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Inhibition of estrogen-mediated uterine gene expression responses by dioxin

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Running Title: Estrogen-TCDD uterine gene expression cross-talk

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Abbreviations
TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DREs, dioxin response elements; EE, ethynyl estradiol; ER, estrogen receptor

ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) exhibits antiestrogenic properties including the inhibition of estrogen-induced uterine growth and proliferation. The inhibition of estrogen-mediated gene expression through ER/AhR crosstalk has been proposed as a plausible mechanism, however, only a limited number of inhibited responses have been investigated which are unlikely to fully account for the anti-uterotrophic effects of TCDD. Therefore, the effects of TCDD on ethynyl estradiol (EE) mediated uterine gene expression were investigated using cDNA microarrays with complementary physiological and histological phenotypic anchoring. Mice were gavaged with either vehicle, 3 daily doses of 10 µg/kg EE, a single dose of 30 µg/kg TCDD or a combination of EE plus TCDD and sacrificed after 4, 12, 24 and 72 hours. TCDD cotreatment inhibited EE induced uterine wet weight by 37, 23 and 45% at 12, 24 and 72 hrs, respectively. TCDD cotreatment also reduced EE-mediated stromal edema, hypertrophy and hyperplasia and induced marked luminal epithelial cell apoptosis. A 2×2 factorial microarray design was used to identify EE- and TCDD-specific differential gene expression responses as well as their interactive effects. Only 133 of the 2,753 EE-mediated differentially expressed genes were significantly modulated by TCDD cotreatment, indicating a gene-specific inhibitory response. The EE-mediated induction of many genes, including trefoil factor 1 and keratin 14, were inhibited by greater than 90% by TCDD. Functional annotation of inhibited responses was associated with cell proliferation, water and ion transport, and maintenance of cellular structure and integrity. These inhibited responses correlate with the observed histological alterations and may contribute to the anti-uterotrophic effects of TCDD.

INTRODUCTION

Estrogens regulate growth, development and reproductive function in both males and females and have been implicated in the etiology of breast and endometrial cancers (Hewitt et al., 2005). Many estrogen effects are mediated through the estrogen receptor (ER), a ligand-activated transcription factor and member of the nuclear receptor superfamily (Nilsson et al., 2001). In the classical mechanism, ligand binding to the ER results in dissociation from heat shock and chaperone proteins, homodimerization, and interaction with regulatory elements near estrogen responsive genes known as estrogen response elements (EREs)(Klinge, 2001). However, the activated ER can also mediate effects via interactions with Fos/Jun at AP-1 sites, Sp1 at GC rich promoter regions (Hall et al., 2001; Nilsson et al., 2001), and through ligand-independent, DNA binding-independent and cell-surface (nongenomic) signaling mechanisms (Hall et al., 2001). These ER-mediated alterations in gene expression and signaling pathways are responsible for the subsequent molecular and physiological responses to estrogens.

Like the ER, the aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor but is a member of the basic-helix-loop-helix-PAS (bHLH-PAS) family of transcription factors. The AhR is responsible for mediating many, if not all, of the toxic and biochemical responses to TCDD and related compounds. These include a wasting syndrome, tumor promotion, teratogenesis, hepatotoxicity, immunotoxicity and modulation of endocrine systems, which are mediated in a tissue-, sex-, age- and species-specific manner (Denison and Heath-Pagliuso, 1998; Poland and Knutson, 1982). The proposed mechanism involves ligand binding to the cytoplasmic AhR and translocation to the nucleus where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the bHLH-PAS family. This complex then binds specific DNA elements, termed dioxin response elements (DREs), in

the regulatory regions of target genes leading to changes in gene expression (Hankinson, 1995). Although the mechanisms of AhR/ARNT-mediated changes in gene expression are well established, how changes in gene expression results in the subsequent physiological and toxicological effects remains poorly understood.

As part of its repertoire of effects, TCDD exhibits antiestrogenic activity in the rodent female reproductive tract including the inhibition of estrogen induced increases in cellular growth and proliferation, uterine wet weight, DNA synthesis, and gene expression responses (Astroff et al., 1991; Buchanan et al., 2002; Gallo et al., 1986; Umbreit et al., 1988). Chronic administration decreased the incidence of both mammary and uterine tumors in female rats suggesting TCDD inhibits the development of estrogen-dependent tumors (Kociba et al., 1978). These effects are not mediated through TCDD binding to the ER (Klinge et al., 1999), but appear to involve AhR-ER cross-talk. Results from *in vitro* and *in vivo* studies have led to a number of proposed antiestrogenic mechanisms for TCDD including increased estrogen metabolism, decreased estrogen receptor levels, induction of inhibitory factors, competition for cofactors, and the inhibition of estrogen-induced gene expression through interactions at estrogen responsive promoters (reviewed in (Safe and Wormke, 2003)).

Only a limited number of estrogen responsive genes which are inhibited by TCDD have been identified to support the ER-AhR gene expression crosstalk mechanism (Duan et al., 1999; Gillesby et al., 1997; Krishnan et al., 1995; Porter et al., 2001). However, a comprehensive assessment of inhibitory gene expression responses and their relationship to *in vivo* antiestrogenic endpoints has not been investigated. Therefore, to further examine the inhibitory effects of TCDD on estrogen-mediated uterine gene expression, temporal gene expression responses to EE, TCDD, and EE plus TCDD were investigated   Results indicate that the

inhibitory effect of TCDD on EE-induced uterotrophy is associated with the selective inhibition of EE-mediated gene expression responses.

MATERIALS AND METHODS

Animal Treatments

Female C57BL/6 mice, ovariectomized on post-natal day 20 were obtained from Charles River Laboratories (Raleigh, NC) on PND 25. Animals were housed in polycarbonate cages containing cellulose fiber chip bedding (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) and maintained at 40-60% humidity and 23°C on a 12 hr dark/light cycle (7am-7pm). Animals were provided free access to de-ionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI), and acclimatized for 4 days prior to treatment.

Animals (n= 5/treatment group/time point) were orally gavaged at time zero with sesame oil vehicle (Sigma Chemical, St Louis, MO), TCDD (provided by S. Safe, Texas A&M University, College Station, TX), 17α-ethynylestradiol (EE, Sigma Chemical) or a combination of EE plus TCDD followed by additional doses of vehicle (vehicle and TCDD groups) or EE (EE and EE+TCDD groups) at 24 and 48 hrs as per the uterotrophic assay (Figure 1). Doses of 10 and 30 µg/kg EE and TCDD, respectively, were empirically determined to elicit an optimal inhibitory effect on the EE-mediated induction of uterine weight in cotreatment studies (data not shown). Mice were sacrificed by cervical dislocation 4, 12, 24 or 72 hrs after dosing. Uterine weights were recorded before (wet) and after (blotted) blotting with absorbent tissue. A section of the left uterine horn was removed for histology and fixed in 10% neutral buffered formalin (NBF, Sigma). The remaining tissue was subsequently snap-frozen in liquid nitrogen and stored at -80°C. All doses were calculated based on average weights of the animals prior to dosing. All

procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

Histological Processing and Assessment

Fixed uteri were embedded in paraffin according to standard histological techniques. Five μm cross-sections were mounted on glass slides and stained with hematoxylin and eosin. Embedding, mounting and staining were performed at the Histology/Immunohistochemistry Laboratory, Michigan State University (http://humanpathology.msu.edu/histology/index.html). Histological slides were evaluated according to standardized National Toxicology Program (NTP) pathology codes. Morphometric analyses were performed for each sample using image analysis software (Scion Image, Scioncorp, Frederick, Maryland) and standard morphometric techniques. The length of basal lamina underlying the luminal epithelium (LE) and corresponding area of the luminal epithelial cells (LECs) was quantified for multiple representative sectors of each section to calculate LEC height.

RNA Isolation

Total RNA was isolated from uteri using Trizol Reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Uteri were removed from -80˚C storage and immediately homogenized in 1 mL Trizol Reagent using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was resuspended in The RNA Storage Solution (Ambion, Austin, TX). RNA concentrations were calculated by spectrophotometric methods (A_{260}) and purity assessed by the A_{260}:A_{280} ratio and visual inspection of 1μg on a denaturing gel.

Microarray Experimental Design and Protocols

Spotted mouse cDNA microarrays were prepared in-house and consist of 13,361 features, representing 7,948 unique genes (Unigene Build #144). Detailed protocols for microarray

construction, labeling of the cDNA probe, sample hybridization and slide washing can be found
at http://dbzach.fst.msu.edu/interfaces/microarray.html. Briefly, PCR amplified DNA was
robotically arrayed onto epoxy coated glass slides (Schott-Nexterion, Duryea, PA) using an
Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 48 (12 x 4) Chipmaker 2 pins
(Telechem) at the Genomics Technology Support Facility at Michigan State University
(http://www.genomics.msu.edu). Changes in uterine gene expression were assessed using a 2 ×
2 factorial design (Figure 2) (Yang and Speed, 2002). In this design, arrow bases represent
samples labeled with Cy3 and arrow heads represent samples labeled with Cy5. Within each
replicate a sample is labeled and hybridized on three independent arrays for a total of 6
arrays/replicate/time point. Three biological replicates were completed at each time point for a
total of 72 microarrays.   A 3DNA Array 900 Expression Array Detection Kit (Genisphere,
Hatsfield, PA) using 1.0 μg of total RNA was used for probe labeling in all microarray
experiments, according to manufacturer's specifications.   Samples were hybridized for 18–24
hrs at 42°C in a water bath. Slides were then washed, dried by centrifugation and scanned at 635
(Cy5) and 532 nm (Cy3) on an Affymetrix 428 Array Scanner (Santa Clara, CA). Images were
analyzed for feature and background intensities using GenePix Pro 5.0 (Molecular Devices,
Union City, CA).

 Array Data Normalization and Statistical Analysis

Data were normalized using a semi-parametric approach (Eckel et al., 2005). Model-
based t-values were calculated from normalized data, comparing treated to vehicle responses per
time-point. Empirical Bayes analysis was used to calculate posterior probabilities of activity
(P1(t)-value) on a per gene and time-point basis using the model-based t-value (Eckel et al.,
2004). Gene lists were filtered for activity based on the P1(t)-value which indicates a greater

likelihood of activity as the value approaches 1.0. A conservative P1(t) cutoff of 0.9999 combined with a differential expression of ±1.5-fold relative to time matched vehicle controls (TMVC) was used to filter the expression data and to define active gene lists. All arrays were subjected to quality control assessment to ensure assay performance and data consistency (Burgoon et al., 2005). Data are stored within dbZach (http://dbzach.fst.msu.edu), a MIAME supportive relational database that ensures proper data management and facilitates data analysis (Burgoon et al., 2006). Complete data sets with annotation and P1(t) values are available in Supplementary Table 1. Gene expression patterns that passed the established threshold criteria were visualized using hierarchical clustering (GeneSpring 6.0, Silicon Genetics, Redwood City, CA).

Quantitative Real-Time PCR (QRTPCR) Analysis

For each sample, 1.0µg of total RNA was reverse transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen). The resultant cDNA (1.0µl) was used as the template in a 30 µl PCR reaction containing 0.1 µM each of forward and reverse gene-specific primers, designed using Primer3 (Rozen and Skaletsky, 2000), 3 mM MgCl$_2$, 1.0mM dNTPs, 0.025 IU AmpliTaq Gold and 1x SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). Gene names, accession numbers, forward and reverse primer sequences and amplicon sizes are listed in Supplementary Table 2. PCR amplification was conducted in MicroAmp Optical 96-well reaction plates (Applied Biosystems) on an Applied Biosystems PRISM 7000 Sequence Detection System using the following conditions: initial denaturation and enzyme activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation protocol was performed to assess the specificity of the primers and the uniformity of the PCR generated products. Each plate contained duplicate

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standards of purified PCR products of known template concentration covering six orders of

magnitude to interpolate relative template concentrations of the samples from the standard curves

of log copy number versus threshold cycle (Ct). No template controls (NTC) were also included

on each plate. Samples with a Ct value within 2 SD of the mean Ct values for the NTCs were

considered below the limits of detection. The copy number of each unknown sample for each

gene was standardized to Rpl7 to control for differences in RNA loading, quality and cDNA

synthesis (Couse et al., 1995). Statistical significance of differentially expressed genes was

determined using two-way ANOVA followed by a Tukey's post hoc test (SAS 9.1, SAS Institute,

Cary, NC). For graphing purposes, the relative expression levels were scaled such that the

expression level of the time-matched vehicle control was equal to one.

RESULTS

Uterine and Hepatic Weights

Increases in uterine weight due to water imbibition, hypertrophy and hyperplasia are well-characterized responses to estrogenic compounds and serve as the basis of the uterotrophic assay (Diel et al., 2002). EE induced an expected increase in uterine wet and blotted weights at 12, 24 and 72 hrs, relative to the TMVC, while TCDD elicited no effect (Figure 3A and B).). Cotreatment of mice with EE and TCDD significantly ($p<0.05$) inhibited EE-mediated induction of uterine wet weight by 37, 23 and 45% at 12, 24 and 72 hrs, respectively (Figure 3A). Blotted uterine weights were also inhibited at levels of 71, 38 and 30% at 12, 24 and 72 hrs, respectively (Figure 3B). These results confirm previous reports of the antiestrogenic effects of TCDD on the inhibition of EE-mediated induction of uterine weight in the standard uterotrophic assay (Astroff et al., 1991; Gallo et al., 1986; Umbreit et al., 1988)

Histopathology and Morphometry

Treatment of mice with EE resulted in the expected complex uterine histology consisting of minimal stromal edema at 4 hrs which progressed to moderate severity by 12 hrs. At 24 hrs moderate epithelial cell hypertrophy and hyperplasia with moderate stromal edema were observed which progressed to marked epithelial and stromal hypertrophy and hyperplasia with mild stromal edema at 72 hrs (Figure 4). Cotreatment of EE and TCDD resulted in comparable histological effects when compared to EE treatment alone with the exception of reduced stromal edema at 12, 24 and 72 hrs, subnuclear vacuolization in epithelial cells at 24 hrs, and reduced stromal hypertrophy and hyperplasia with marked luminal epithelial cell (LEC) apoptosis at 72 hrs (Figure 4). The LEC layer effects are consistent with previous reports of TCDD in the

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murine uterus (Gallo et al., 1986). These alterations in uterine histology may contribute to the associated decreases in uterine weight observed with TCDD cotreatment.

Treatment with TCDD alone exhibited negligible effects when compared to vehicle controls. Inconsistent responses of minimal stromal edema at 12 hrs, minimal stromal hypertrophy at 24 hrs and minimal subnuclear vacuolization at 12 and 24 hrs were noted. No significant differences were noted between TCDD and vehicle samples at 4 and 72 hrs.

Increased luminal epithelial cell height (LECH) is a well-recognized marker of estrogen exposure and has been used to assess the estrogenicity of a number of structurally diverse ligands (Nakagawa and Tayama, 2001; O'Connor et al., 1996). EE-induced LECH at 24 and 72 hrs, as previously reported (Kwekel et al., 2005). Cotreatment with TCDD did not inhibit this response. The inability to detect antiestrogenic effects on LECH may indicate that TCDD does not influence this response. Alternatively, it may be attributed technical difficulties in the measurement of such a change due to the complex pseudostratified nature of the proliferating LE cells combined with the histopathological alterations induced by TCDD on this cell layer.

Microarray Data Filtering and Clustering

Microarray analyses were preformed using a 2×2 factorial design which allowed for the identification of differentially expressed genes following EE and TCDD treatment alone as well as the interactive effects of EE+TCDD when compared to the single treatment groups (Yang and Speed, 2002). A conservative statistical P1(t) cutoff of 0.9999 combined with a differential expression of ±1.5 fold relative to TMVCs was used to identify lists of differentially active genes. Gene expression responses to EE alone displayed the expected complex transcriptional profile as previously reported (Fertuck et al., 2003; Kwekel et al., 2005) with a total of 3,746 features, representing 2,753 unique genes, identified as differentially expressed at one or more

time points. The number and magnitude of uterine gene expression responses elicited by TCDD were modest compared to EE. 793 features representing 628 unique genes were found to be differentially expressed in response to TCDD. EE+TCDD cotreatment resulted in an overall gene expression response similar to that of EE alone, with a total of 3,631 features representing 2,647 unique genes identified as differentially expressed at one or more time points.

To compare the global gene expression responses of EE, TCDD and EE+TCDD, hierarchical clustering was performed on features which were differentially expressed in any of the treatment groups at any time point relative to the TMVCs. Visualization of the global responses for each treatment group revealed that the temporal expression pattern of EE+TCDD was essentially indistinguishable from EE alone (Figure 5A). In addition, TCDD displayed similarities to EE, consistent with our previous studies describing the estrogen-like gene expression profile of TCDD (Boverhof et al., 2006). Clustering by treatment and time point further revealed the temporal similarity of the EE and EE+TCDD treatment groups as each of their gene expression time points clustered with one another (Figure 5B). The 12 hr gene expression responses to TCDD, EE and EE+TCDD also clustered together, further demonstrating the estrogen-like response to TCDD at this time point.

Although these clustering approaches illustrate the similarity of gene expression patterns across treatments, they do not adequately demonstrate variations in the magnitudes of the responses between treatments, an important consideration when examining the inhibition of EE-mediated gene expression by TCDD. Scatter plots of the log_2 expression ratios of EE versus EE+TCDD revealed that most responses were of the same magnitude (Figure 6A). However, a small subset of genes were more highly expressed in the EE treated group, suggesting inhibition upon cotreatment with TCDD. Similar comparisons between EE and TCDD groups revealed

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low correlations, indicating differences in the magnitudes of the response despite similar expression patterns (Figure 6B).

Genes Differentially Regulated by EE and EE+TCDD Treatments

Genes identified as differentially expressed by EE+TCDD cotreatment when compared to EE treatment alone were identified and investigated further. To be considered in this category two successive criteria were required to be met. First, these genes needed to exhibit differential expression following: EE treatment relative to TMVCs, and second, these genes needed to exhibit differential gene expression following EE+TCDD cotreatment relative to EE. This approach identified genes regulated by EE which were subsequently modulated upon cotreatment with TCDD and identified 163 features representing 133 EE-regulated genes at one or more time points. In many cases, EE-mediated gene expression responses were inhibited by greater than 80% upon cotreatment with TCDD. The data also indicate that only a select number of EE-mediated gene expression responses experience inhibition following cotreatment with TCDD with most EE responsive genes being unaffected by the cotreatment. On a per time point basis, 9, 23 , 32 and 130 features representing 5, 21, 28, and 106 genes were inhibited by TCDD cotreatment at 4, 12, 24 and 72 hrs, respectively. This indicates a time-dependent increase in the inhibitory effects of TCDD on EE-mediated gene expression responses suggesting direct early primary responses may subsequently mediate more extensive secondary and tertiary indirect inhibitory responses.

A small number of gene expression responses were differentially expressed between the EE+TCDD and EE groups but were not EE-regulated responses, rather they were differentially expressed due to TCDD alone. This included the well-characterized induction of Cyp1a1 as well as the induction of inhibitor of growth 1 (Ing1), karyopherin alpha 6 (Kpna6), and

replication protein A2 (Rpa2). The induction of these genes cannot be dismissed as a contributing factor to the antiestrogenic effects of TCDD as the induction of inhibitory factors is a previously proposed mechanism (Rogers and Denison, 2002).

Functional Categorization of Microarray Data

Functional annotation of gene expression responses was performed using data extracted from public databases and published literature. The functions of EE differentially expressed genes have been associated with transcription factors, mRNA and protein synthesis, cell cycle regulation, cellular proliferation, energetics and structural constituents (Fertuck et al., 2003; Kwekel et al., 2005; Moggs et al., 2004). Functional annotation of EE-mediated gene expression responses which were inhibited upon cotreatment with TCDD were associated with the regulation of cell proliferation and growth, water/ion transport and the maintenance of cellular structural architecture (Table 1). Cellular growth and proliferation genes included branched chain aminotransferase 1 (Bcat), serine proteinase inhibitor B5 (Serpinb5), sestrin 1 (Sesn1), stratifin (Sfn), and trefoil factor 1 (Tff1). Inhibition of this functional category is consistent with previous reports of decreased cellular growth responses in breast and endometrial cancer cell lines (Castro-Rivera et al., 1999; Puga et al., 2000; Wang et al., 1998; Wormke et al., 2000) and uterine tissue (Buchanan et al., 2002). TCDD inhibited water and ion transport genes included aquaporins 1 and 3, (Aqp1 and 3) solute carriers 4a2, 38a3 and 40a1 (Slc4a2, 38a3 and 40a1), and FXYD ion transport regulator 4 (Fxyd4). Inhibition of these responses may contribute to TCDD-mediated decreases in stromal edema and uterine wet weight. Desmocollin 2 (Dsc2), keratins 4, 7, 14 and 19 (Krt2-4, Krt2-7, Krt1-14 and Krt1-19), macrophage receptor with collagenous structure (Marco), TP53 apoptosis effector (Perp), and small proline-rich protein 2A (Sprr2a) represent structural genes inhibited by TCDD. Collectively, the inhibition of these EE-

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mediated responses may contribute to the antiestrogenic effects of TCDD on uterine histology, growth and LEC integrity.

Verification of Microarray Results

QRTPCR was used to verify changes in transcript levels for a selected subset of EE-inducible genes inhibited by TCDD (Figure 7). There was good agreement between the microarray and QRTPCR results although compression of the gene expression response was observed in the microarray data, which has been previously reported when comparing microarray analysis to other methods (Yuen et al., 2002). QRTPCR revealed Tff1 transcripts were induced greater than 400-fold by EE at 12 and 24 hrs while cotreatment with TCDD inhibited this response by greater than 90%. Similar confirmatory responses were noted for Dsc2, Krt1-14, Sprr2a, and Sfn which were maximally induced 20-, 171-, 206- and 6.8 –fold by EE treatment and inhibited 95, 90, 83 and 93 %, respectively, by TCDD. QRTPCR was also used to verify the EE induction of proliferating cell nuclear antigen (Pcna) and solute carrier family 25, member 5 (Slc25a5) as microarray analysis indicated these genes were not affected by TCDD cotreatment. Induction levels of Pcna and Slc25a5 by EE and EE+TCDD were comparable, verifying that TCDD inhibited select EE induced responses.

Four estrogen induced genes, including Fos (Duan et al., 1999), Ctsd (Krishnan et al., 1995), Hsp27 (Porter et al., 2001), and Tff1 (also known as pS2) (Gillesby et al., 1997), have been reported to be inhibited following TCDD cotreatment in human MCF-7 cells. The dramatic inhibition of EE-induced uterine Tff1 transcript levels by TCDD suggests cross-species (human to mouse), cross-model (*in vivo* to *in vitro*), and cross-tissue (breast to uterus) conservation of this response. To further investigate the conservation of these responses, the effect of TCDD on the remaining three transcripts was also investigated by QRTPCR. EE significantly induced Fos,

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Ctsd, and Hsp27 transcript levels in the mouse uterus, however, TCDD cotreatment did not inhibit the induction of these genes (data not shown), suggesting the inhibitory effects of TCDD on these genes may be model specific.

DISCUSSION

The present study was conducted to further elucidate the anti-uterotrophic effects of TCDD. TCDD significantly inhibited EE induced uterine weight and altered the integrity of the LEC layer, consistent with previous reports (Astroff and Safe, 1988; Gallo et al., 1986; Umbreit et al., 1988). Comparison of EE and EE+TCDD gene expression responses revealed that the majority of EE-mediated changes were unaffected by cotreatment. However, a subset of EE-responsive genes was inhibited upon cotreatment with TCDD suggesting a gene-specific inhibitory response. The inhibited genes are involved in cell proliferation, growth and differentiation, water and ion transport, and maintenance of cellular structure and integrity and were consistent with the observed histological alterations.

Inhibition of Cellular Growth and Proliferation Responses

Estrogen induction of uterine weight involves a coordinated proliferative response which is mediated through a well-orchestrated series of changes in gene expression (Fertuck et al., 2003; Kwekel et al., 2005; Moggs et al., 2004). Cotreatment with TCDD disrupted several EE-induced genes with important functions in cell cycle regulation, growth and proliferation. For example, EE-mediated induction of Bcat and Sfn, important regulators of cell cycle progression, was inhibited by TCDD between 12 and 72 hrs. Bcat regulates G1 to S phase transition and cells with reduced expression exhibit faster growth rates, a shorter G1 stage and an increased frequency of mutations (Schuldiner et al., 1996). Sfn serves as a G2 checkpoint component as a

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positive mediator of growth-factor-induced cell cycle progression (Hermeking et al., 1997; Zhang et al., 2004). TCDD also inhibited EE induction of Serpinb5 at 12, 24 and 72 hrs which plays an essential role in development, as exhibited by embryonic lethality in knockout mice (Gao et al., 2004), while decreased expression results in reduced cellular proliferation and adhesion (Gao et al., 2004). Sesn1 is a positive regulator of cell growth and protects against apoptosis (Budanov et al., 2004; Velasco-Miguel et al., 1999), and was also inhibited by TCDD cotreatment. Additional EE-induced genes implicated in cellular growth, proliferation and development included retinol binding protein 2, Tnfsf8 and Vezf1, which were also inhibited upon cotreatment between 12 and 72 hrs. The inhibition of genes involved in regulating cell cycle progression is consistent with a previous study, however, the inhibition of estrogen-induced cyclin transcripts was not detected which may be attributed to different experimental treatments and time points (Buchanan et al., 2002). Overall, the alteration of these responses may be a contributing factor to the observed reduction in stromal cell hypertrophy and hyperplasia as well as the marked LEC apoptosis.

One of the most dramatic TCDD-inhibited responses was that of Tff1 which plays a fundamental role in epithelial maintenance, protection and regeneration (Lefebvre et al., 1996; Playford et al., 1996). Tffs block p53-dependent and independent pathways of apoptosis and promote growth and regeneration by allowing cells to break attachments with the basement membrane to replace epithelial defects without cell death (Hoffmann et al., 2001). Tff peptides also have anti-inflammatory actions and protect the epithelial mucous layers (Hoffmann et al., 2001; Vieten et al., 2005). Therefore, TCDD's inhibition of EE-induced Tff1 by greater than 90% may play an important role in the increased LEC degeneration and apoptosis.

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Tff1 is a also a prognostic marker in human breast cancer and is an estrogen responsive gene in breast cancer cells and the human endometrium (Gillesby and Zacharewski, 1999; Punyadeera et al., 2005). The inhibitory effect of TCDD on estrogen induction of Tff1 has been characterized in human MCF-7 breast cancer cells and is dependent on an inhibitory DRE (iDRE) which interferes with AP-1 and ERE mediated transcriptional activation (Gillesby et al., 1997). Tff1 induction and inhibition by TCDD in human MCF-7 breast cancer cells and the mouse uterus indicates that this mechanism may be conserved across sensitive species and tissues. Examination of the mouse promoter region for Tff1 identified a variant ERE at – 475 as well as an AP-1 site at –998 relative to the transcriptional start site. Although a DRE does not overlap with the AP-1 site, two putative DREs are located further upstream at –1920 and –2637 which may play a role in mediating the inhibition.

Water/Ion Transport Responses

EE induced stromal edema which was significantly inhibited by TCDD. Several EE-regulated genes involved in water and ion transport were inhibited by TCDD including the EE-mediated down-regulation of Aqp1 and up-regulation of Aqp3. Isoform-specific regulation of aquaporins plays an integral role in mediating the water imbibition in the uterus (Richard et al., 2003). EE-regulated transcripts involved in sodium and chloride transport were also inhibited by TCDD including Slc4a2, Slc38a3 and Fxyd4 (Garty et al., 2003; Quentin et al., 2004). Collectively, the inhibition of these EE-mediated gene expression responses may have contributed to reductions in uterine wet weight following cotreatment with TCDD.

Inhibition of Structural Constituents

The uterus undergoes extensive cytoarchitectural changes to accommodate the dramatic proliferation and growth response to estrogen which involves numerous structural, adhesion and

extracellular matrix genes including a number of keratins, actins, procollagens, tubulins, desmocollins, and small proline-rich proteins in mice, rats and humans (Hewitt et al., 2003; Moggs et al., 2004; Watanabe et al., 2003; Watanabe et al., 2002). TCDD cotreatment inhibited a number of these EE-mediated responses which likely contributed to its anti-uterotrophic effects as well as increases in apoptosis.

In this study, the EE induction of keratins 4, 7, 14 and 19 was significantly inhibited by TCDD cotreatment. Keratins are involved in the formation of the cytoskeleton which consists of an extensive array of filamentous networks. Their disruption results in epithelial cell fragility and lysis (Sorensen et al., 2003; Wong et al., 2005). Keratins 18 and 19 are estrogen inducible transcripts whose induction is blocked by TCDD in MCF-7 cells (Chen et al., 2001). Inhibition of uterine keratin 18 and 19 suggests that this may represent a conserved response between rodents and humans. Moreover, the inhibition of multiple keratin genes suggests that TCDD may disrupt signaling at a common regulatory region as the basic (Krt2-2 through -8) and acidic (Krt1-9 through -19) keratin genes are encoded in a tandem array on chromosomes 15 and 11, respectively (Chu and Weiss, 2002).

Desmocollin 2 (Dsc2), a component of desmosomes, is primarily expressed in epithelial cells and serves an integral role in cell adhesion by forming links with the intermediate filament network (Marsden et al., 1997) and was inhibited by TCDD. TCDD also inhibited Perp induction, which promotes desmosomal complex assembly (Ihrie et al., 2005). EE induction of small proline rich protein 2a (Sprr2a) was also inhibited by cotreatment with TCDD. The Sprr2 family consists of 11 genes (Sprr2a-2k) which are important structural components of epithelial cells due to their ability to form extensive cross-links (Hong et al., 2004). Several Sprr2 genes are up-regulated in the luminal epithelial cells of the uterus in response to estrogen where they

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are important for cytoarchitectural changes (Hong et al., 2004). TCDD also inhibited the induction of other structural molecules including Marco, procollagen 6a2, troponin T1, and tubulin beta 6. Together, the inhibition of these structural constituents could compromise the rapid proliferation and growth induced by EE resulting in altered histology, increased apoptosis and overall decreased uterine growth.

TCDD as estrogen and antiestrogen

Reports have indicated that TCDD elicits an estrogen-like, ER-dependent gene expression profile in the uterus (Boverhof et al., 2006; Ohtake et al., 2003; Watanabe et al., 2004). The regulation of similar genes by EE and TCDD suggests these responses may represent targets for inhibition. However, these genes were largely unaffected by TCDD including well-characterized estrogen responsive genes such as Pcna, Slc25a5, cell division cycle 2 homolog A (Cdc2a) and ornithine decarboxylase (Odc). Instead, many of the inhibited responses were unaffected by TCDD treatment alone, consistent with previous reports of estrogen/TCDD gene expression crosstalk (Porter et al., 2001; Safe and Wormke, 2003; Zacharewski et al., 1994). These data suggest that the anti-uterotrophic effects of TCDD are independent of its weak estrogenic activity.

Decreased ER levels (Romkes et al., 1987) and increased estrogen metabolism (Spink et al., 1994) have also been proposed as mechanisms for the antiestrogenic effects of TCDD. However, several reports indicate that these mechanisms do not account for the antiestrogenic effects of TCDD. TCDD does not increase estrogen metabolism *in vivo* (DeVito et al., 1992; Petroff and Mizinga, 2003) and uterine ER levels were unaffected by TCDD (DeVito et al., 1994; White et al., 1995). In the present study, TCDD inhibited only a select subset of EE responses further indicating that increased metabolism and decreased ER levels are not the

primary mechanisms as these would be expected to elicit a more global effect on gene expression. The gene-specific inhibitory effects may involve an iDRE mechanism, alteration in the activity or availability of gene-specific cofactors, or induction/inhibition of an upstream regulatory protein that are likely to be tissue and species-specific.

Summary

The present study has identified a small subset of EE- induced uterine gene expression responses that are inhibited by TCDD. Moreover, the repressed functional categories can be related to the observed histological and physiological responses and therefore represent potential mediators of TCDD's anti-uterotrophic effect. Select responses, including Tff1, are also in agreement with *in vitro* studies, indicating the potential conservation of these responses between different models and species. Furthermore, the results indicate that the estrogenic and antiestrogenic gene expression effects of TCDD are independent. The importance of these genes in the uterotrophic response as targets for the antiestrogenicity of TCDD can be addressed through comparative studies with rats and mice and through receptor- and gene-specific null mice. Additional research is also required to fully delineate the mechanism of the gene specific inhibitory response and should employ molecular approaches including receptor interaction (fluorescence resonance energy transfer), chromatin immunoprecipitation, promoter dissection/reporter gene assays and siRNA approaches in appropriate *in vitro* models (e.g., uterine based cell lines, primary cells or co-cultured primary systems) that closely simulate *in vivo* conditions. These approaches will more comprehensively elucidate the dual nature of TCDD as an estrogenic and antiestrogenic compound.

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Acknowledgements

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References

Astroff B, Eldridge B and Safe S (1991) Inhibition of the 17 beta-estradiol-induced and constitutive expression of the cellular protooncogene c-fos by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the female rat uterus. *Toxicol Lett* **56**(3):305-315.

Astroff B and Safe S (1988) Comparative antiestrogenic activities of 2,3,7,8-tetrachlorodibenzo-p-dioxin and 6-methyl-1,3,8-trichlorodibenzofuran in the female rat. *Toxicol Appl Pharmacol* **95**(3):435-443.

Boverhof DR, Kwekel JC, Humes DG, Burgoon LD and Zacharewski TR (2006) Dioxin induces an estrogen-like, estrogen receptor-dependent gene expression response in the murine uterus. *Mol Pharmacol* **69**(5):1599-1606.

Buchanan DL, Ohsako S, Tohyama C, Cooke PS and Iguchi T (2002) Dioxin inhibition of estrogen-induced mouse uterine epithelial mitogenesis involves changes in cyclin and transforming growth factor-beta expression. *Toxicol Sci* **66**(1):62-68.

Budanov AV, Sablina AA, Feinstein E, Koonin EV and Chumakov PM (2004) Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* **304**(5670):596-600.

Burgoon LD, Boutros PC, Dere E and Zacharewski TR (2006) dbZach: A MIAME-Compliant Toxicogenomic Supportive Relational Database. *Toxicol Sci* **90**(2):558-568.

Burgoon LD, Eckel-Passow JE, Gennings C, Boverhof DR, Burt JW, Fong CJ and Zacharewski TR (2005) Protocols for the assurance of microarray data quality and process control. *Nucl Acids Res* **33**(19):e172-.

Castro-Rivera E, Wormke M and Safe S (1999) Estrogen and aryl hydrocarbon responsiveness of ECC-1 endometrial cancer cells. *Mol Cell Endocrinol* **150**(1-2):11-21.

Chen I, Hsieh T, Thomas T and Safe S (2001) Identification of estrogen-induced genes downregulated by AhR agonists in MCF-7 breast cancer cells using suppression subtractive hybridization. *Gene* **262**(1-2):207-214.

Chu PG and Weiss LM (2002) Keratin expression in human tissues and neoplasms. *Histopathology* **40**(5):403-439.

Couse J, Curtis S, Washburn T, Lindzey J, Golding T, Lubahn D, Smithies O and Korach K (1995) Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. *Mol Endocrinol* **9**(11):1441-1454.

Denison MS and Heath-Pagliuso S (1998) The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bull Environ Contam Toxicol* **61**(5):557-568.

DeVito MJ, Ma X, Babish JG, Menache M and Birnbaum LS (1994) Dose-response relationships in mice following subchronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: CYP1A1, CYP1A2, estrogen receptor, and protein tyrosine phosphorylation. *Toxicol Appl Pharmacol* **124**(1):82-90.

DeVito MJ, Thomas T, Martin E, Umbreit TH and Gallo MA (1992) Antiestrogenic action of 2,3,7,8-tetrachlorodibenzo-p-dioxin: tissue-specific regulation of estrogen receptor in CD1 mice. *Toxicol Appl Pharmacol* **113**(2):284-292.

Diel P, Schmidt S and Vollmer G (2002) In vivo test systems for the quantitative and qualitative analysis of the biological activity of phytoestrogens. *J Chromatogr B Analyt Technol Biomed Life Sci* **777**(1-2):191-202.

Duan R, Porter W, Samudio I, Vyhlidal C, Kladde M and Safe S (1999) Transcriptional activation of c-fos protooncogene by 17beta-estradiol: mechanism of aryl hydrocarbon receptor-mediated inhibition. *Mol Endocrinol* **13**(9):1511-1521.

Eckel JE, Gennings C, Chinchilli VM, Burgoon LD and Zacharewski TR (2004) Empirical bayes gene screening tool for time-course or dose-response microarray data. *J Biopharm Stat* **14**(3):647-670.

Eckel JE, Gennings C, Therneau TM, Burgoon LD, Boverhof DR and Zacharewski TR (2005) Normalization of two-channel microarray experiments: a semiparametric approach. *Bioinformatics* **21**(7):1078-1083.

Fertuck KC, Eckel JE, Gennings C and Zacharewski TR (2003) Identification of temporal patterns of gene expression in the uteri of immature, ovariectomized mice following exposure to ethynylestradiol. *Physiol Genomics* **15**(2):127-141.

Gallo MA, Hesse EJ, Macdonald GJ and Umbreit TH (1986) Interactive effects of estradiol and 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic cytochrome P-450 and mouse uterus. *Toxicol Lett* **32**(1-2):123-132.

Gao F, Shi HY, Daughty C, Cella N and Zhang M (2004) Maspin plays an essential role in early embryonic development. *Development* **131**(7):1479-1489.

Garty H, Lindzen M, Fuzesi M, Aizman R, Goldshleger R, Asher C and Karlish SJ (2003) A specific functional interaction between CHIF and Na,K-ATPase: role of FXYD proteins in the cellular regulation of the pump. *Ann N Y Acad Sci* **986**:395-400.

Gillesby BE, Stanostefano M, Porter W, Safe S, Wu ZF and Zacharewski TR (1997) Identification of a motif within the 5' regulatory region of pS2 which is responsible for AP-1 binding and TCDD-mediated suppression. *Biochemistry* **36**(20):6080-6089.

Gillesby BE and Zacharewski TR (1999) pS2 (TFF1) levels in human breast cancer tumor samples: correlation with clinical and histological prognostic markers. *Breast Cancer Res Treat* **56**(3):253-265.

Hall JM, Couse JF and Korach KS (2001) The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem* **276**(40):36869-36872.

Hankinson O (1995) The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35**:307-340.

Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW and Vogelstein B (1997) 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* **1**(1):3-11.

Hewitt SC, Deroo BJ, Hansen K, Collins J, Grissom S, Afshari CA and Korach KS (2003) Estrogen Receptor Dependent Genomic Responses in the Uterus Mirror the Biphasic Physiological Response to Estrogen. *Mol Endocrinol* **17**(10):2070-2083.

Hewitt SC, Harrell JC and Korach KS (2005) Lessons in estrogen biology from knockout and transgenic animals. *Annu Rev Physiol* **67**:285-308.

Hoffmann W, Jagla W and Wiede A (2001) Molecular medicine of TFF-peptides: from gut to brain. *Histol Histopathol* **16**(1):319-334.

Hong SH, Nah HY, Lee JY, Lee YJ, Lee JW, Gye MC, Kim CH, Kang BM and Kim MK (2004) Estrogen regulates the expression of the small proline-rich 2 gene family in the mouse uterus. *Mol Cells* **17**(3):477-484.

Ihrie RA, Marques MR, Nguyen BT, Horner JS, Papazoglu C, Bronson RT, Mills AA and Attardi LD (2005) Perp Is a p63-Regulated Gene Essential for Epithelial Integrity. *Cell* **120**(6):843-856.

Klinge CM (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res* **29**(14):2905-2919.

Klinge CM, Bowers JL, Kulakosky PC, Kamboj KK and Swanson HI (1999) The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements. *Mol Cell Endocrinol* **157**(1-2):105-119.

Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA and Humiston CG (1978) Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. *Toxicol Appl Pharmacol* **46**(2):279-303.

Krishnan V, Porter W, Santostefano M, Wang X and Safe S (1995) Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in MCF-7 cells. *Mol Cell Biol* **15**(12):6710-6719.

Kwekel JC, Burgoon LD, Burt JW, Harkema JR and Zacharewski TR (2005) A Cross-Species Analysis of the Rodent Uterotrophic Program: Elucidation of Conserved Responses and Targets of Estrogen Signaling. *Physiol Genomics*.

Lefebvre O, Chenard M-P, Masson R, Linares J, Dierich A, LeMeur M, Wendling C, Tomasetto C, Chambon P and Rio M-C (1996) Gastric Mucosa Abnormalities and Tumorigenesis in Mice Lacking the pS2 Trefoil Protein. *Science* **274**(5285):259-262.

Marsden MD, Collins JE, Greenwood MD, Adams MJ, Fleming TP, Magee AI and Buxton RS (1997) Cloning and transcriptional analysis of the promoter of the human type 2 desmocollin gene (DSC2). *Gene* **186**(2):237-247.

Moggs JG, Tinwell H, Spurway T, Chang HS, Pate I, Lim FL, Moore DJ, Soames A, Stuckey R, Currie R, Zhu T, Kimber I, Ashby J and Orphanides G (2004) Phenotypic anchoring of gene expression changes during estrogen-induced uterine growth. *Environ Health Perspect* **112**(16):1589-1606.

Nakagawa Y and Tayama K (2001) Estrogenic potency of benzophenone and its metabolites in juvenile female rats. *Arch Toxicol* **75**(2):74-79.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M and Gustafsson J-A (2001) Mechanisms of Estrogen Action. *Physiol Rev* **81**(4):1535-1565.

O'Connor JC, Cook JC, Craven SC, Van Pelt CS and Obourn JD (1996) An in vivo battery for identifying endocrine modulators that are estrogenic or dopamine regulators. *Fundam Appl Toxicol* **33**(2):182-195.

Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanagisawa J, Fujii-Kuriyama Y and Kato S (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* **423**(6939):545-550.

Petroff BK and Mizinga KM (2003) Pharmacokinetics of ovarian steroids in Sprague-Dawley rats after acute exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Reprod Biol* **3**(2):131-141.

Playford RJ, Marchbank T, Goodlad RA, Chinery RA, Poulsom R and Hanby AM (1996) Transgenic mice that overexpress the human trefoil peptide pS2 have an increased resistance to intestinal damage. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **93**(5):2137-2142.

Poland A and Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22**:517-554.

Porter W, Wang F, Duan R, Qin C, Castro-Rivera E, Kim K and Safe S (2001) Transcriptional activation of heat shock protein 27 gene expression by 17beta-estradiol and modulation by antiestrogens and aryl hydrocarbon receptor agonists. *J Mol Endocrinol* **26**(1):31-42.

Puga A, Barnes SJ, Dalton TP, Chang C-y, Knudsen ES and Maier MA (2000) Aromatic Hydrocarbon Receptor Interaction with the Retinoblastoma Protein Potentiates Repression of E2F-dependent Transcription and Cell Cycle Arrest. *J Biol Chem* **275**(4):2943-2950.

Punyadeera C, Dassen H, Klomp J, Dunselman G, Kamps R, Dijcks F, Ederveen A, de Goeij A and Groothuis P (2005) Oestrogen-modulated gene expression in the human endometrium. *Cell Mol Life Sci* **62**(2):239-250.

Quentin F, Eladari D, Frische S, Cambillau M, Nielsen S, Alper SL, Paillard M and Chambrey R (2004) Regulation of the Cl-/HCO3- exchanger AE2 in rat thick ascending limb of Henle's loop in response to changes in acid-base and sodium balance. *J Am Soc Nephrol* **15**(12):2988-2997.

Richard C, Gao J, Brown N and Reese J (2003) Aquaporin water channel genes are differentially expressed and regulated by ovarian steroids during the periimplantation period in the mouse. *Endocrinology* **144**(4):1533-1541.

Rogers JM and Denison MS (2002) Analysis of the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human ovarian carcinoma BG-1 cells. *Mol Pharmacol* **61**(6):1393-1403.

Romkes M, Piskorska-Pliszczynska J and Safe S (1987) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic and uterine estrogen receptor levels in rats. *Toxicol Appl Pharmacol* **87**(2):306-314.

Rozen S and Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers, in *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (Krawetz S MS ed) pp 365-386, Humana Press, Totowa, NJ.

Safe S and Wormke M (2003) Inhibitory aryl hydrocarbon receptor-estrogen receptor alpha cross-talk and mechanisms of action. *Chem Res Toxicol* **16**(7):807-816.

Schuldiner O, Eden A, Ben-Yosef T, Yanuka O, Simchen G and Benvenisty N (1996) ECA39, a conserved gene regulated by c-Myc in mice, is involved in G1/S cell cycle regulation in yeast. *Proc Natl Acad Sci U S A* **93**(14):7143-7148.

Sorensen CB, Andresen BS, Jensen UB, Jensen TG, Jensen PK, Gregersen N and Bolund L (2003) Functional testing of keratin 14 mutant proteins associated with the three major subtypes of epidermolysis bullosa simplex. *Exp Dermatol* **12**(4):472-479.

Spink DC, Hayes CL, Young NR, Christou M, Sutter TR, Jefcoate CR and Gierthy JF (1994) The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on estrogen metabolism in MCF-7 breast cancer cells: evidence for induction of a novel 17 beta-estradiol 4-hydroxylase. *J Steroid Biochem Mol Biol* **51**(5-6):251-258.

Umbreit TH, Hesse EJ, Macdonald GJ and Gallo MA (1988) Effects of TCDD-estradiol interactions in three strains of mice. *Toxicol Lett* **40**(1):1-9.

Velasco-Miguel S, Buckbinder L, Jean P, Gelbert L, Talbott R, Laidlaw J, Seizinger B and Kley N (1999) PA26, a novel target of the p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes. *Oncogene* **18**(1):127-137.

Vieten D, Corfield A, Carroll D, Ramani P and Spicer R (2005) Impaired mucosal regeneration in neonatal necrotising enterocolitis. *Pediatr Surg Int* **21**(3):153-160.

Wang W, Smith I, Roger and Safe S (1998) Aryl Hydrocarbon Receptor-Mediated Antiestrogenicity in MCF-7 Cells: Modulation of Hormone-Induced Cell Cycle Enzymes,. *Archives of Biochemistry and Biophysics* **356**(2):239-248.

Watanabe H, Suzuki A, Goto M, Ohsako S, Tohyama C, Handa H and Iguchi T (2004) Comparative uterine gene expression analysis after dioxin and estradiol administration. *J Mol Endocrinol* **33**(3):763-771.

Watanabe H, Suzuki A, Kobayashi M, Takahashi E, Itamoto M, Lubahn DB, Handa H and Iguchi T (2003) Analysis of temporal changes in the expression of estrogen-regulated genes in the uterus. *J Mol Endocrinol* **30**(3):347-358.

Watanabe H, Suzuki A, Mizutani T, Khono S, Lubahn DB, Handa H and Iguchi T (2002) Genome-wide analysis of changes in early gene expression induced by oestrogen. *Genes Cells* **7**(5):497-507.

White TE, Rucci G, Liu Z and Gasiewicz TA (1995) Weanling female Sprague-Dawley rats are not sensitive to the antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Toxicol Appl Pharmacol* **133**(2):313-320.

Wong P, Domergue R and Coulombe PA (2005) Overcoming Functional Redundancy To Elicit Pachyonychia Congenita-Like Nail Lesions in Transgenic Mice. *Mol Cell Biol* **25**(1):197-205.

Wormke M, Castro-Rivera E, Chen I and Safe S (2000) Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. *J Steroid Biochem Mol Biol* **72**(5):197-207.

Yang YH and Speed T (2002) Design issues for cDNA microarray experiments. *Nat Rev Genet* **3**(8):579-588.

Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ and Sealfon SC (2002) Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. *Nucleic Acids Res* **30**(10):e48.

Zacharewski TR, Bondy KL, McDonell P and Wu ZF (1994) Antiestrogenic effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on 17 beta-estradiol-induced pS2 expression. *Cancer Res* **54**(10):2707-2713.

Zhang Y, Karas M, Zhao H, Yakar S and LeRoith D (2004) 14-3-3sigma mediation of cell cycle progression is p53-independent in response to insulin-like growth factor-I receptor activation. *J Biol Chem* **279**(33):34353-34360.

Footnotes

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Figure Legends

Figure 1.
Experimental design for EE+TCDD cotreatment time course study.
An *in vivo* time course study was performed in which immature ovariectomized C57BL/6 mice were orally administered vehicle (sesame oil), 10 µg/kg ethynyl estradiol (EE), 30 µg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or a mixture of EE and TCDD at time zero followed by doses of vehicle (vehicle and TCDD groups) or EE (EE and EE+TCDD groups) at 24 and 48 hrs as per the uterotrophic assay. Mice were sacrificed 4, 12, 24 or 72 hrs after the initial dose at which time uterine tissues were harvested.

Figure 2.
2 × 2 Factorial design utilized for the microarray experiments
A 2×2 factorial design was used to investigate the effects of EE and TCDD alone while also testing for interactive effects between EE and TCDD. Each arrow represents a microarray with the arrow bases representing Cy3 labeled samples and arrow heads Cy5 labeled samples. This design was applied at each of the four time points with each biological replicate consisting of 6 arrays (6 arrows). Three biological replicates (18 arrays per time point) were completed for a total of 72 microarrays.

Figure 3.
EE, TCDD and EE+TCDD effects on temporal uterine weights. EE induced the expected increases in uterine wet (A) and blotted (B) weights at 12, 24 and 72 hrs while TCDD (T) had no effect. Cotreatment with TCDD inhibited EE-mediated increases in wet (72 hrs) and blotted (12 and 72 hrs) uterine weights. Data are expressed as fold-change in uterine weight (normalized to body weight) for each treatment relative to the TMVC group. * = p<0.05 when compared to TMVC. a = p<0.05 when compared to time matched EE-treated animals. BW= Body weight

Figure 4
Comparison of uterine histology at 72 hours after vehicle, TCDD, EE, or EE+TCDD treatment.
Relative to the time matched vehicle control (A), TCDD did not induce any alterations in uterine histology (B). EE induced marked epithelial and stromal hypertrophy and hyperplasia with mild stromal edema at 72 hrs (C and E). Cotreatment of EE plus TCDD exhibited histology comparable to EE treatment alone at 72 hrs with the exceptions of reduced stromal edema, decreased stromal hypertrophy and hyperplasia and marked luminal epithelial cell (LEC) apoptosis (circled) (D and F). Bars = 10 microns

Figure 5.
Comparison of global gene expression responses to EE, TCDD and EE+TCDD.
A. Comparison of temporal gene expression profiles indicates the similarity of EE and EE+TCDD patterns while TCDD were minimal by comparison with the exception of the 12 hr time point. B. Hierarchical clustering further illustrates the temporal similarity between EE and EE+TCDD groups as each treatment/time point clustered together. The 12 hr TCDD samples clustered with the 12 hr EE and EE+TCDD samples indicating estrogen-like expression. The 24

hr TCDD sample exhibited modest similarity to the 24 and 72 hr EE and EE+TCDD groups while the 4 and 72 hr TCDD groups clustered separately. Comparisons were performed on features which were differentially expressed in any of the treatment groups at any time point relative to time matched vehicle controls (E = EE, T = TCDD and M = EE+TCDD mixture).

Figure 6.
Scatter plot comparisons of gene expression magnitudes between treatment groups
A. Scatter plots of the $\log_2$ expression ratios of EE versus EE+TCDD at the 12 hour time point revealed that the majority of the responses were of the same magnitude relative to the time matched vehicle controls (TMVCs) with a correlation of 0.95. This graph also reveals a small subset of genes which were more highly expressed in the EE treated group, suggesting inhibition upon cotreatment with TCDD. D. A similar comparison between EE and TCDD groups at 12 hrs revealed a low correlation of 0.064, indicating high variation between the magnitudes of the response despite similar expression patterns. Similar responses were noted at the other time points (data not shown.)

Figure 7.
Quantitative real-time PCR verification of the selective inhibition of EE-induced gene expression responses by TCDD.
TCDD cotreatment inhibited the EE-mediated induction of Dsc2, Krt1-14, Sfn, Sprr2a and Tff1 but did not affect the induction of Pcna. The same RNA used for cDNA microarray analysis was examined by QRTPCR. All fold changes were calculated relative to time matched vehicle controls. Genes are indicated by official gene symbols and results are the average of 5 biological replicates. Error bars represent the SEM for the average fold change.
* = $p<0.05$ for treatment groups relative to time matched vehicle controls
a = $p<0.05$ for EE+TCDD when compared to time matched EE controls

Table 1. Functional Categorization of EE-regulated genes inhibited by TCDD cotreatment as determined by microarray analysis

Gene Name	Gene Abbrev	Entrez Gene ID	GenBank Accession	EE-mediated Response Time point (hr)[a]				% Inhibition of EE Response by TCDD Time point (hr)[b]			
				4	12	24	72	4	12	24	72
Growth and Proliferation											
branched chain aminotransferase 1	Bcat1	12035	AA003372	**3.41**	**3.71**	**2.36**	**5.80**	0.84	48.77	65.33	**60.43**
serine proteinase inhibitor B 5	Serpinb5	20724	BF021354	0.90	**4.02**	**3.56**	**2.31**	–	**86.92**	**89.73**	**93.07**
sestrin 1	Sesn1	140742	AA154829	1.14	**1.80**	**1.55**	**4.74**	–	8.73	48.08	**56.18**
stratifin	Sfn	55948	AA009229	1.06	**5.47**	**4.89**	**3.31**	–	**83.36**	**89.46**	75.12
trefoil factor 1	Tff1	21784	NM_009362	0.91	**5.70**	**12.08**	**1.82**	–	**100.00**	**92.31**	19.62
tumor necrosis factor (ligand) superfamily, member 8	Tnfsf8	21949	NM_134131	0.76	**3.11**	**3.29**	**2.68**	–	**79.65**	**85.95**	72.57
vascular endothelial zinc finger	Vezf1	22344	NM_016686	0.81	1.41	1.33	**5.36**	–	–	–	**64.87**
Water/Ion Transport											
aquaporin 3	Aqp3	11828	AI788487	0.76	**2.29**	**2.66**	**2.85**	–	**100.00**	65.28	60.15
FXYD domain-containing ion transport regulator 4	Fxyd4	108017	BG072055	1.02	**0.61**	**0.55**	**1.60**	–	**100.00**	**84.73**	–
solute carrier family 38, member 3	Slc38a3	76257	NM_023805	1.15	1.07	**0.60**	0.95	–	–	83.55	–
solute carrier family 40, member 1	Slc40a1	53945	BG074144	0.71	1.32	1.44	**6.19**	–	–	–	**62.32**
solute carrier family 4, member 2	Slc4a2	20535	AA048952	0.92	**1.95**	1.43	**1.59**	–	**85.49**	–	88.74
Structural Function											
desmocollin 2	Dsc2	13506	BG063370	1.08	**3.83**	**4.01**	**2.55**	–	**59.44**	**73.53**	70.63
keratin complex 1, acidic, gene 14	Krt1-14	16664	NM_016958	0.86	**13.63**	**13.94**	**9.35**	–	**83.73**	**91.96**	**90.38**
keratin complex 1, acidic, gene 19	Krt1-19	16669	BG064706	0.87	**2.06**	**1.53**	**3.28**	–	52.18	**100.00**	**66.04**
keratin complex 2, basic, gene 4	Krt2-4	16682	W98341	0.71	**4.95**	**4.66**	**3.25**	–	**83.93**	52.56	0.00
keratin complex 2, basic, gene 7	Krt2-7	110310	AA014127	1.04	**4.97**	**2.52**	**4.00**	–	46.12	**78.99**	**64.88**
macrophage receptor with collagenous structure	Marco	17167	NM_010766	1.01	**3.05**	**4.93**	**3.11**	–	**68.35**	**80.67**	**89.22**
TP53 apoptosis effector	Perp	494479	NM_022032	0.41	**4.54**	**4.13**	**2.75**	–	**79.92**	73.48	54.14
small proline-rich protein 2A	Sprr2a	20755	AI596101	1.11	**4.85**	**8.89**	**18.75**	–	22.02	59.23	**71.55**
troponin T1, skeletal, slow	Tnnt1	21955	AA637201	1.06	**1.92**	**3.37**	1.12	–	**100.00**	98.58	0.00

a- Values in bold indicate active genes based on statistical criteria of ± 1.5 fold induction and P1(t) ≥ 0.9999
b- Only values statistically significant for EE are indicated. Values in bold indicate significantly inhibited responses based on statistical criteria of ± 1.5 fold induction and P1(t) ≥ 0.9999
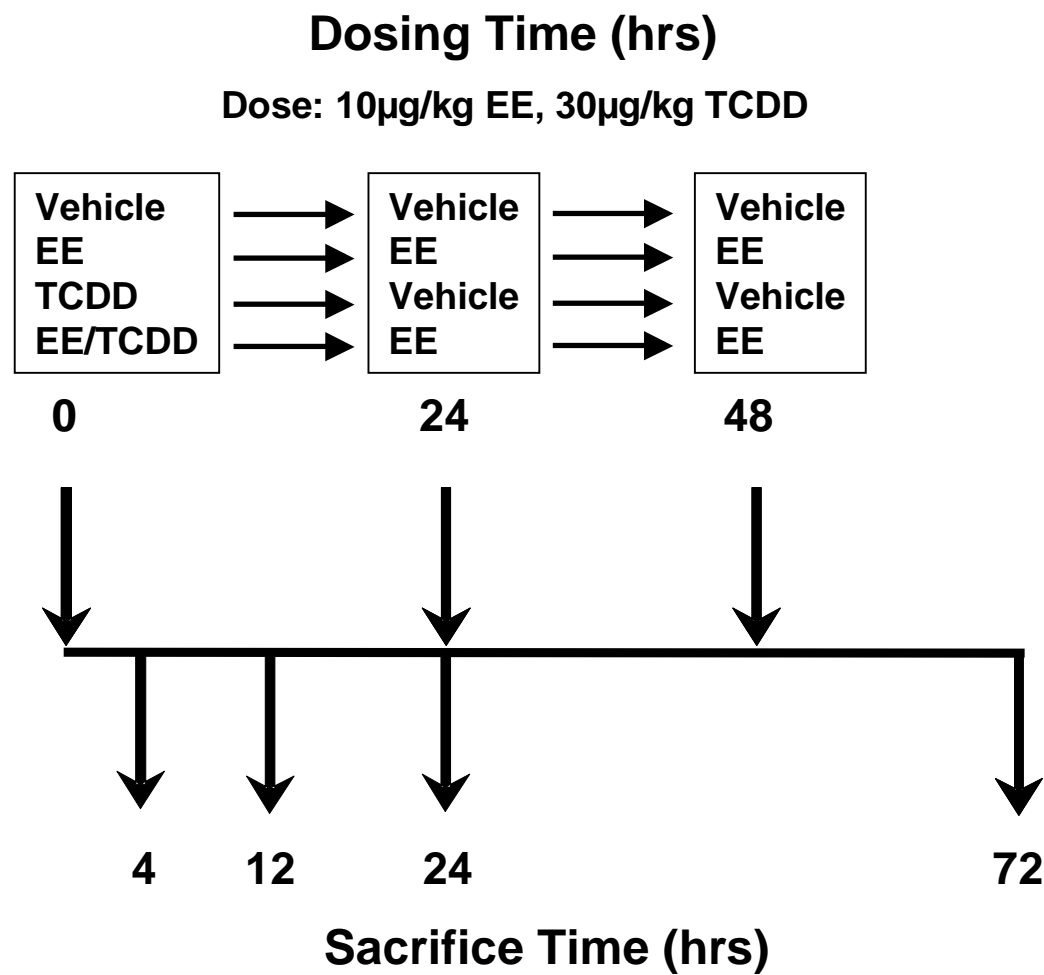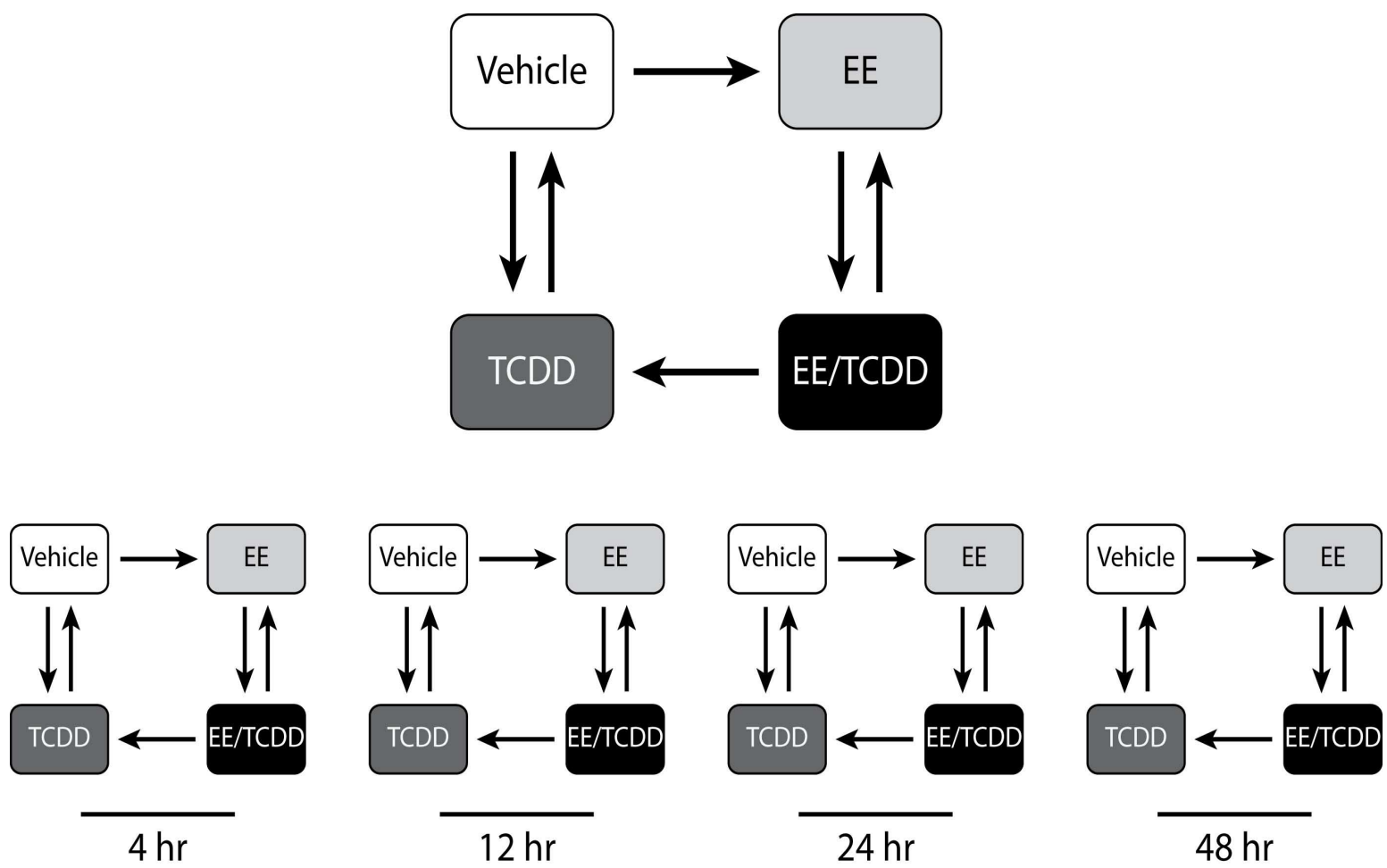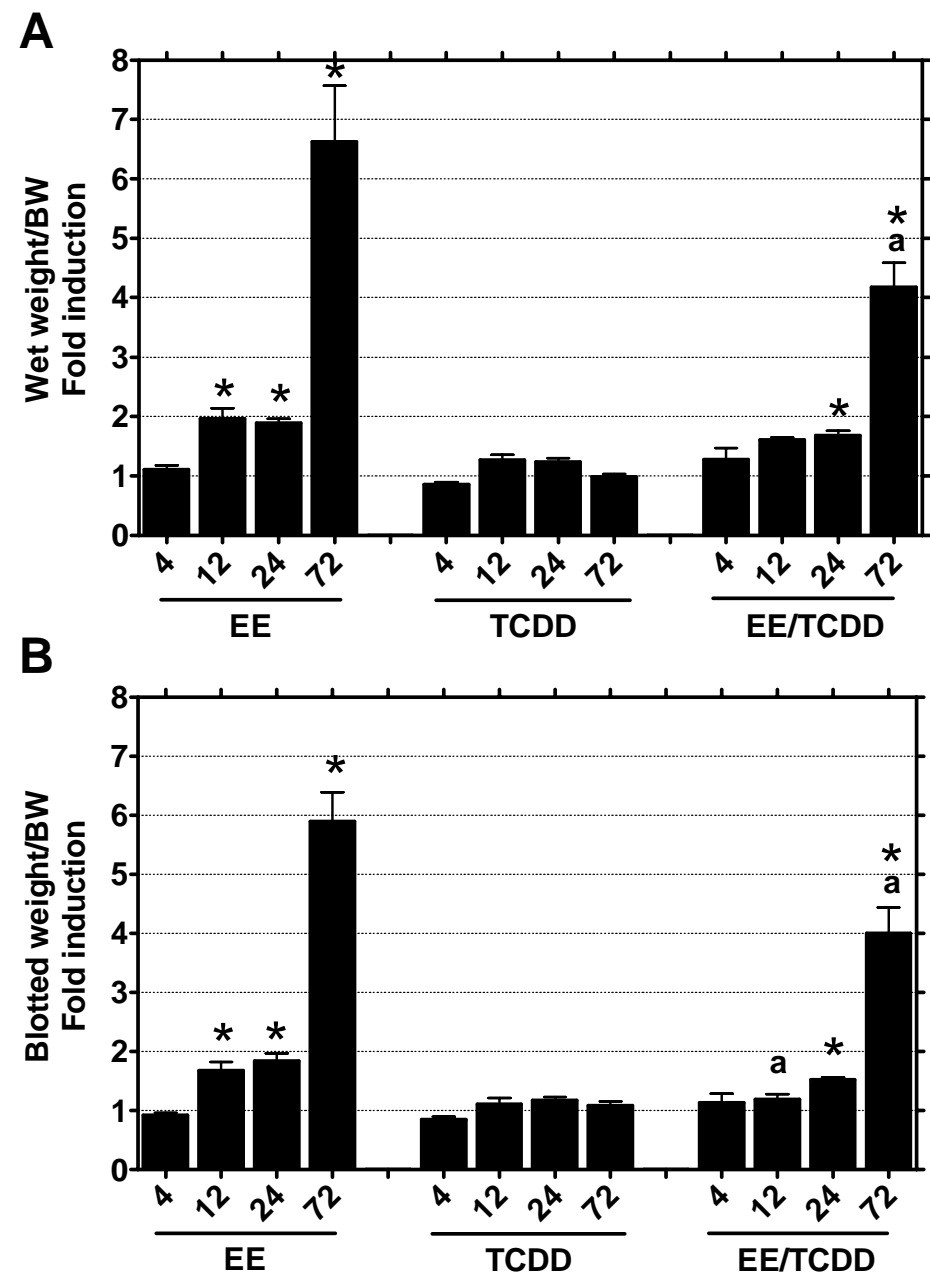
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Figure 1

Dosing Time (hrs)

Dose: 10μg/kg EE, 30μg/kg TCDD

Vehicle		Vehicle		Vehicle
EE		EE		EE
TCDD		Vehicle		Vehicle
EE/TCDD		EE		EE

0   **24**   **48**

4   **12**   **24**   **72**

Sacrifice Time (hrs)

Figure 2

Figure 3

Figure 4
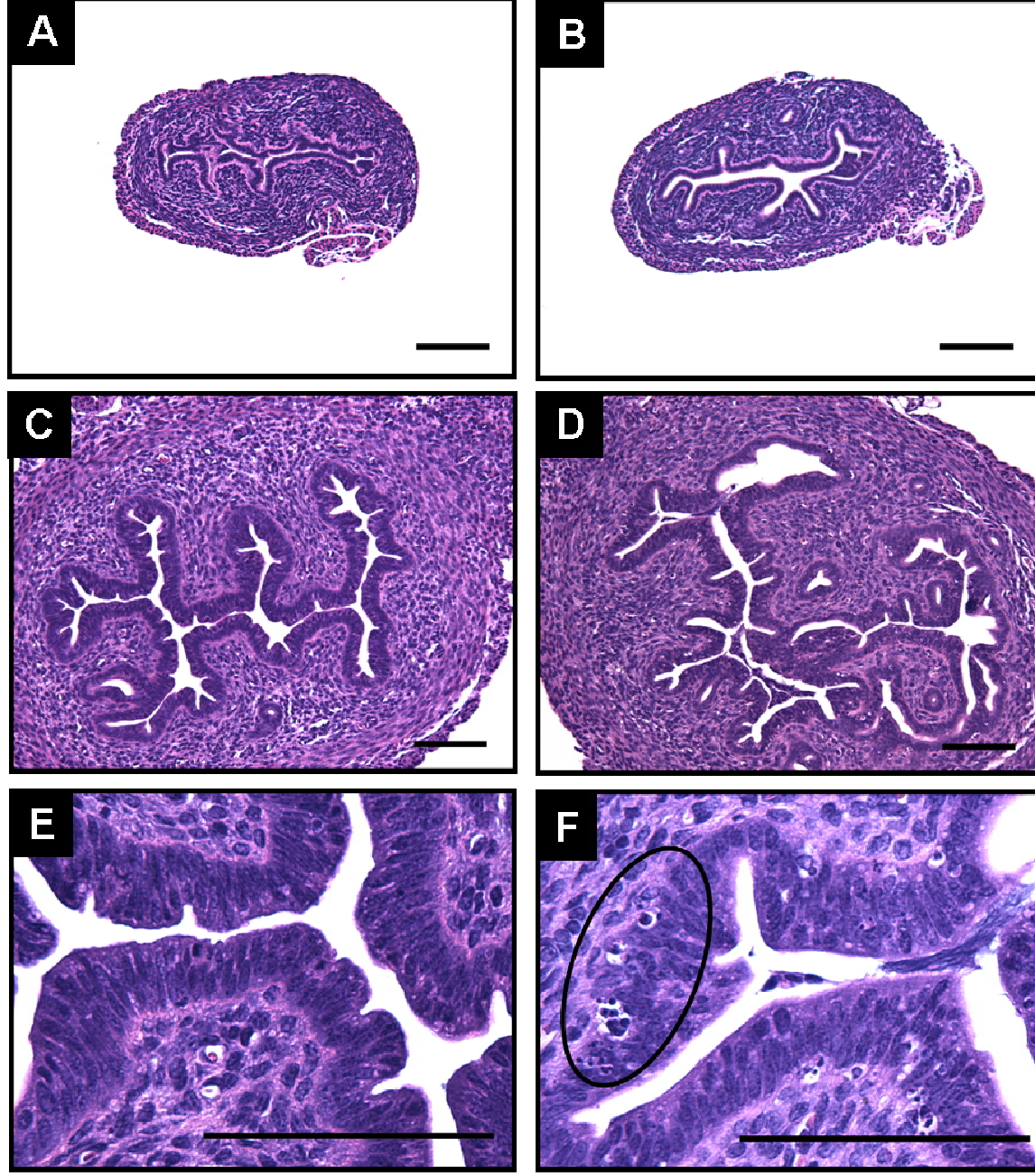
Figure 5

Figure 6

Figure 7