessed by three investigators and scored as 0, 1, 2, or 3, corresponding to the presence of negative, weak, intermediate, or intense staining, respectively. The average of percent positive

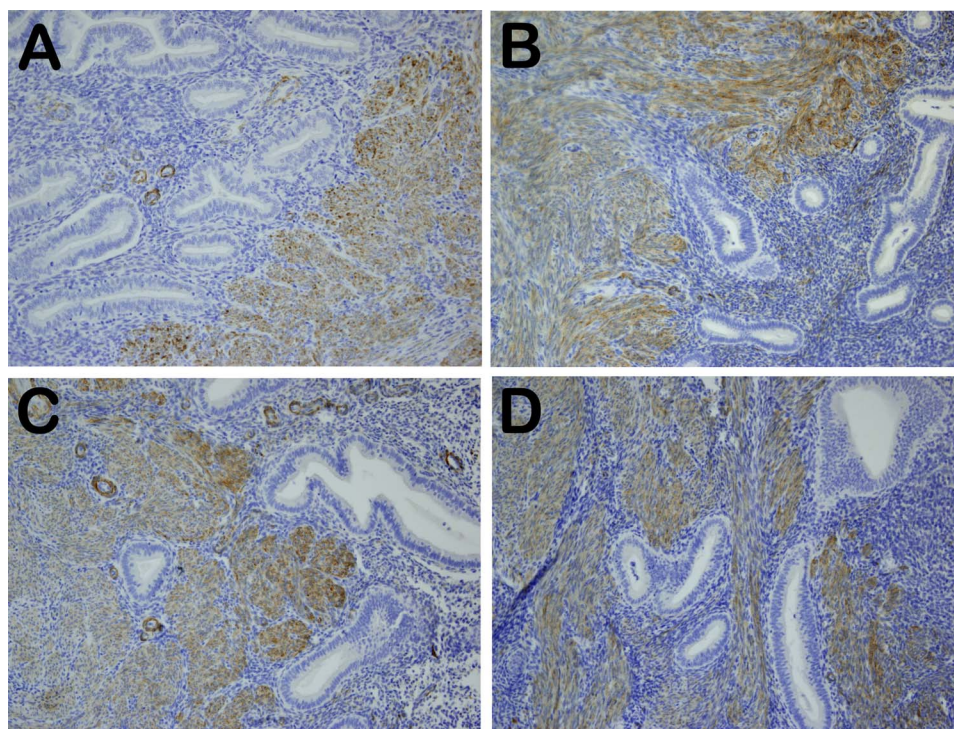


FIG. 1. Presence and histologic morphology of adenomyosis in patients with endometriosis. Formalin-fixed, paraffin-embedded tissues obtained from women with endometriosis were stained using an antibody to SMA to aid in identifying potential adenomyotic lesions. Eight of Ten endometriosis patients were determined to additionally have adenomyosis. Interestingly, in all patients with adenomyosis, we identified areas that exhibited a morphologically normal myometrial-endometrial interface (A). These histologically normal areas were assigned a score of 0. Areas in which the myometrium appeared to intrude into the endometrium, but without completely surrounding glands, were also scored as stage 0 (B), because the significance of these changes is currently unknown. Similar myometrial-endometrial interfaces that also included rare glands/stroma embedded within the muscle were given a score of stage 1 (C). Finally, large nests of endometrial glands and stroma deep within the muscle were considered stage 2 disease (D). All photomicrographs, magnification $\times 200$.

cells at each level of intensity was determined, and the H-score was calculated based on the formula:

$$\text{H-score} = (\% \text{ of cells stained at level 1 intensity} \times 1) + (\% \text{ of cells stained at level 2 intensity} \times 2) + (\% \text{ of cells stained at level 3 intensity} \times 3).$$
 An H-score of between 0 and 300 was obtained, where a score of 300 is equivalent to 100% of cells maximally stained. The interobserver variation was less than 25% across all samples.

The H-score from each observer (three observers) per field was averaged; thus, 10 numbers were plotted for each group.

Microvessel Density

Microvessels from each tissue were enumerated by standard methodology [27, 28] by three individuals blinded to sample identity. Briefly, each slide was scanned at low power (40 \times) to identify areas of dense vascularization. Of these areas, three random fields were selected per tissue and photographed at 400 \times , and vWF-positive vessels were counted. For consistency between observers, a vessel was counted only if a lumen could be identified, whereas staining without a visible lumen was not counted. Although this approach likely results in some vessels being counted more than once per field (due to a vessel weaving in and out of the observation plane), this method limits observer variability. Following these guidelines, the interobserver variation was less than 10% across all samples. The MVD number obtained from each observer (three observers) per field was averaged; thus, three numbers were plotted for individual animals (see Fig. 3E). Methods used for statistical analysis are described below.

Development of a Staging System for Adenomyosis

Prior to examining uteri from control and toxicant-exposed mice, we identified archived specimens of full-thickness uteri, obtained from women who had undergone hysterectomy for endometriosis. To enhance identification of adenomyosis, muscle cells were stained with SMA. Consistent with previous reports, adenomyosis was frequently found in patients known to have endometriosis [4, 5] (8 of 10 samples examined; data not shown). Staining

for SMA (Fig. 1) revealed that in addition to the presence of deep adenomyosis, uterine samples from these patients exhibited areas of varying histologic phenotypes, which we have scored as: stage 0, smooth myometrial-endometrial interface (A) or areas exhibiting a disordered interface, but without endometrial glands/stroma embedded within the myometrium (B); stage 1, transitional adenomyosis with occasional glands/stroma embedded in muscle (C); and stage 2, deep/complex adenomyosis (D). This scoring system was used to assess the presence, absence, or degree of adenomyosis within our murine samples.

RT-PCR Analysis of *Esr1*/*Esr2*

Samples of flash-frozen, whole uterine tissues from control (n = 8) and F(1) (n = 10) mice were identified from previous studies. Total RNA was extracted from approximately 10 mg of uterine tissues lysed in TRI-ZOL reagent (Invitrogen) using Direct-zol RNA MiniPrep (Zymo Research), following the manufacturer's instructions. A total of 1 μ g of RNA was used to generate cDNA with High-Capacity cDNA Reverse Transcription kits (Applied Biosystems). Quantitative PCR reactions were performed in duplicate with PowerUp SYBR Green Master Mix (Applied Biosystems) in an Applied 7300 Real-Time PCR System (Applied Biosystems). Each reaction was repeated in duplicates. Sequences of primer pairs *Esr1*, *Esr2*, and 18S rRNA (18S) were obtained from National Institutes of Health quantitative PCR primer depot (<https://mouseprimerdepot.nci.nih.gov>). The SYBR Green assay was used for *Esr1*, *Esr2*, and 18S. 18S values were used for normalization. Primers were as follows: *Esr1*: forward, 5'-TCATGGTCATGGTAAGTGGC-3'; reverse, 5'-CCCTCCTCTGCCATTGTCTA-3'; *Esr2*: forward, 5'-TACACTGATTCGTGGCTGGA-3'; reverse, 5'-TTACGGTGTCTGGTCCTGTG-3'; and 18S: forward, 5'-CGCGTTCTATTTTGTGGT-3'; reverse, 5'-AGTCGGCATCGTTATATGGTC-3'.

Statistical Analysis

Statistical analysis was performed using JMP 11 (SAS) software. For MVD, H-scores, and ratios of *Esr1*:*Esr2*, the statistical difference between groups was determined by nonparametric testing for nonnormally distributed

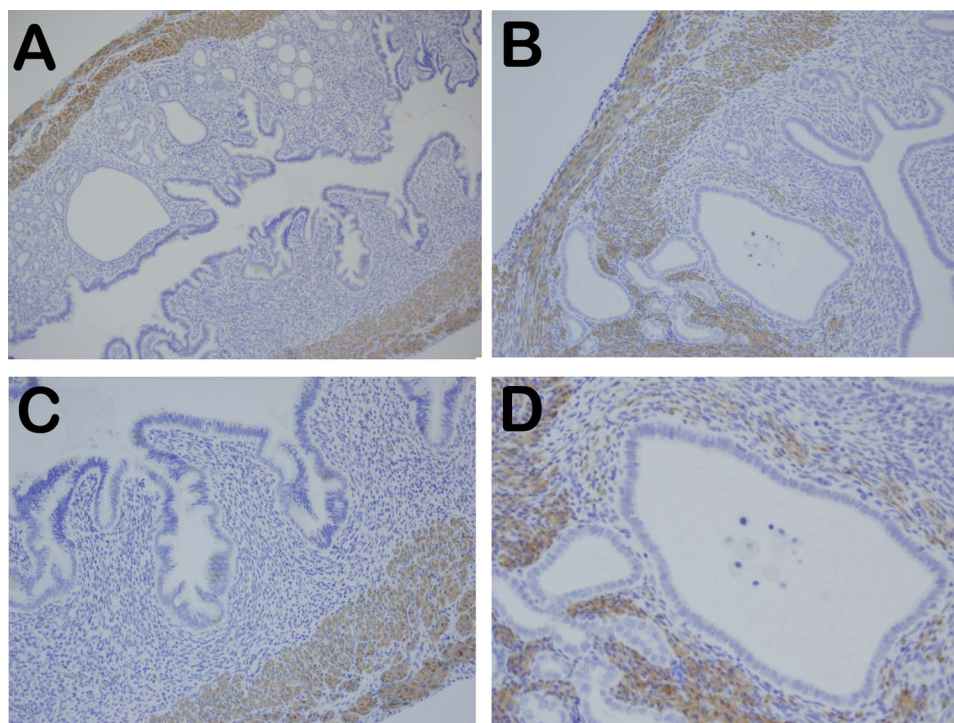


FIG. 2. Presence and histologic morphology of adenomyosis in mice with a direct (F1) history of TCDD exposure. Muscle cells within formalin-fixed, paraffin-embedded tissues obtained from control and F1 animals were identified using an antibody against SMA. Unexposed control animals exhibited primarily a smooth myometrial-endometrial interface (A and C). Adult offspring (F1) of mice exposed to TCDD during pregnancy also exhibited areas of morphologically normal myometrial-endometrial interfaces, but large nests of endometrial glands and stroma embedded within the muscle were frequently observed (B). Higher magnification of the image shown in B reveals the presence of muscle cells beginning to surround a large gland within the endometrium (D). Original magnifications $\times 100$ (A and B) and $\times 200$ (C and D).

data using Kruskal-Wallis test followed by pairwise comparisons with controls using the Steel method. Analysis of stages 1 and 2 adenomyosis was determined by Fisher exact test. $P < 0.05$ was considered significant.

RESULTS

Adenomyosis in Mice with the Endometriosis-like Uterine Phenotype

Our previous studies have identified multiple aberrations in the uterine phenotype of mice with a direct (F1–F2) or indirect (F3) history of developmental TCDD exposure, many of which have also been noted in women with endometriosis [20, 21]. Herein, we conducted a retrospective analysis of control females and three generations of our mice with a history of direct or indirect TCDD exposure. Uteri from all groups of mice were subjected to SMA staining and were assessed microscopically. As in women with endometriosis, we identified adenomyotic lesions in most of our animals with a history of direct (F1–F2) or indirect (F3) TCDD exposure. However, although 70% ($n = 10$) of F1 animals exhibited deep adenomyosis, the incidence of advanced disease was slightly lower in F2 mice (63%; $n = 11$) and F3 animals (56%; $n = 9$). Although 60% of control mice exhibited areas of disordered endometrial-myometrial interface and muscle cell bundling in addition to areas of smooth endometrial-myometrial interfaces (stage 0, no disease), none of the control animals were found to have endometrial cell nests embedded within the muscle. In contrast, most of the F1–F3 mice exhibited both stage 1 and stage 2 disease, although areas of normal endometrial-myometrial interfaces were also frequently identified in these same animals. The frequency and degree of adenomyosis in mice were scored based on the system we developed following

the assessment of the human tissues (Fig. 1) and are summarized in Table 1. Representative photomicrographs of SMA staining in control and F1 mice are shown in Figure 2.

Immunohistologic Assessment of MVD in TCDD-Associated Murine Adenomyosis

Schindl et al. [29] reported an increase in MVD in adenomyosis tissues compared with endometrium from the same patient as well as compared with the myometrium and endometrium of disease-free women. Similarly, Li et al. [10, 30] reported an increase in MVD within the myometrium of mice with tamoxifen-induced adenomyosis compared with control animals. Herein, we subjected uterine sections to immunohistochemical staining with vWF, which binds normal and neoplastic endothelial cells. As shown in Figure 3, MVD assessment of the endometrial-myometrial junction of control mice (A) revealed significantly ($P < 0.0001$) fewer vessels compared with mice with any history of TCDD exposure (B–D). The MVD for each group is graphically represented in E.

Presence of Muscle Cells Within the Endometrium

To distinguish between muscle cells surrounding vessels and myometrial cells otherwise present within the endometrium, we examined sister sections from all groups of animals stained with both SMA and vWF. Although myometrial cells were not found in the endometrium of control mice, myometrial cells could be identified within the endometrium of 100% of animals with a direct exposure to TCDD (F1–F2) and in 75% of mice with an indirect exposure (F3 animals). Representative images from an F1 animal are shown in Figure 4.

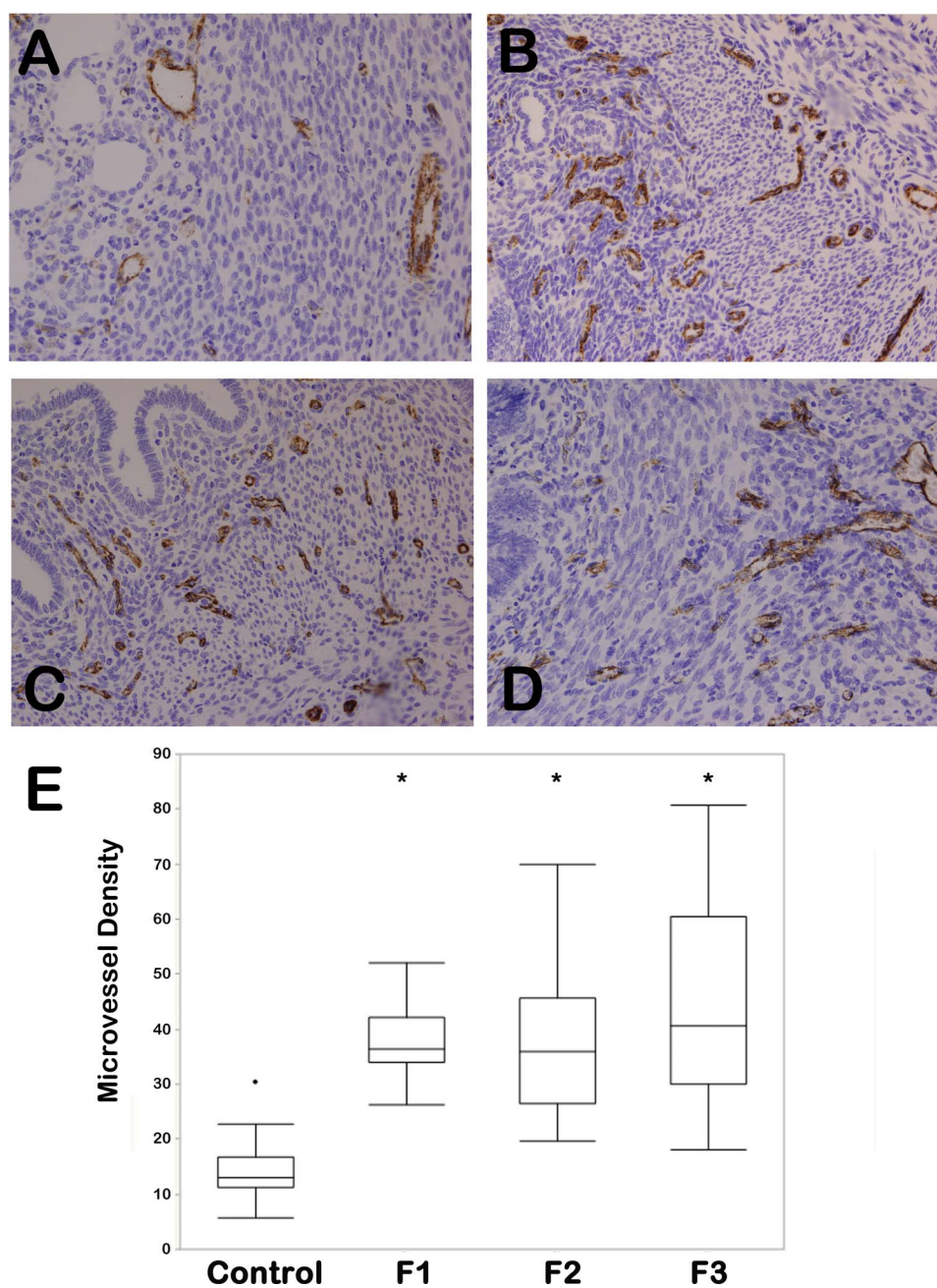


FIG. 3. Assessment of MVD via immunohistochemical localization of vWF. Endothelial cells within formalin-fixed, paraffin-embedded tissues obtained from control and all generations of experimental animals were identified using an antibody against vWF. As expected within these highly vascular tissues, unexposed control animals exhibited numerous small vessels at the myometrial-endothelial interface (A). Assessment of MVD in uterine tissues of F1 mice revealed an increased number of vessels (B). Similarly, F2 tissues (C) and F3 tissues (D) also revealed a greater number of vessels compared with controls. Original magnification $\times 400$. E) Box-and-whisker plot of MVD for each animal within each treatment group. Center lines indicate the median of each group. Columns and vertical bars indicate the 25th–75th percentiles and 10th–90th percentiles, respectively. *Statistically significant difference compared with control animals ($P < 0.05$).

Immunolocalization of ESR1/ESR2 in Mice with and Without Toxicant Exposure

Several studies have suggested a role for exposure to estrogenic compounds and the subsequent development of adenomyosis in mice [31, 32] and in humans [14, 33]. Interestingly, altered expression of ESR1 and ESR2 has been associated with adenomyosis in women [14, 34], whereas polymorphisms in these genes appear to influence fertility [35, 36]. Therefore, we examined the adult uterine expression of estrogen receptor α (ESR1) and estrogen receptor β (ESR2) in

TABLE 1. Percent of mice with histologic changes associated with adenomyosis.^a

Mouse lineage	n	Adenomyosis stage, %			<i>P</i> compared with control		
		0	1	2	0	1	2
Control	9	100	0	0			
F1	10		100	70	0.001		0.005
F2	11		73	63	0.005		0.003
F3	9		67	56	0.01		0.03

^a Most animals exhibited more than one phenotype across the uterus.

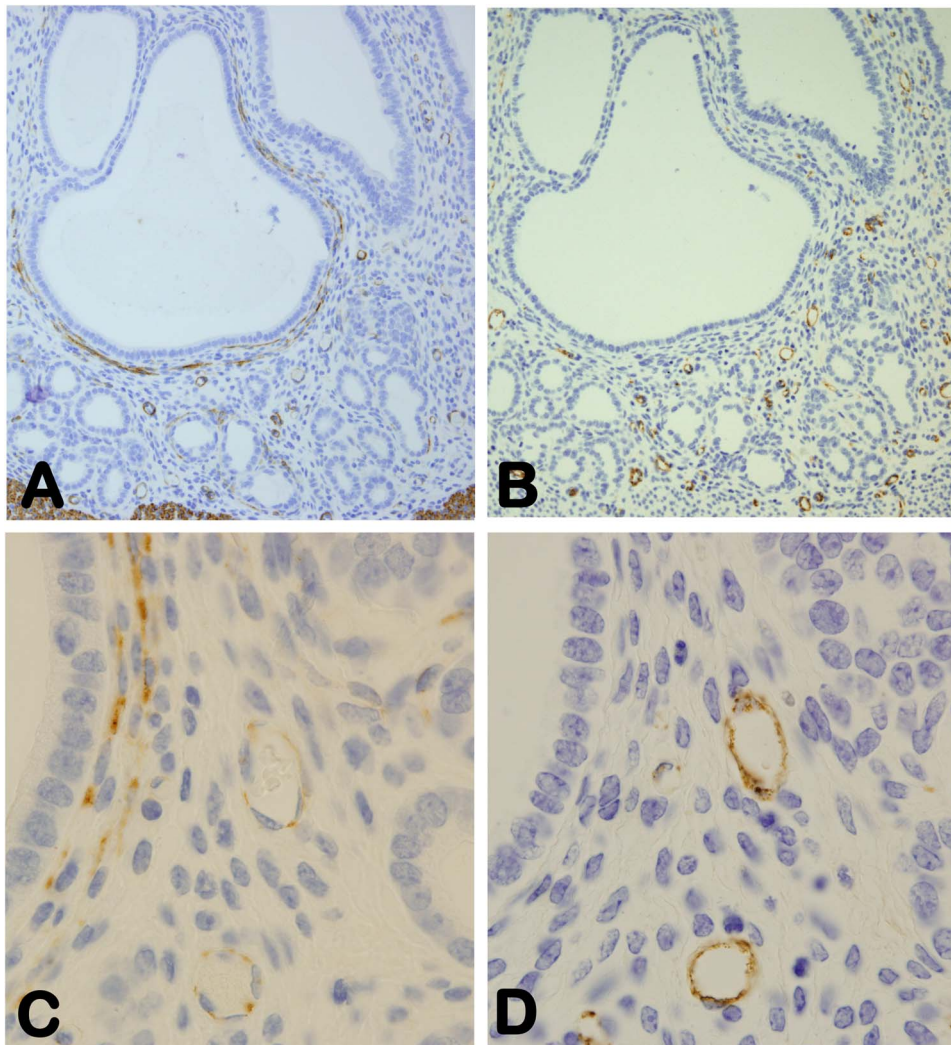


FIG. 4. Presence of muscle cells within the endometrium of an F(1) animal. To distinguish between muscle cells surrounding vessels and myometrial cells otherwise present within the endometrium, we stained sister sections with SMA (A and C) and vWF (B and D). The large gland in A exhibits a thin layer of SMA-stained muscle cells surrounding it, but these cells do not exhibit colocalization of vWF, suggesting these cells are not associated with vessels. Original magnifications $\times 400$ (A and B) and $\times 1000$ (C and D).

control mice and all generations of toxicant-exposed mice (F1–F3). As shown in Figure 5, we observed strong expression of ESR1 protein in all control samples, whereas expression of this protein varied widely among F1–F3 mice. Interestingly, among mice with a direct or indirect toxicant exposure immunostaining of ESR1, this protein was found to be lowest in mice known to be infertile (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org). With regard to ESR2 expression (Fig. 6), immunostaining for this protein was low or absent in both epithelium and stroma of control uteri. Within the F1–F3 uteri, staining was typically stronger, but it was highly variable and with no clear association to fertility status. Notably, although ESR2 staining was localized to the nucleus of granulosa cells within the ovaries of all mice (Supplemental Fig. S2) and in the control uteri (Fig. 6A), this protein exhibited largely cytoplasmic staining in the uterine glands of toxicant-exposed mice (Fig. 6B). Stromal cells exhibited both nuclear and cytoplasmic staining (Fig. 6).

RT-PCR of *Esr-1/Esr-2*

To further evaluate the potential toxicant influence on uterine estrogen receptor expression in F1 mice compared with

controls, we identified archived, frozen whole uterine samples from both groups. Tissues were subjected to RNA extraction and quantitative RT-PCR. Expression of both *Esr1* and *Esr2* was highly variable in F1 animals, with overall expression of both genes increased in these mice compared with control animals ($Esr1 = 192.9 \pm 44.0$, $P = 0.05$; $Esr2 = 628.5 \pm 158.4$, $P = 0.001$; expressed as a percent of control). As shown in Figure 7, compared with control ($n = 8$) mice, we identified a significant decrease in the *Esr1:Esr2* ratio in F1 animals ($n = 10$; control = 205.6 ± 55.2 ; F1 = 114.2 ± 46.9 ; $P = 0.0012$), consistent with the increased levels of ESR2 protein expression noted above.

DISCUSSION

Adenomyosis is frequently observed in women with endometriosis [4, 5], and, perhaps not surprisingly, these conditions have overlapping symptomology and reproductive consequences [22]. These diseases are known to have a familial component, and several genetic polymorphisms have been associated with both endometriosis and adenomyosis [37]. Although the precise etiology of adenomyosis is unknown, this potentially invasive disease, like endometriosis, is estrogen

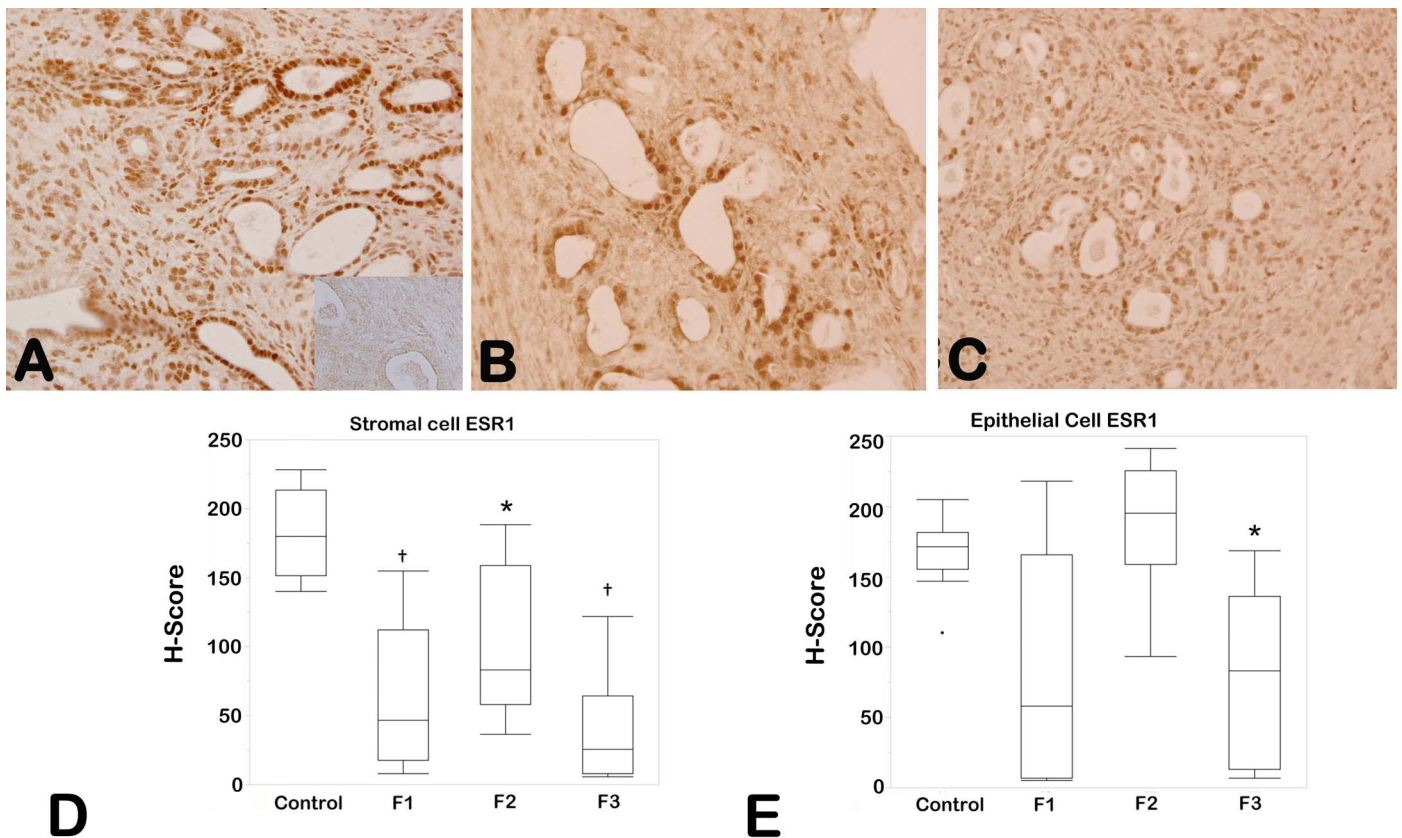


FIG. 5. Immunolocalization of uterine ESR1 in control and toxicant-exposed mice. Control mice exhibited robust expression of ESR1 (A), which was blocked by addition of the immune antigen (inset). Expression of ESR1 in F1 (B) and F3 (C) mice varied widely among animals; however, semiquantitative assessment of staining, determined by H-score, revealed a significant reduction in all generations of experimental mice (D and E) and is presented as a box-and-whisker plot of ESR1 for each animal within each treatment group. Center lines indicate the median of each group. Columns and vertical bars indicate the 25th–75th percentiles and 10th–90th percentiles, respectively (* $P < 0.05$, † $P < 0.01$). Original magnification $\times 200$.

dependent and frequently resolves after menopause [12, 13]. Consequently, to advance our understanding of early events associated with the development of adenomyosis, several investigators have developed animal models of this disease using estrogenic compounds, such as tamoxifen [32, 38, 39] and toremifene [32]. Of note, following neonatal exposure of CD-1 mice to tamoxifen, Parrott et al. [32] concluded that alteration and disorganization of the myometrial layer likely preceded, and perhaps facilitated, endometrial tissue invasion into the muscle. Additionally, in response to tamoxifen, CD-1 mice have been found to develop early-onset adenomyosis (by 6 wk of age) [24]. In this same study, although adenomyosis was not observed at this early age in tamoxifen-treated C57BL/6 mice, which do not develop spontaneous disease, disruptions of the myometrial-endometrial interface were observed [39]. It is tempting to speculate that if these animals had been examined at a later time, after numerous estrus cycles, deep adenomyotic lesions may have developed as a consequence of the disordered endometrial-myometrial interface coupled with cycles of estrogen exposure. These published studies are consistent with our current findings, which also suggest that changes to the myometrium may be an early event in disease pathogenesis associated with TCDD exposure. Regarding disease development in women, current clinical perspectives consider that establishment of adenomyotic lesions is a consequence of epithelial cell migration and invasion into the myometrium [40]. Clearly, additional studies will be necessary to precisely determine the cellular mechanisms that lead to adenomyosis in mice with different genetic backgrounds and

whether these murine models are relevant to disease development in women.

To our knowledge, only one previous study has experimentally examined the potential role of TCDD exposure in the development of adenomyosis. In that study, young adult female goats were treated with a short course (three exposures over 6 days) of a relatively high dose of TCDD and euthanized 16 days later. The uteri of exposed animals exhibited cystic glandular hyperplasia and adenomyosis, leading the authors to conclude TCDD was inducing estrogenic effects on this tissue [41]. In an epidemiologic study, Heilier and colleagues [42] assessed the adult body burden of TCDD and dioxin-like compounds in healthy women compared with women with peritoneal or deep (adenomyotic) endometriosis. Their examination revealed a significant increased risk of either disease in association with the highest body burden of toxicants. Our current murine study demonstrates that direct in utero TCDD exposure was associated with the adult development of adenomyosis, but it was also associated with an increased risk of disease in subsequent generations. Additionally, it is interesting to note that although it was not the intent of this retrospective, morphological analysis to examine the relationship between adenomyosis and infertility, we did observe an apparent relationship because most animals were of known fertility status. Specifically, segregating animals by fertility status and combining all generations of mice (F1–F3), we identified deep adenomyosis in 3 of 12 animals (25%) with a history of successful pregnancy. In contrast, 100% of infertile mice ($N = 10$, all generations combined) were found to have

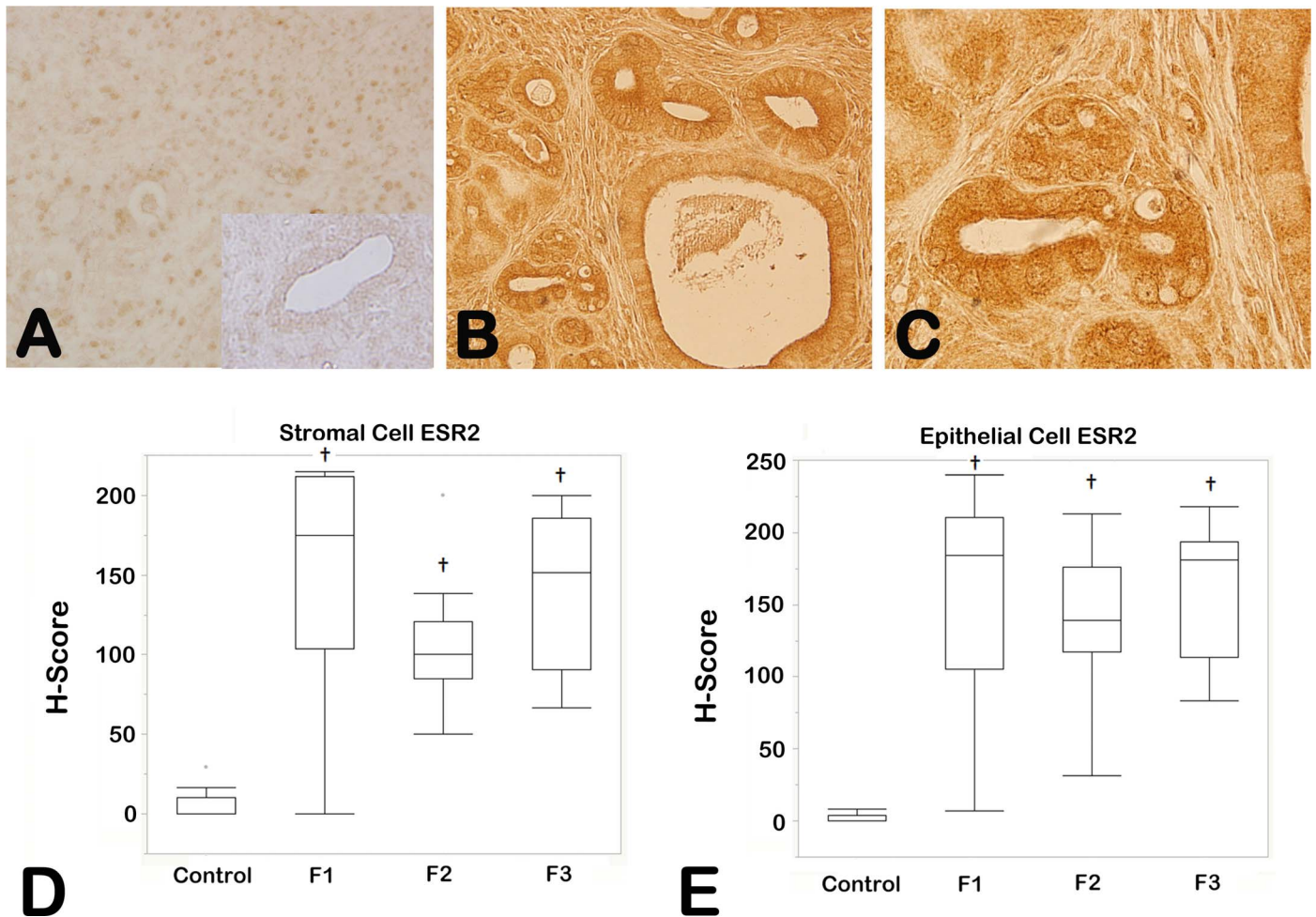


FIG. 6. Immunolocalization of uterine ESR2 in control and toxicant-exposed mice. Control mice exhibit minimal expression of ESR2, which always immunolocalizes to the nucleus of both epithelial and stromal cells (A). Coincubation of the primary antibody with the immune antigen neutralized staining (inset). Mice with a direct (F1; B) or indirect (F3; C) TCDD exposure exhibit variable but generally robust staining, with ESR2 localizing the both the nucleus and cytoplasm of stromal cells but primarily limited to cytoplasmic expression within the epithelium. Semiquantitative assessment of staining was determined by H-score (D and E; † $P < 0.01$). ESR2 expression is presented as a box-and-whisker plot for each animal within each treatment group. Center lines indicate the median of each group. Columns and vertical bars indicate the 25th–75th percentiles and 10th–90th percentiles, respectively. Original magnification $\times 400$.

deep adenomyotic lesions. These data suggest a potential role for adenomyosis in the infertility previously observed in our toxicant-exposed animals [20] and are reminiscent of the reduced fertility observed in women with this disease [5, 22].

As we have previously reported, mice with a history of TCDD exposure exhibit both diminished uterine PGR and reduced fertility [20, 21, 25]. Because estrogen response is also known to be critical for successful mammalian reproduction (reviewed by Adams and DeMayo [43]), herein we examined uterine expression of ESR1 in mice with and without TCDD exposure. We also assessed uterine expression of ESR2, which is normally minimally expressed in both mice and humans but has been found to be overexpressed in the endometrium of women with endometriosis as well as in experimental models of this disease [34, 44, 45]. Consistent with previous reports, our study revealed robust ESR1 expression in control uteri; however, across all generations of experimental mice, expression of this protein varied widely, with expression in some mice to be virtually absent. Also in agreement with published studies, ESR2 expression was minimal in control mice, with expression limited to the nucleus. Interestingly, not only was expression of this protein much greater in toxicant-exposed

mice, but the cellular distribution pattern was also altered. Specifically, within F1–F3 uteri, immunohistochemical staining for ESR2 was observed in the epithelial cytoplasm and in both the nucleus and cytoplasm of stromal cells. This change in ESR2 distribution may reflect expression of different ESR2 protein subtypes and/or be a consequence of the pathologic process. Specifically, at least five subtypes of ESR2 have been reported [46], which can be expressed in either the cytoplasm or nucleus, depending on the tissue type. For example, multiple human studies have described cytoplasmic localization of ESR2 within breast and other cancers, and such localization has been correlated with a poor clinical prognosis [46–48]. However, determining the potential significance of cytoplasmic ESR2 in the uterus of our toxicant-exposed mice will require additional studies.

Finally, the persistence of adenomyosis in the F3 generation, the first without direct toxicant exposure, strongly implicates epigenetic processes associated with the development of this disease. Indeed, several studies have demonstrated altered epigenetic marks as well as altered expression of epigenetic regulatory enzymes in women with adenomyosis compared with healthy women [9, 11, 38, 49]. Although in our

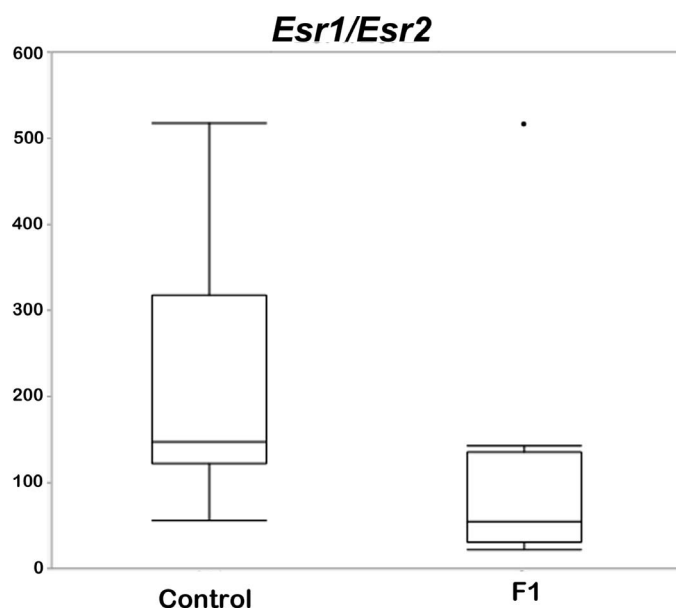


FIG. 7. Reverse transcription PCR of *Esr1/Esr2* in whole uteri. RNA from whole uteri was extracted and quantitative RT-PCR was performed to evaluate *Esr1* and *Esr2* expression (normalized to 18S). Compared with control animals, the *Esr1:Esr2* ratio of F1 mice was significantly ($P = 0.0012$) lower, with enhanced expression of *Esr2* in these animals.

retrospective study we did not examine the epigenetic status of any gene, we have previously identified hypermethylation of the *Pgr* in association with development of the endometriosis-like uterine phenotype in mice exposed to TCDD either in utero (F1–F2) or ancestrally (F3) [8]. Loss of progesterone action in these mice was associated with a hyperinflammatory uterine and peritoneal microenvironment [7]. Herein, we identified significant changes in *Esr1/Esr2* gene expression in toxicant-exposed F1 mice. Like *Pgr*, the estrogen receptors have also been found to be susceptible to epigenetic modification [50, 51]. Relevant to the current study, Bulun and colleagues [44, 52] have presented evidence that loss of *ESR2* hypermethylation promotes both the progesterone resistance and inflammatory phenotype associated with endometriosis. Not surprisingly, inflammatory processes, in addition to estrogen action, have been proposed to play a role in the development of adenomyosis [53]. Therefore, an estrogen-dominant, hyperinflammatory environment associated with TCDD-mediated loss of progesterone action may represent an interactive mechanism for development of adenomyosis in our model. Our hypothesis is consistent with a recent human study by Mehaseb and colleagues [34] that demonstrated that enhanced *ESR2* expression coupled with a reduced progesterone responsiveness correlated with the presence of adenomyosis. Nevertheless, additional studies are clearly needed to fully identify mechanisms associated with potential environmental exposures that may be associated with the development of this disease.

Our data, presented herein, add to the growing list of endocrine-disrupting compounds that have been associated with the presence of adenomyosis in mice (DES, BPA, tamoxifen). Whether similar exposures in women can also promote the development of adenomyosis is currently unknown, but this has been suggested by at least one epidemiology study [42], described above. We believe that our model, which exhibits both an endometriosis-like uterine phenotype and adenomyotic lesions, may be a useful model in which to examine the early events associated with disease

pathogenesis and may be useful as a preclinical model for identifying new therapeutic strategies.

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