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Reproductive Toxicology

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Abstract: Humans and other animals are exposed to a wide array of man-made toxicants, many of which act as endocrine disruptors that exhibit differential effects across the lifespan. Although adult exposure effects in humans are known for some compounds, the impact of developmental exposure to endocrine disrupting chemicals (EDCs) is more difficult to ascertain. Animal studies have revealed that exposure to EDCs prior to puberty can lead to adult reproductive disease and dysfunction. Specifically, in adult female mice with an early life exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), we demonstrated a transgenerational occurrence of several reproductive diseases that have been linked to endometriosis in women. Herein, we review the evidence for TCDD-associated development of adult reproductive disease as well as known epigenetic alterations associated with TCDD and/or endometriosis. We will also introduce new "Organ-on-Chip" models which, combined with our established murine model, are expected to further enhance our ability to examine alterations in gene-environment interactions that lead to heritable disease.

Abstract

Humans and other animals are exposed to a wide array of man-made toxicants, many of which act as endocrine disruptors that exhibit differential effects across the lifespan. Although adult exposure effects in humans are known for some compounds, the impact of developmental exposure to endocrine disrupting chemicals (EDCs) is more difficult to ascertain. Animal studies have revealed that exposure to EDCs prior to puberty can lead to adult reproductive disease and dysfunction. Specifically, in adult female mice with an early life exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), we demonstrated a transgenerational occurrence of several reproductive diseases that have been linked to endometriosis in women. Herein, we review the evidence for TCDD-associated development of adult reproductive disease as well as known epigenetic alterations associated with TCDD and/or endometriosis. We will also introduce new “Organ-on-Chip” models which, combined with our established murine model, are expected to further enhance our ability to examine alterations in gene-environment interactions that lead to heritable disease.

Key Words: TCDD, transgenerational, endometriosis, adenomyosis, preterm birth, epigenetics

March 29, 2016

Jerry Heindel, PhD
Thad Schug, PhD
Reproductive Toxicology, guest editors

Dear Jerry and Thad,

Thank you for inviting us to contribute an article to the special DOHaD edition of Reproductive Toxicology. Our submission, "Exposure to Environmental Endocrine Disrupting Chemicals and Human Reproductive Dysfunction: Translating Lessons from Murine Models" is enclosed and we look forward to the review process.

Best wishes,



Kaylon L. Bruner-Tran, PhD
On behalf of all authors.

Exposure to Environmental Endocrine Disrupting Chemicals and Human Reproductive Dysfunction: Translating Lessons from Murine Models

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Abstract

Humans and other animals are exposed to a wide array of man-made toxicants, many of which act as endocrine disruptors that exhibit differential effects across the lifespan. Although adult exposure effects in humans are known for some compounds, the impact of developmental exposure to endocrine disrupting chemicals (EDCs) is more difficult to ascertain. Animal studies have revealed that exposure to EDCs prior to puberty can lead to adult reproductive disease and dysfunction. Specifically, in adult female mice with an early life exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), we demonstrated a transgenerational occurrence of several reproductive diseases that have been linked to endometriosis in women. Herein, we review the evidence for TCDD-associated development of adult reproductive disease as well as known epigenetic alterations associated with TCDD and/or endometriosis. We will also introduce new “Organ-on-Chip” models which, combined with our established murine model, are expected to further enhance our ability to examine alterations in gene-environment interactions that lead to heritable disease.

Key Words: TCDD, transgenerational, endometriosis, adenomyosis, preterm birth, epigenetics

1. Introduction

Endometriosis, the presence of endometrial glands and stroma growing outside the uterus, is a common and debilitating gynecologic disease of uncertain etiology [1]. Women with this disease frequently suffer from chronic pelvic pain, subfertility and, as recently noted, an enhanced risk of spontaneous preterm birth (sPTB) [1, 2]. Common co-morbidities among these patients include adenomyosis, adhesive disease and inflammatory diseases such as interstitial cystitis and inflammatory bowel disease [3-5]. However, understanding the natural history of endometriosis, as well as the myriad of equally poorly understood diseases that often accompany it, has proven to be elusive. Multiple theories for development of endometriosis have been proffered, including ectopic implantation of retrograde menstruation, coelomic metaplasia and activation of embryonic cell rests (reviewed in [6]). Endometriosis is also known to have a familial component and several genetic polymorphisms have been associated with occurrence of this disease [1, 7], but as yet, no gene has been definitively linked to the development of this complex disease [8].

Following the occurrence of severe, life-threatening endometriosis in a colony of rhesus monkeys previously exposed to the common, widespread environmental toxicant and endocrine disruptor TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) [9], numerous human and animal studies examining the relationship between this endocrine disrupting chemical (EDC) and endometriosis ensued. Although animal studies [10-14] and in vitro analysis of human tissues [15, 16] supported a potential role of this toxicant in the development of endometriosis, several epidemiology studies failed to link adult body burden to the presence of disease (reviewed by [17-19]). However, a growing body of evidence suggests that numerous adult diseases may have their origin within the environment of early pregnancy (reviewed by [20]). TCDD and other dioxins readily cross the placenta; thus, fetal exposure to these EDCs is likely the norm rather than the exception [21]. For these reasons, our research group has explored the potential role of in utero TCDD exposure on

the subsequent development of adult reproductive tract disease, with a specific focus on the uterine phenotype(s) associated with endometriosis.

Unlike humans and other primates, mice do not menstruate and thus, do not naturally develop ectopic lesions which are the pathological hallmark of endometriosis. Nevertheless, our studies revealed that the eutopic uterine phenotype of mice with a developmental exposure history was markedly similar to that of women with endometriosis. Specifically, TCDD exposed mice, like women with endometriosis, exhibit reduced endometrial progesterone receptor (PR) expression as well as altered expression of transforming growth factor- β 2 [22], proteins which are critical for successful establishment and maintenance of pregnancy. Additional studies utilizing this murine model, detailed below, indicated that reproductive tract disease associated with TCDD exposure is not limited to the endometriosis phenotype nor to only the first generation of offspring following maternal exposure. Furthermore, our studies and those from other groups provide evidence that reproductive tract disease and dysfunction may be a consequence of epigenetic alterations induced by a previous exposure to bioactive chemical contaminants [10, 12, 23-26]. Significantly, experimental animal studies indicate that epigenetic alterations occurring in either the male or female germline are capable of negatively affecting reproductive health for multiple generations [27-29] and underscore the urgent need to better understand the pathogenic mechanisms and functional impact of toxicant-mediated cellular changes in humans so that appropriate therapeutic intervention and/or lifestyle-related preventive strategies can be developed.

Herein, we will review our studies in mice with a history of developmental TCDD exposure, detailing the observed adult reproductive effects that may be translatable to humans. We will also review relevant in vivo observations in women as well as in vitro cell studies, which link TCDD and related EDCs to adult reproductive dysfunction, with a specific focus on the role that inflammation plays in the pathogenesis of endometriosis and related disorders.

2. Environmental Endocrine Disruptors

Thousands of chemicals are released into our environment each year, but current regulations do not require prospective risk assessment of these multiple, potentially interactive chemicals on human health [30]. Nevertheless, it has been demonstrated that some of these chemicals can act as disruptors of endocrine and immune system development, potentially compromising not only adult reproductive health but also promoting cancer, obesity and metabolic syndrome [20, 31-34]. The endometrium, which undergoes cyclical patterns of endocrine and immune signaling that regulate its growth and function during the reproductive years, is particularly sensitive to these endocrine disrupting chemicals (EDCs). In order to begin to assess the impact of early life EDC exposure on this tissue, we have utilized TCDD as a prototypical endocrine/immune disrupting toxicant for our studies due to the well-established toxicity and high affinity binding characteristics of this compound to the aryl hydrocarbon receptor (AhR). The AhR, an orphan nuclear receptor, is abundantly expressed within human and murine reproductive tissues [35, 36]. Significantly, male and female Ahr knockout mice exhibit reduced fertility and pregnancy loss, implicating the presence of endogenous ligands for this receptor in normal reproductive function [37]. Indeed, the AhR gene is highly conserved across all mammalian species examined and is considered to be important for normal embryonic development [38]. Although the primary physiologic ligand remains currently unclear, a number of endogenous compounds (indirubin, bilirubin, lipoxin A4, prostaglandin G and several dietary carotenoids) have been found to bind the AhR and act as weak agonists [39-42] or antagonists (7-ketocholesterol, resveratrol and chrysoeriol) [43-45]. Exogenous AhR agonists of environmental concern include TCDD and benz(a)pyrene, products of industrial processes and cigarette smoke which are known disruptors of male and female reproductive function in multiple species [46-48]. Furthermore, numerous experimental studies reveal sex-specific effects of developmental TCDD exposure with regard to

adult behavior, metabolism and endocrine action [49-52], which may be related to variations in AhR expression patterns [49]. Thus, a person's age, diet, smoking status and gender can each influence the impact of exposure to a toxicant such as TCDD, further complicating human epidemiology studies related to reproductive health.

2.1. TCDD and related compounds

TCDD belongs to the dioxin/dioxin-like family of environmental contaminants which includes polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), co-planar polychlorinated biphenyls (PCBs) and mono-ortho-substituted PCBs [2, 3]. Among the numerous environmental toxicants which comprise the dioxin/dioxin-like family, TCDD is considered the most toxic and has been shown to disrupt steroid receptor levels as well as steroid metabolism and transport [36, 53-55]. The known biological effects of TCDD are numerous, but the consequences of chronic low-dose exposure to this toxicant on mechanistic pathways within the reproductive system remain poorly understood. The cellular effects of TCDD exposure are primarily mediated through high affinity binding to the AhR, which subsequently forms an activated heterodimeric complex with the structurally related Aryl Hydrocarbon Nuclear Transport protein (ARNT)[56]. The activated AhR-ARNT complex binds to specific DNA enhancer sequences known as dioxin response elements (DREs) which subsequently affects the expression of specific genes, including multiple steroid receptors. TCDD also stimulates expression of CYP1A1, a drug metabolizing liver enzyme, but TCDD is a chemically stable toxicant that is not readily metabolized by biological pathways in most species [57, 58].

TCDD is introduced to our environment largely as an unwanted by-product of manufacturing processes, incineration and burning of fossil fuels, although volcanic eruptions and forest fires also contribute to the overall environmental burden of dioxins. Among human and

animal populations, ingestion of contaminated food is the primary source of dioxin exposure [59-62]. Unfortunately, as noted above, these compounds are resistant to either chemical or biological degradation and thus they exhibit a significant degree of bioaccumulation and environmental persistence [63]. Nevertheless, stricter emission standards and reduced pollution in first world countries has led to a significant decline in TCDD exposure in residents of the United States, Canada, Germany and France over the past 30 years. It is estimated that the current TCDD body burden of citizens in these countries is approximately two parts per trillion-lipid adjusted (ppt), down from an estimated 20 ppt in the early 1970s [64]. In contrast, emission of TCDD and other AhR agonists is not declining in many developing nations, as a consequence of incomplete burning of trash, burning of electronic waste and indoor cooking habits; thus, the body burdens in less developed countries can be significantly higher than those noted above [65, 66]. Importantly, normal weather patterns can carry airborne contaminants far from their site of origin [67], reducing the ability of any one nation to eliminate these toxicants completely from their environment. Furthermore, numerous EDCs have been identified as having “nonmonotonic dose response curves”, which simply means that low dose effects cannot be predicted by extrapolating from effects observed at a high dose. Many EDCs, including TCDD, not only negatively impact human and animal systems at high doses, but also can have equally damaging, albeit different, effects at low doses. Thus, *a toxicant demonstrated to be “safe” at a high concentration, cannot be assumed to be equally safe at all lower doses* (comprehensively reviewed by [68]). For this reason, an unintentional “human experiment” may be underway as industrialized countries make significant strides in reducing emission of toxicants, leading to a decline in the human body burden.

2.2. Human Exposure and Reproductive Consequences in Women

In 1976, a chemical plant explosion near Seveso, Italy led to the highest known exposure of residential populations to TCDD [69], and since that time, women living near the site have been carefully monitored with regard to reproductive tract disease and pregnancy outcomes. To date, these studies have revealed a modest increased risk of endometriosis, a non-significant increased risk of sPTB and an increase in time to pregnancy among the most heavily exposed individuals compared to unexposed women [70-74]. More recently, several studies have linked environmental exposures not associated with industrial accidents, such as airborne particulate matter, to reduced fertility and sPTB in women [75-77]. For example, a recent epidemiology study of women living within 4 kilometers of a municipal solid waste incinerator in Italy identified a significant increased risk for sPTB [78]. As noted previously, cigarette smoke is replete with a wide array of toxicants, including TCDD [79], and has long been associated with reduced fertility and low birthweight due to either primary or secondary exposures [80-82].

2.3. Human Exposure and Reproductive Consequences in Men

Although the primary focus of the current review is EDC exposure and development of endometriosis and related reproductive disorders in women, it should be noted that the male population is not immune to reproductive consequences of toxicant exposure. For example, following the explosion in Seveso Italy described above (section 2.2), Mocarelli and colleagues examined sperm quality and hormone concentrations in men whose mothers were living in the exposure area compared to sons of unexposed women born in the same time frame. Their studies revealed reduced sperm count and reduced motility in adult sons, leading the authors to conclude that in utero/lactational exposure of males to TCDD can permanently reduce semen quality [83]. Indeed, a recent report from the European Union examining sperm counts in healthy young men reveals a significant decline in human populations over the recent past [84]. Equally concerning,

the rates of testicular cancer and prostate cancer are on the rise in industrialized nations, potentially implicating environmental exposures in their development (reviewed by [85]).

2.3. Human Exposures Associated with Military Action

In addition to the consequences of industrialization, human toxicant exposures have long been associated with military service. During the American war in Vietnam, thousands of pounds of the highly toxic herbicide and defoliant known as Agent Orange were sprayed over large areas of central and South Vietnam. Agent Orange, a 50:50 mixture of 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) and 2,4-Dichlorophenoxyacetic acid (2,4-D), was manufactured by the Monsanto Company and Dow Chemical for use in "Operation Ranch Hand". However, as a consequence of contamination during the manufacturing process, TCDD was also unintentionally present in Agent Orange. Over a 10 year period (1961-1971), the U.S. military sprayed the dense jungles of Vietnam with Agent Orange, destroying the ground cover it provided to enemy troops [86]. Unfortunately, agriculture crops and many inhabited villages were also sprayed, resulting in the unintentional exposure of South Vietnamese residents, many of whom continue to experience significant body burdens and a wide range of health issues, including high rates of early pregnancy loss, sPTB, congenital birth defects and serious health problems, including cancer, in surviving children [62, 87-90]. After a study in 1970 found that 2,4,5-T could cause birth defects in laboratory animals, its use in Vietnam was discontinued and the herbicide was soon banned altogether.

The tragic legacy of Agent Orange is not limited to the Vietnamese population. It is estimated that up to 1.5 million American servicemen were also exposed to TCDD as a consequence of Operation Ranch Hand. Some of these veterans reported skin rashes (chloracne), cancer, psychological symptoms, birth defects in their children, and other health problems [91]. In

an effort to determine a potential relationship between the TCDD present in Agent Orange and the reported health problems among Veterans, a series of scientific studies was conducted. These studies implicated TCDD in many, (though not all) adverse disease outcomes and eventually led to a successful class action lawsuit against Monsanto and Dow Chemical [92]. While it might be easy to dismiss Agent Orange exposures as an unfortunate consequence of the unique, forested landscape of Vietnam, Veterans of World War II and current servicemen (and women) have also been affected by exposure to TCDD and other EDCs over the course of their service. Specifically, the crematoriums associated with the Nazi regime in Germany would have produced substantial amounts of dioxin and dioxin-like PCBs [93]; exposing both the “residents” of the concentration camps as well as local civilians and military personnel in the surrounding area. Today’s military personnel serving in Afghanistan and Iraq are also exposed to EDCs via the burn pits used to destroy military refuse as well as from the oil fires accidentally or intentionally set [94-97]. Thus, exposure to dioxins and other EDCs continues to be an unintentional, additional threat to the health of our military personnel and local populations within war zones. The potential, long-term reproductive consequences of such exposures have yet to be determined to any of these affected individuals and populations.

Although the environmental exposures detailed in this section centered on examples of “high dose” exposures, there can be little doubt that chronic low dose exposures must also occur at sites distal to the primary event (via weather patterns, contamination of food and water etc). Acute, high dose effects of TCDD exposure in humans are well-documented (chloroacne, pregnancy loss and teratogenic effects), but the consequences of variable, low dose exposures are still being determined. Furthermore, although the potential consequence of *developmental exposures* are difficult to identify in humans, mounting experimental evidence indicates that TCDD and other EDCs may have transgenerational effects on not only adult reproductive function but child health as well.

3. Transgenerational Reproductive Dysfunction in a Mouse Model

The overwhelming evidence from numerous epidemiology studies, several of which are discussed above (section 2), implicates a likely role for environmental EDC exposure and adverse reproductive consequences in humans. However, carefully controlled, mechanistic studies cannot be prospectively conducted in humans; thus we have utilized an animal model known to be sensitive to TCDD. For our studies, pregnant 10-12 wk old female C57BL/6 mice (F0, or founding generation) were exposed to TCDD (10µg/kg) by gavage on embryonic day 15.5 (E15.5), resulting in in utero and lactational exposure of offspring. As illustrated in **Figure 1**, mice which are exposed to TCDD during pregnancy are designated F0, or founding generation. The feti present with the uteri are directly exposed and identified as the F1 generation. Germ cells residing in the F1 feti, which have the potential to become the F2 generation, are also directly exposed. Within this rodent model, adult female F1 mice were examined in initial studies for endometrial progesterone responsiveness, fertility and pregnancy outcomes, measurements related to the consequence of endometriosis in women. Additional studies conducted in F2-F4 mice examined these same endpoints, in order to determine the potential for multi- and transgenerational effects of TCDD exposure in females. As detailed below, we not only identified the occurrence of an endometriosis-like reproductive phenotype among F1-F4 female mice, many of these animals also exhibited histological and functional evidence of co-morbidities which are common in women diagnosed with endometriosis .

3.1. *The Endometriosis-related Phenotype in Female Mice*

Using our developmental toxicant exposure model (**Figure 1**), we initially reported that early life/in utero exposure of female mice to TCDD leads to an adult uterine phenotype in animals which mimics the reduced uterine progesterone responsiveness observed in women with endometriosis [22]. More specifically, we found that F1 females exhibit reduced uterine PR and progesterone-

sensitive TGF- β 2 expression, proteins essential for establishment and maintenance of pregnancy. Thus, we were not surprised to find that subfertility was also common among F1 females mated to control breeder males. Approximately 50% of female F1 mice failed to exhibit signs of pregnancy (weight gain/nipple prominence) despite multiple matings (4+) and observation of vaginal plugs. In addition to subfertility, among females which achieved pregnancy, a high rate of sPTB was observed, representing a unique experimental model system to explore this condition [10, 98]. An equally important finding was that F2-F4 female mice continued to exhibit endometriosis-like histological and functional phenotypes in the absence of additional toxicant exposure. Specifically, following a single developmental exposure to TCDD, we identified both multi-generational (F1-F2) and transgenerational (F3-F4) occurrence of reproductive disorders that are similar to those encountered by endometriosis patients. As outlined in **Table 1**, our murine studies support a potential role for a developmental origin of endometriosis; a disease which currently remains one of the most poorly understood conditions affecting not only women's reproductive potential, but their overall quality of life. Addressing the potential medical consequences of an inherited risk of developing endometriosis due to an ancestral toxicant exposure will likely challenge our current clinical practices related to the management of this disease.

3.2. Adenomyosis

Adenomyosis, the presence of endometrial glands and stroma embedded within the uterine muscle, is frequently identified in women undergoing hysterectomy as a surgical treatment for endometriosis [99]. Adenomyosis, like endometriosis, has been associated with reduced fertility, pelvic pain, heavy menstrual bleeding and dysmenorrhea [4, 100]. The causes of adenomyosis are currently unknown, although both human and animal studies have suggested a role of inflammatory processes in the development of this disease [101-104]. In our murine model, we recently reported the transgenerational occurrence of adenomyosis in mice exhibiting the

endometriosis-like uterine phenotype as a consequence of developmental TCDD exposure of F1 animals [101]. Within this recent study, we conducted a retrospective analysis of uteri from TCDD exposed F1 female mice and two generations of their offspring to determine whether histological evidence of adenomyosis was present. Although none of the control mice examined exhibited adenomyotic lesions, we identified deep, adenomyotic lesions in the majority of mice with a history of direct (F1-F2) or indirect (F3) TCDD exposure. Specifically, 0/10 controls had adenomyosis while 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) (**Table 1**). Interestingly, although current clinical perspectives on adenomyosis in women is primarily focused on identifying endometrial invasion into the muscle [105], our murine model clearly demonstrated the presence of myometrial cell nests embedded within the endometrium. As shown in **Figure 2**, staining for smooth muscle actin revealed the presence of individual muscle cells within the endometrial stroma in both human and murine samples exhibiting deep adenomyosis suggesting that the myometrial compartment may participate in the pathogenesis of this disease. While the initiating events leading to the initial development of adenomyosis are not currently known, the occurrence of this disease as a co-morbidity in women endometriosis, fibroids and menorrhagia, supports a potential role of inflammation in the pathogenesis of this disease as well. Finally, the transgenerational presence of adenomyosis in our murine model of developmental TCDD exposure in a single generation provides evidence that epigenetic alterations affecting steroid sensitive immune-endocrine crosstalk within the uterus may underlie the development of this, and perhaps other, reproductive disorders.

3.3. Heightened Sensitivity to Inflammation following TCDD Exposure

We have documented a heightened sensitivity to a secondary pro-inflammatory signal both within our murine model of developmental TCDD exposure [106] and following acute in vitro

exposure to TCDD using human endometrial cells. Specifically, following a 6-10 day exposure period to TCDD, the anti-inflammatory effects of progesterone are lost in isolated endometrial stromal cells acquired from control tissue donors with a concomitant increased reactivity to pro-inflammatory signals [16, 19]. The potential in vivo significance of altered immune-endocrine crosstalk to reproductive success emerged when we observed a *doubling* of the sPTB rate in TCDD exposed F1 and F3 mice in response to an unexpected parvovirus (MPV) outbreak in our colony. Importantly, pregnant, unexposed control mice within the same colony, which was housed in Vanderbilt University's general use, non-barrier facility, were not affected [10]. This serendipitous finding further suggested to us that the *TCDD mediated decrease in the anti-inflammatory action of progesterone may have less of a negative impact on the length of pregnancy in the absence of an additional pro-inflammatory stressor*. In order to experimentally confirm the "dual-hit" etiology for increased sPTB in our model, we established a new colony of animals within Vanderbilt's Specific Pathogen Free Barrier Mouse Facility, which is known to be free of MPV and other common mouse pathogens. Following establishment of this colony, we identified the lowest dose of LPS (lipopolysaccharide; 200 ug/kg), which did not independently cause PTB in control C57bl/6 mice within our colony. Mice were subjected to intraperitoneal injection of 200 ug/kg LPS on E15.5 of pregnancy. Although control mice were not affected, PTB was observed within 24 hrs in 100% of C57bl/6 female mice with a history of direct (F1) or ancestral (F3) exposure to TCDD (**Table 2**). Interestingly, we noted that the *sPTB* rate in TCDD exposed F1/F3 mice was slightly (but not significantly) lower in the Barrier facility, suggesting that mouse pathogens that are commonly present within a general use facility may have also contributed to sPTB in mice with a history of early life toxicant exposure.

While a similar prospective, in vivo human TCDD exposure study would not be possible, our murine data suggests that early life TCDD exposure may significantly alter the negative impact of either viral or bacterial infection on adult reproductive success. Our murine studies also support our

previous in vitro observations using isolated human endometrial stromal cells treated with estrogen and progesterone to induce decidualization as occurs in vivo in preparation for pregnancy. Using this in vitro model, we have documented the effects of TCDD exposure alone and in combination with LPS. Using quantitative RT-PCR, we found that TCDD exposure alone only minimally affected PR mRNA expression levels during in vitro decidualization, however; the combination of LPS and TCDD exposure markedly reduced PR expression [16]. Since disrupting progesterone action will induce pregnancy termination in mice or women, the ability of LPS to accelerate the negative impact of TCDD on PR expression levels likely explains the enhanced incident of PTB we observe within our mouse colony in response to infective agents. Furthermore, our observations utilizing isolated human cells in vitro suggest that women may be equally sensitive to a secondary inflammatory event following a developmental or adult exposure to an environmental endocrine disruptor. Developing a better understanding of the role that systemic inflammation plays on the development of diseases that negatively affect the function of the adult reproductive system would potentially allow the use of preventative therapies. In order to understand the marked increase in sensitivity of toxicant exposed mice to a secondary inflammatory challenge related to an infection, we examined the omentum within the peritoneal cavity of our mice with and without a history of toxicant exposure. The omentum is a fatty tissue that connects the spleen, stomach, pancreas and colon [107]. In both humans and mice, the omentum contains clusters of leukocytes, termed “milky spots” [108, 109], leading some to suggest that this tissue acts as an immune cell reservoir for the movement of cells in and out of the peritoneal cavity. For example, chronic inflammation associated with obesity and diseases such as HIV are known to increase the size and immune cell content of the omentum [110, 111]. Compared to disease-free women, Williams et al [112] observed alterations in omental proteinase expression of women with endometriosis, which correlated with increased body burden of several EDCs (although TCDD was not examined). As shown in **Figure 3**, compared to control animals, F1 and F3 mice exhibit a marked accumulation of

immune cells within the omentum, supporting our hypothesis that these animals have a heightened “baseline” inflammatory phenotype.

While it is well established that immune and endocrine dysregulation play a role in endometriosis, the mechanisms potentially linking the pathogenesis of endometriosis to adenomyosis or other related inflammatory co-morbidities have not been elucidated. However, our murine model and in vitro human cell models suggest that environmental toxicants like TCDD may contribute to the etiology of multiple diseases by disrupting normal endocrine-immune cell crosstalk both locally and systemically. Supporting our early life exposure model, other investigators have shown that adult exposure of mice to TCDD led to a rapid, systemic increase in leukocyte markers, chemokines and macrophage infiltration, promoting a chronic inflammatory state [113, 114]. Endometriosis is currently being viewed by many investigators as a chronic, systemic inflammatory disease that is characterized not only by the presence of enhanced numbers of activated, peritoneal immune cells but also by the negative impact of activated immune cells to overall reproductive success [115, 116]. However, at this juncture, whether altered systemic inflammation is the result or a driver for reproductive tract disease pathogenesis is not currently known. In an elegant review, Kobayashi et al [116] hypothesized that endometriosis may be a consequence of immune cell response to an infection in conjunction with oxidative stress associated with menstruation, resulting in a chronic, sterile inflammation which ultimately promotes disease. Our murine studies support this theory, but also extend it as our studies suggest that an early life exposure to an EDC such as TCDD may *mimic* an infection; thereby leading to chronic inflammation and an increased risk of disease development.

3.4. Adhesive Disease

Patients with endometriosis frequently undergo surgery to remove ectopic disease or for hysterectomy in an attempt to prevent disease recurrence [1]. Unfortunately, compared to the

general population, these women are at a higher risk of not only surgery-associated adhesions, but also for the development of spontaneous adhesive disease [117]. The basic pathophysiology of postsurgical adhesion development is known to involve inflammatory processes that occur during normal wound healing. Certainly, macrophages and neutrophils play key roles in the initiation of inflammation related to wound healing, by releasing both inflammatory cytokines and proangiogenic factors. Women with endometriosis are known to exhibit dysregulated immune cell function, which likely contributes to the increased risk of developing adhesive disease [115]. In an effort to explore the pathogenesis of these co-morbidities, we examined the development of spontaneous adhesive disease in a chimeric mouse model of human experimental endometriosis [118, 119]. For this model, we mimicked retrograde menstruation by injecting human endometrial tissue fragments intraperitoneally into ovariectomized, immunocompromised nude mice treated with estradiol. As reported in these published studies, we noted a cooperative effect of the presence of endometrial tissue fragments on the development of post-surgical adhesions when tissue fragments were introduced into the peritoneum within 16 hours of ovariectomy. Sham surgery (removal of the fat pad surrounding ovary, but not the ovary) or tissue injection in the absence of experimental endometriosis *was not* associated with increased adhesive disease [118, 119]. Thus, within the peritoneal cavity, immune cell responses to injury, similar to infection or radiation therapy, may promote formation of adhesions [120].

Additionally, over the course of our studies utilizing TCDD exposed mice, we noted that while adhesions were rarely observed in control mice at necropsy, adhesive disease was common among F1-F3 mice. As described above in section 3.3, immune cell numbers and LPS responsiveness was enhanced in mice with a history of TCDD exposure, suggesting that toxicant exposed mice may exhibit an increased risk for development of post-surgical adhesions due to their altered peritoneal phenotype. In order to examine the risk of adhesive disease in TCDD-exposed mice, we established a syngeneic endometriosis model in control and F1 mice within 16

hours of a surgical ovariectomy similar to the approach with the chimeric adhesion model described earlier in this section [118, 119]. As shown in **Table 3** and **Figure 4**, injection of uterine fragments into the peritoneal cavity of TCDD-exposed F1 mice within 16 hours of peritoneal surgery led to the development of both multiple adhesions and ectopic sites of endometrial growth. Interestingly, injection of uterine tissue fragments derived from either toxicant-exposed F1 mice or control animals led to a similar degree of adhesion development within the peritoneal cavity of F1 recipient animals (**Table 3**). Notably, control recipients exhibited minimal lesions or adhesions, regardless of whether the donor tissue was obtained from a control or F1 animal. These data suggest that the inflammatory peritoneal environment may be more relevant to the risk of post-surgical adhesive disease than that of the uterine phenotype. In future experiments it will be necessary to examine whether there is a transgenerational risk for developing adhesive following early life TCDD exposure as we noted in our endometriosis and adhesive disease models.

4. TCDD-Mediated Epigenetic Events

As we discussed in a previous review [101], reduced fertility and an increased risk of sPTB in female mice across multiple generations following a single, developmental exposure to TCDD strongly suggests toxicant mediated epigenetic modifications have occurred. Epigenetic marking of DNA is now recognized as an important mechanism by which gene expression can be altered without a change to the DNA sequence. The primary role of epigenetic modification is to control DNA accessibility, i.e. the ability of the cellular machinery to act as a control switch for gene expression by activating or inhibiting transcription. The best described mechanisms of epigenetic changes are methylation and acetylation of DNA and histones [121]. Hypermethylation of DNA is generally associated with gene silencing while hyperacetylation of histones leads to chromatin

relaxation and unwinding which promotes gene transcription. Appropriate patterns of methylation and acetylation are necessary for normal expression and regulation of certain genes. Thus, in addition to information present within an individual's specific genetic code (DNA sequence), epigenetic alterations can modulate how this otherwise hard-wired genetic information is utilized within a cell. Significantly, although such epigenetic changes are stable, they are themselves subject to modification [122]. For example, while lifestyle choices such as smoking can promote epigenetic changes which can accelerate aging, it is theoretically possible that epigenetic therapies can reverse these changes in order to combat certain cancers and other diseases [123-125]. Additionally, epigenetic marks occurring within the germline are inheritable [126, 127] and can positively or negatively affect offspring. For this reason, lifestyle choices that preserve a healthy epigenome may dramatically reduce not only individual's risk of disease, but may promote the long term health of future offspring.

4.1. Studies linking abnormal epigenetic marks to endometriosis in women.

Numerous studies have reported alterations in epigenetic marks of multiple endometrial genes from samples obtained from women with endometriosis compared to disease-free control subjects (for example, [128-130]). Perhaps predictably, studies have also identified differential methylation/acetylation profiles between the eutopic endometrium and ectopic sites of tissue growth in women with endometriosis (for example, [131-133]). Several functional studies linked altered decidualization capacity in women with endometriosis to abnormal epigenetic marks (ie, [134, 135]). For example, subjecting primary cultures of stromal cells, obtained from women with or without endometriosis, to a standard decidualization protocol, Bulun and colleagues identified a failure of decidualization-associated downregulation of DNMT3B hormones [136]. These

investigators concluded that endometriosis “may contribute to an aberrant epigenetic fingerprint that misdirects gene expression and contributes to its altered response to steroids” [136]. In our laboratory, using methylation-specific PCR, we identified hypermethylation of the PR gene in endometrial tissues obtained from women with endometriosis compared to disease-free women (**Figure 5A**). Significantly, whereas isolated endometrial stromal cells obtained from women without endometriosis do not exhibit methylation of the PR gene, short-term treatment of these cells with interleukin 1 (IL-1), an inflammatory cytokine produced by both immune and endometrial cells is associated with hypermethylation (**Figure 5B**). Further implicating inflammatory processes in development of the endometriosis phenotype, Wu et al [137] demonstrated that treatment of isolated, control human endometrial epithelial cells with TNF- α led to hypermethylation of the PR promoter and a loss of PR-B protein expression. Using the human endometriotic epithelial cell line 12Z and stromal cell line 22B, the Arosh laboratory revealed that selective inhibition of prostaglandin E2 (PGE2) receptors EP2 and EP4 modulates DNA methylation and histone modification machinery proteins, suggesting these receptors may be useful for therapeutic targets. However, findings between the numerous studies investigating the potential relevance of epigenetic alterations and disease development are not always in agreement. For example, both hypomethylation of HOXA10 [128] and hypermethylation of HOXA10 [131] have been described in women with endometriosis compared to controls. Although differences in patient selection, variation among control populations and sample size likely contribute to the inconsistencies among various studies, Saare et al, [138] recently described a variation in cycle-dependent methylation patterns in healthy women compared to endometriosis patients. These investigators found only minimal differences between endometriosis patients and controls, while differences between cycle phases, in particular, between the late secretory phase and menstruation, were more robust. Therefore, future studies attempting to assess epigenetic differences between healthy women and

those with endometriosis will need to carefully consider the cycle phase during which the tissues are obtained as an additional variable.

4.2. Studies linking TCDD-mediated epigenetic events to reproductive disease in mice

We have previously demonstrated that in vitro TCDD exposure of human endometrial cells suppresses expression of both PR-B mRNA and protein, which may be mediated by the local action of toxicant-induced inflammatory cytokines [15, 19]. As described above (section 3.3), F1-F3 mice within our developmental TCDD exposure study were found to exhibit both reduced PR protein expression within the uterus and a heightened peritoneal inflammatory response. These data, taken together with the in vitro human cell data, suggested to us that the loss of progesterone sensitivity associated with developmental exposure of mice to TCDD may be due to an epigenetic modification, mediated by inflammatory processes. Therefore, we recently examined the methylation status of the PR gene in uterine samples from our female mice. As discussed earlier, DNA hypermethylation can mediate gene silencing and is an important mechanism by which heritable epigenetic modifications may occur [139]. As we previously reported, examination of murine uteri by methylation-specific PCR (MS-PCR) revealed partial methylation of the PR in 60% of tissues removed from F1 females (exposed to TCDD in utero) while this gene was largely unmethylated in similar tissue samples acquired from control mice [102]. In order to determine if PR gene hypermethylation is a transient or stable (heritable) effect, we similarly examined uterine tissues removed from F3 females, revealing partial methylation of the PR in 40% of these animals [102]. These data, presented in **Table 1**, suggest that the transgenerational infertility phenotype and associated risk of sPTB in these animals may be due to epigenetic silencing of PR as a consequence of ancestral TCDD exposure. Although mice utilized for methylation analysis were not mated, it is interesting to note that the frequency of hypermethylation observed in these mice

strongly correlated with the frequency of infertility among previously examined F1 and F3 mice (**Table 1**).

5. Developing a New Human Model for Reproductive Toxicology

Despite the fidelity of our developmental TCDD exposure model to endometrial disease and dysfunction associated with endometriosis in women, these animals do not menstruate and thus do not spontaneously develop ectopic disease. Additionally, despite the universal necessity of progesterone in mammalian pregnancy, the role and expression patterns of specific progesterone receptors vary across species [140]. Furthermore, differences between the murine and human inflammatory responses [141, 142] may complicate human translation of TCDD toxicity studies conducted in mice. Thus, it is critically important to validate the murine results using models which better recapitulate the in vivo human condition, in order to accurately define the impact and mechanisms of action of environmental toxicants such as TCDD.

Clearly, intentional exposure of reproductive age women to TCDD or other EDCs is neither ethical nor feasible. Therefore, our traditional in vitro studies utilized human endometrial stromal and epithelial cells maintained in static culture models for analysis of cellular responses to acute TCDD. As noted above (section 3.3), these in vitro studies revealed that this toxicant acts to disrupt the anti-inflammatory action of progesterone, leading to an increase in endometrial cell sensitivity to inflammatory stimuli [16]. Nevertheless, mechanistic interpretation of these results remains limited as a consequence of a non-physiological experimental setting [143-145]. Multi-cell culture assays, including organ cultures, co-cultures and tissue recombinants, clearly demonstrate the significant role of cell-cell communication in normal endometrial physiology [15, 146, 147].

Furthermore, using co-cultures of murine stromal and epithelial cells, Buchanan et al [148] revealed that TCDD's negative effect on the epithelium is largely mediated by the stromal cells. Together, these findings demonstrate the significant contribution of the cellular microenvironment to normal organ function and underscore the need to incorporate multiple cells into studies attempting to understand the mechanism of action of EDCs. Because the in vivo physiological function of the endometrium is maintained by spatial and temporal cues mediated through complex endocrine, paracrine and immune components, it becomes critical to mimic these facets in a more robust in vitro model.

Technical challenges constrain the utility of static cell culture models to identify how TCDD disrupts cell-cell communication and immune-endocrine regulation, ultimately leading to reproductive dysfunction. However, recent advances in microfabrication technologies have enabled researchers to mimic the physiological microenvironment of human tissues. These tissue engineered devices or "Organs-on-Chips" (OoC) offer the ability to perform multi-cellular co-cultures in a microfluidic system to mimic the physiological nature of the tissue and examine the crosstalk between cells and distal organs [149]. Several funding agencies including the NIH's National Center for Advancing Translational Sciences (NCATS) and the Environmental Protection Agency understand this issue and have prioritized initiatives for the development of 3D devices capable of mimicking the structure and function of human organs. Long term culture stability, appropriate high resolution microscopy techniques, use of microporous biomaterials, cell compartmentalization and continuous perfusion and sampling allow for qualitative and quantitative analysis of real time interactions between cells [150, 151]. Individualized perfusion of cells additionally allows for introduction of immune cells or test agent (ie, therapy or EDC). Significantly, OoC can be interconnected, allowing organ to organ communication. For example, placing the "liver-on-chip" [152] upstream of the endometrium on a chip would potentiate the ability to

physiologically address how EDCs, xenobiotics and their metabolites impact human endometrial homeostasis.

With regard to the reproductive tract, several groups are pursuing the use of microfluidic OoC devices as both research tools and for assisted reproductive technology applications [153, 154]. Using these techniques, our group is also currently developing an Organ-on-Chip microfluidic model of both the eutopic and ectopic human endometrium to further advance our understanding of the mechanisms by which TCDD leads to endometrial dysregulation and disease (**Figure 6**). The ability to interconnect the OoC devices will dramatically enhance our ability to examine the downstream consequences of ectopic disease on the eutopic endometrium. These models will also serve as a platform to test and screen therapeutic agents in order to identify high efficacy candidates prior to testing in animal models. Although the OoC models are still at the prototype phase, we expect that in the near future, these models will be routinely utilized for pathological, toxicological and biological studies and will significantly transform our in vitro modeling capabilities.

Summary/Conclusions

The emerging evidence that many diseases and conditions affecting adults are influenced by the prevailing in utero environment during development [20] suggests that reproductive health may also be negatively influenced by disruption of normal fetal development. Within this review, we have discussed our findings in a murine model that demonstrates an epigenetic link between early life exposure to TCDD and the development of reduced progesterone sensitivity within the adult reproductive tract across multiple generations. Using both in vivo and in vitro models, we have shown that the loss of endometrial progesterone sensitivity, a well-recognized component of endometriosis, is biologically linked to an inflammatory-like pattern of cell-cell communication

within the uterus [6-8]. *Whether reduced reproductive tract responsiveness to progesterone leads to the development of endometriosis or emerges as a consequence of the inflammatory nature of the disease is currently unknown.* Nevertheless, once in place, a loss in the differentiation promoting, anti-inflammatory action of progesterone would be expected to not only compromise reproductive success [10], but potentially promote the development of common comorbidities (ie, adenomyosis, adhesive disease).

6. Methodology

6.1 In utero TCDD Exposure:

Virgin female and male C57bl/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) 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 22-24°C and a relative humidity of 40-50% on a 12-h light:dark schedule. Experiments described herein were approved by Vanderbilt University Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act.

C57bl/6 females (N=20), aged 10-12 weeks, 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). Pregnant mice (F0) were exposed to TCDD (10µg/kg) in corn oil or vehicle alone by gavage at 1100 hours CST on E15.5 (when organogenesis is complete). This in utero plus lactational exposure paradigm results in direct exposure of the feti (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 to

humans and is well below the LD50 for adult mice of this strain (230 μ g/kg) [155]. 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.

6.2 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 4 positive vaginal plugs with no subsequent pregnancy. Second generation (F2) animals were weaned at 4 weeks of age at which time male pups were removed. At 10-12 weeks, F2 females were mated to control males of similar age and monitored as above. Note: As previously described, like their siblings, F1 and F2 males exhibit subfertility [10, 156]. Perhaps as a consequence of reduced fertility in directly exposed animals, to date, mating of non-sibling F1 male and female mice or non-sibling F2 male and female mice has not resulted in offspring [102]; thus, toxicant exposed mice are necessarily mated to control partners.

6.3 Euthanasia:

Adult mice were euthanized between 6 and 7 months of age by cervical dislocation after anesthetic overdose (isoflurane). Animals were typically euthanized during the estrus phase as indicated by vaginal smear.

6.4 Immunohistochemistry/Histochemistry:

Hematoxylin and eosin staining was performed on formalin-fixed, paraffin-embedded tissues by standard methods. Immunohistochemical staining of murine tissues for smooth muscle actin (SMA) was performed after heat activated antigen retrieval using anti-SMA (Cat.#RB-9010-P1, Thermo) for one hour at a 1:500 dilution: The Bond Polymer Refine detection system was used for visualization. Slides were then dehydrated, cleared and coverslipped for morphological analysis. Histopathological assessments were performed using an Olympus BX51 microscope system and images captured using an Olympus DP71 digital camera.

6.5 Syngeneic Experimental Endometriosis in Mice:

Experimental endometriosis was established in control and F1 female mice by intraperitoneal injection of minced uterine tissues from donor mice (control or F1). All donor mice were in estrus at the time of euthanasia and collection of tissues, while recipient mice were ovariectomized and provided a slow release capsule containing estradiol 16 hrs prior to tissue injection. Recipient mice were euthanized 5 days after injection of syngeneic tissues and examined for adhesive disease and experimental endometriosis. Adhesion/lesion scores were determined by standard methodology as previously described [119].

6.6 Methylation-Specific PCR

Endometrial biopsies were obtained from women with endometriosis after providing written, informed consent. DNA was extracted from whole tissue samples or from isolated endometrial stromal cells as previously described [15]. Using non-quantitative, methylation-specific PCR, bisulfite-modified DNA was assessed for PR-B methylation status using primers specific for methylated and unmethylated DNA. Experiments utilizing human tissues and described herein were reviewed and approved by Vanderbilt's Institutional Review Board

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Figure Legends:

Figure 1: Developmental TCDD Exposure Model. Pregnant C57BL/6 mice (F0 generation) are exposed to a single, acute TCDD dose (10 μ g/kg) by gavage on embryonic day 15.5 (E15.5), when organogenesis is complete. However, at the time of exposure, fetal germ cells are present within the F1 fetal gonads. Since these cells have the potential to become the F2 generation, TCDD treatment of a pregnant dam (F0) ultimately results in direct exposure of three generations (F0-F2).

Figure 2: Smooth Muscle Actin Stain of Human and Murine Uteri. We recently assessed the presence of adenomyosis, nests of endometrial glands and stroma within the muscle, in archived samples of human and murine uteri [101]. Using smooth muscle actin (SMA) immunohistochemistry, which localizes to both myometrial cells and muscle cells surrounding some vessels, we frequently identified muscle cells within the endometrial layer of women with endometriosis and within tissues obtained from mice with a history of direct (F1-F2) or ancestral (F3) TCDD exposure. Representative images from this recent study are presented herein. (A) SMA staining in uterine tissue from a woman with surgically confirmed endometriosis. Muscle cells within the endometrial layer are denoted by an arrow. An adenomyotic lesion (denoted "a") is also visible within this image. (B) SMA staining of uterine tissue from an F3 mouse reveals a large gland within the endometrial layer that is surrounded by a layer of muscle cells. In both images, muscle cells appear brown and vessels are marked by an arrowhead. Original magnification 400x.

Figure 3: Hematoxylin and Eosin staining of Murine Omentum. Immune cells present within the omentum are readily apparent due to uptake of hematoxylin (dark blue). Immune cells are rarely observed in the omentum of control mice (A), while large numbers of immune cells are visible in both F1 (B) and F3 (C) omentum. Original magnification, 100x; Inset, 200x.

Figure 4: Gross Morphology of Adhesive Disease. Experimental endometriosis was established in control (A) and F1 (B) mice by intraperitoneal injection of minced uterine tissues from donor mice with the same phenotype as the recipient. Recipient mice were euthanized 5 days after injection of syngeneic tissues and examined for adhesive disease. As shown in (A), adhesions developing in control mice were typically thin and fragile (arrows), with single point attachment sites. In contrast, adhesions developing in toxicant exposed mice were frequently dense, with multiple attachment sites (B).

Figure 5: Hypermethylation of the Endometrial Progesterone Receptor Gene in Women with Endometriosis

DNA was extracted by standard methodology from whole endometrial tissues obtained during the proliferative phase from women with and without endometriosis (A) and from endometrial stromal cells isolated from proliferative phase biopsies obtained from disease-free donors (B). Using non-quantitative, methylation-specific PCR, bisulfite-modified DNA was assessed for PR-B methylation status. As shown in Panel A, the endometrial PR-B gene from women without endometriosis is completely unmodified, while tissues from women with endometriosis exhibit partial methylation. Partial methylation of PR-B was induced in healthy stromal cells following 48 hr treatment with IL-1 (B). N \geq 3 for all groups. U=unmethylated; M=methylated, WT: whole tissue (untreated).

Figure 6: Schematic of a Microfluidic “Organ-on-Chip” Model of the Endometrium (EndoChip). The endometrial microenvironment plays a critical role in both the homeostasis of tissue function and etiology of diseases. To recreate the organ architecture on a chip, the major cellular components of the endometrium (i.e. the immune, vascular, stromal and epithelial components) are isolated and then reassemble in microfluidic devices fabricated with biocompatible materials (i.e. polydimethylsiloxane, PDMS) and hydrogels. These devices 1) reduce the need for large cell numbers, 2) individually compartmentalize each cell type within independent chambers, 3) maintain short and long term growth and 4) are sensitive enough for biochemical and imaging analysis. Perfusion of immune cells, with control over temporal endocrine signaling and physiological mechanical stimuli (e.g. shear stresses), further enhances the physiological performance of *in vitro* cell culture.

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Table 1: Cumulative Overview of Reproductive Characteristics in Control and Toxicant Exposed Female Mice¹

Mouse History	Pregnancy Rate	Term Delivery²	Adenomyosis	Methylation of PR³
Control	95-100%	100%	0%	0%
F1	46	37	70	66
F3	50	60	56	33

¹Data compiled from references 10, 101 and 102.

²Delivery was considered term when occurring E19-E21 (plug date is considered E0.5).

³Whole uteri collected from non-pregnant mice at estrus.

Table 2: Incidence of preterm birth by animal care facility and in response to a secondary inflammatory challenge

Mouse History	Non-Barrier Facility ¹	Non-Barrier +MPV	Barrier Facility ²	Barrier + Low dose LPS
Control	0	0	0	0
F1	36	63	28	100
F3	25	40	20	100

¹The **Non-Barrier Facility** is a general use mouse housing area that may contain common mouse pathogens.

²The **Barrier facility** is a Specific Pathogen Free mouse housing facility that is known to be free of specific mouse pathogens, including MPV.

Table 3: Endometriosis-Associated Adhesive Disease

Recipient/Donor	N	% with Adhesions	Adhesion Score	<i>p</i> *	%with lesions	Lesion score	<i>p</i> *
Control/Control	10	30	0.4		80	1.3	
F1/Control	3	33	0.7	NS	33	0.33	NS
Control/F1	3	100	2.4	0.03	66	1.7	NS
F1/F1	7	100	2.9	<0.002	86	2.6	<0.005

*compared to Control mice receiving Control tissue.

NS: Not-significant

Figure 1

Pregnant Dam
(F0 generation)



F1 fetus
(contains the F2
germ cells)

Figure
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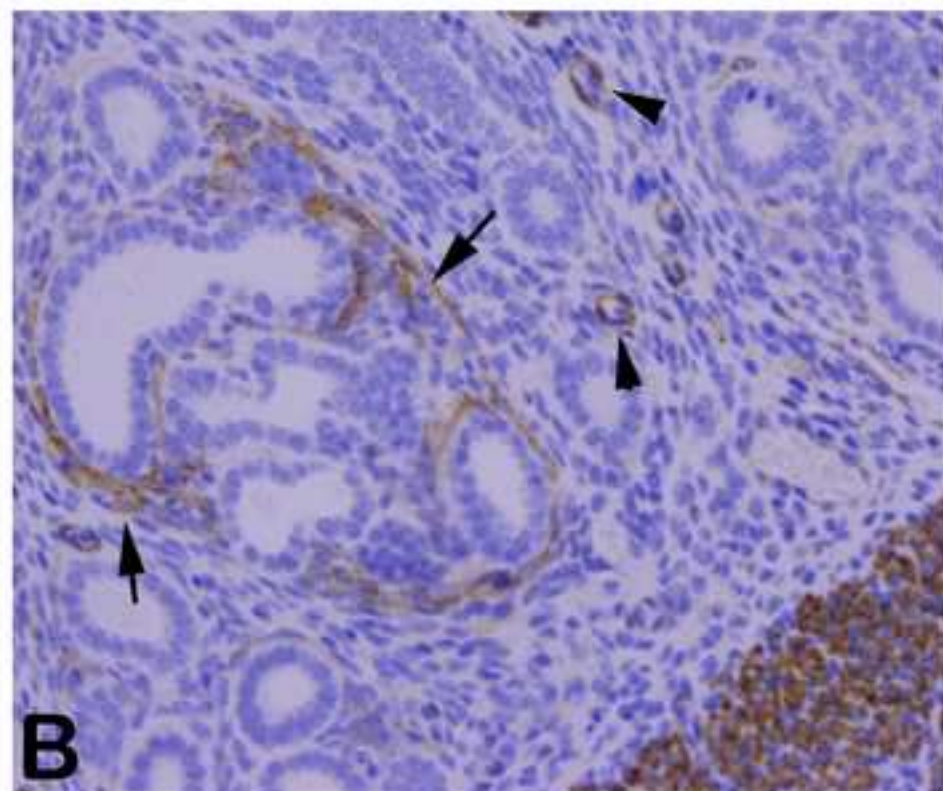
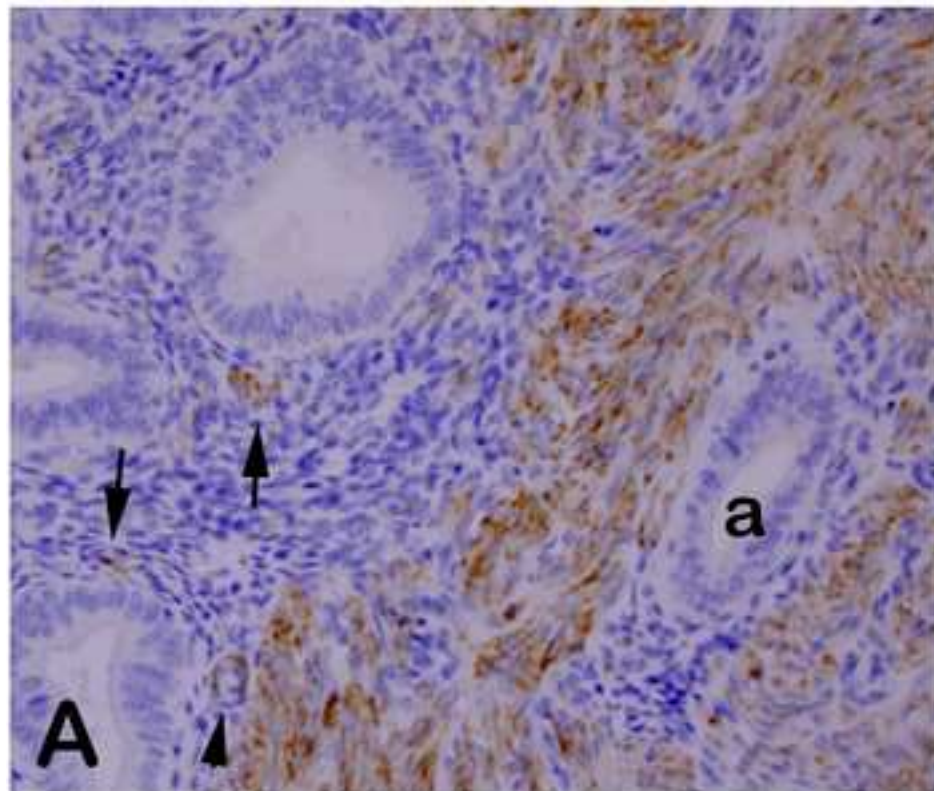


Figure 3
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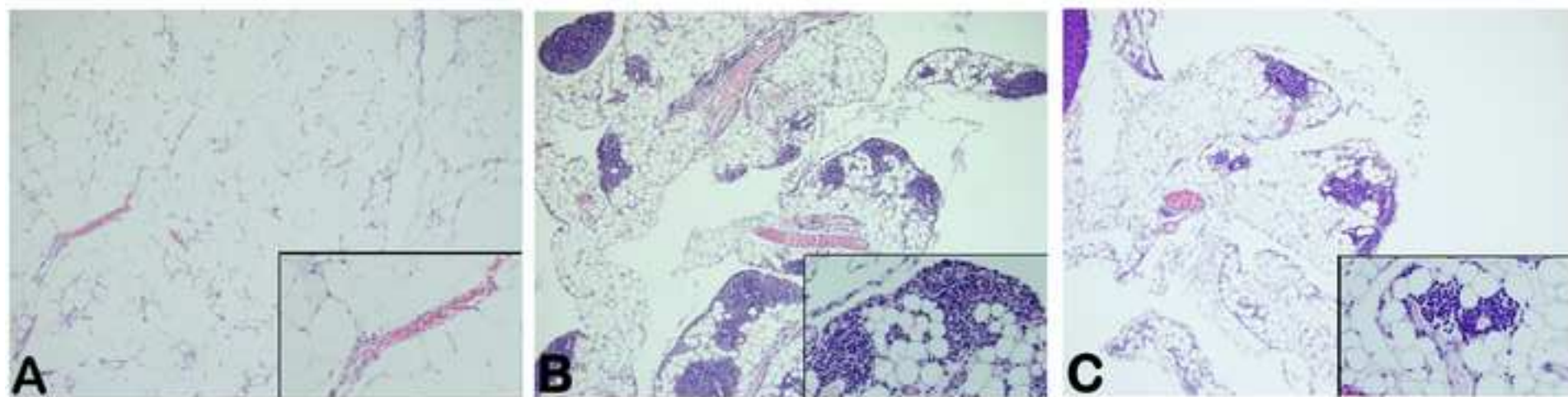
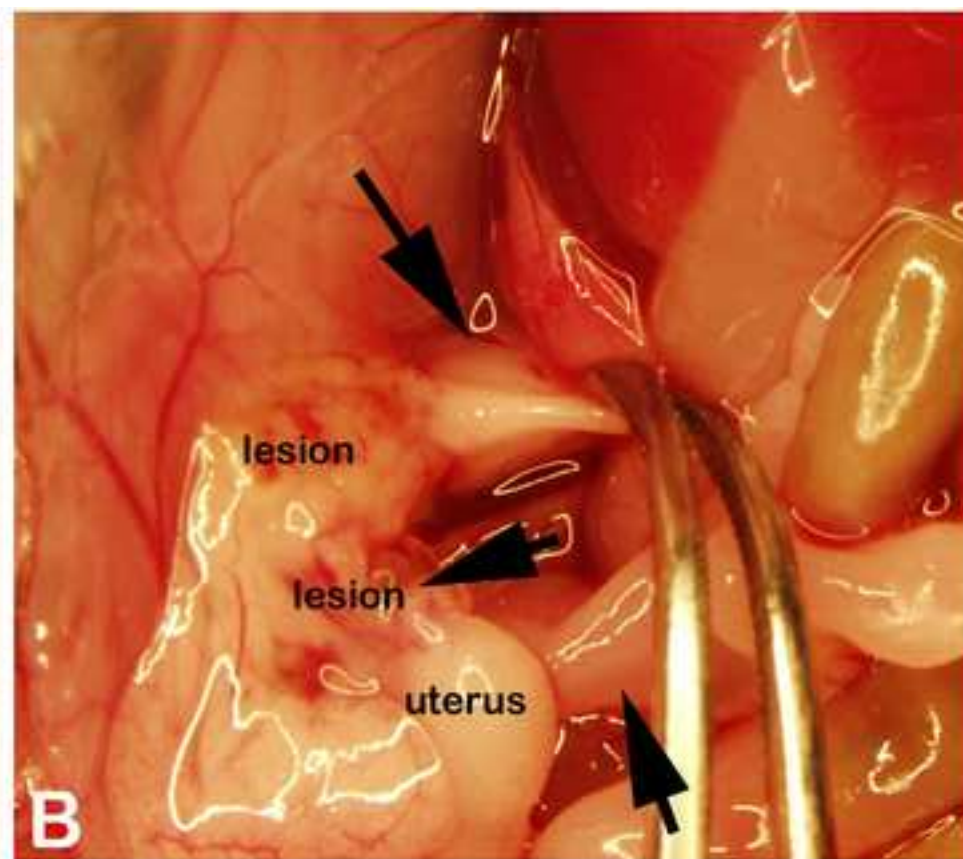
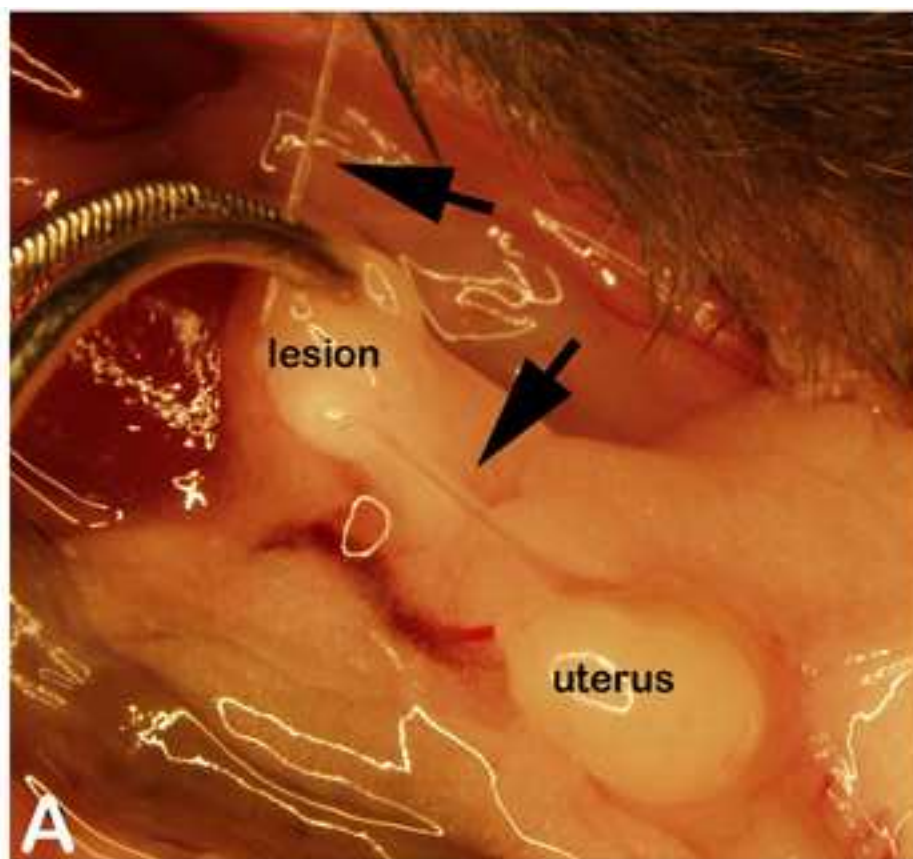
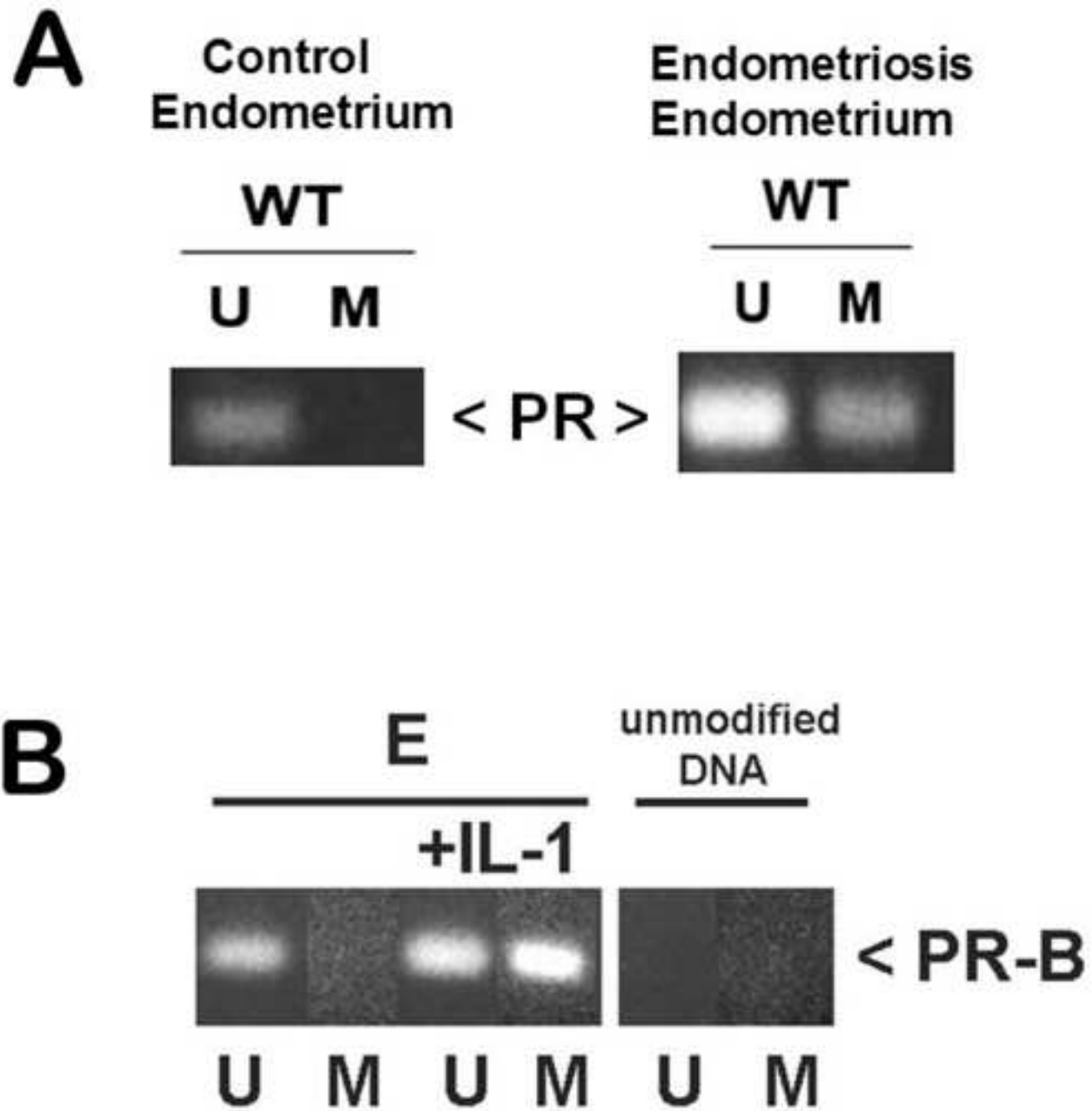


Figure 4
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