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# Activation of the Aryl Hydrocarbon Receptor during Different Critical Windows in Pregnancy Alters Mammary Epithelial Cell Proliferation and Differentiation

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**Exposure to the aryl hydrocarbon receptor (AhR) agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during pregnancy causes severe defects in mammary gland development and function; however, the underlying mechanism remains unclear. Alterations in epithelial cell proliferation, differentiation, and apoptosis during pregnancy-related mammary development can lead to failed lactogenesis. To determine which of these processes are affected and at what time periods, we examined proliferation, differentiation and apoptosis in mammary glands following exposure to TCDD during early, mid or throughout pregnancy. Although AhR activation throughout pregnancy did not cause early involution, there was a 50% decrease in cell proliferation, which was observed as early as the sixth day of pregnancy (DP). TCDD treatment on the day of impregnation only reduced development and proliferation in early and mid-pregnancy, followed by partial recovery by DP17. However, when AhR activation was delayed to DP7, developmental impairment was not observed in mid-pregnancy, but became evident by DP17, whereas proliferation was reduced at all times. Thus, early exposure to TCDD was neither necessary nor sufficient to cause persistent defects in lactogenesis. These varying outcomes in mammary development due to exposure at different times in pregnancy suggest there are critical windows during which AhR activation impairs mammary epithelial cell proliferation and differentiation.**

***Key Words:*** **TCDD; mammary gland development; lactogenesis; ductal branching; alveologenesis.**

The majority of the development of the mammary gland occurs during postnatal life with the most dramatic morphological and physiological changes occurring with the beginning of pregnancy, when the functional state of the gland is realized upon synthesis of milk following the onset of lactogenesis. With impregnation, the gland undergoes a proliferative phase, which consists of epithelial cell proliferation, ductal branching, and elongation (Hovey *et al.*, 1999). This is followed by the initiation of secretory differentiation, which involves formation of lobulo-alveolar structures (Brisken, 2002). Secretory activation begins later in pregnancy, or upon parturition, and is marked by milk synthesis and secretion. All of these changes take place under the influence of a wide range soluble regulators that are produced both locally in the mammary gland and by many other tissues and glands (Hennighausen and Robinson, 2001). Some of the signals that regulate the early events of development, such as ductal branching morphogenesis, also control processes that occur later in pregnancy, such as lobule formation and their differentiation into alveoli (alveologenesis). However, many factors participate in only stage specific processes (Brisken and Rajaram, 2006). Deregulation of the well-orchestrated process of mammary development during pregnancy by extrinsic variables such as nutrition and environmental pollutants can impair ductal branching, alveologenesis or both events and cause decreased ability to produce milk.

Our laboratory made a novel discovery that exposure to the pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) impairs the normal development of mammary glands during pregnancy to such an extent that dams failed to nutritionally support their offspring (Vorderstrasse *et al.*, 2004). This discovery is of interest because it demonstrates very clearly that a known and abundant pollutant has a profound and adverse effect on pregnancy-associated glandular differentiation. Moreover, TCDD is a ligand for the aryl hydrocarbon receptor (AhR), a member of the per-arnt-sim (PAS) family of basic-helix-loop-helix transcription factors. The AhR directs the expression of many detoxification genes and functions as a modulator of cellular signaling pathways critical for cell proliferation, differentiation and apoptosis. In vertebrates, the AhR is found in the cytosol in association with HSP90 chaperones, several HSP90 accessory proteins and immunophilin-like proteins (XAP2/ARA9/AIP). Upon ligand binding, the AhR translocates to the nucleus where it associates with AhR nuclear translocator/hypoxia-inducible transcription factor-1β (ARNT/HIF-1β). The AhR/ARNT complex stimulates transcription of several genes, such as those in the cytochrome P450 CYP1

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family (Gonzalez and Fernandez-Salguero, 1998; Gu *et al.*, 2000; Puga *et al.*, 2005). Mammary glands express AhR and ARNT protein (Safe *et al.*, 2000); however, the precise gene targets of AhR in mammary tissue are not known.

Some of the regulatory pathways that are involved in the development of the mammary gland are affected by TCDD (and other AhR ligands) in other model systems. For example, exposure to AhR ligands altered levels of circulating endocrine hormones in rodents (De Krey *et al*., 1994; Jones *et al*., 1987), and other reports suggest that TCDD can act as a modulator of reproductive hormones (Sarkar *et al*., 2000; Tanaka *et al*., 2007). Yet, the severe impairment in mammary differentiation caused by AhR activation during pregnancy did not correlate with diminished levels of circulating estradiol, progesterone, or prolactin (Vorderstrasse *et al*., 2004). However, numerous other factors that are locally and systemically produced drive mammary development during pregnancy (Brisken, 2002). Therefore, there are many other pathways that are potential targets of AhR, and may mediate the disruptive effects of TCDD on mammary gland development. In addition, increasing evidence indicates that there are multiple mechanisms through which AhR regulates cell proliferation and apoptosis in different cell types (Camacho *et al*., 2001; Jin *et al*., 2004; Lei *et al*., 1998; Miller *et al*., 1996; Mitchell *et al*., 2006; Singh *et al*., 2008). Although the molecular mechanisms by which AhR ligands impact these processes are not known, they show that regulation of apoptosis and cell cycle arrest mechanisms are targets of AhR.

Given that mammary gland development during pregnancy involves proliferation and differentiation, and that TCDD affects these processes in other system, the failed lactogenesis caused by exposure to TCDD could be due to a decrease in epithelial cell proliferation, impaired cell differentiation into alveolar structures, premature induction of apoptosis, or a combination of these events. The objectives of the present study were to determine whether: (1) AhR activation by TCDD affects mammary development during early pregnancy and is associated with reduced induction of milk protein gene expression, (2) exposure to TCDD during early pregnancy impairs lactogenesis by suppressing mammary epithelial cell proliferation or by increasing apoptosis, and (3) AhR activation during very early pregnancy (i.e., the proliferative phase) is necessary and sufficient to cause the defects in mammary gland development observed at parturition.

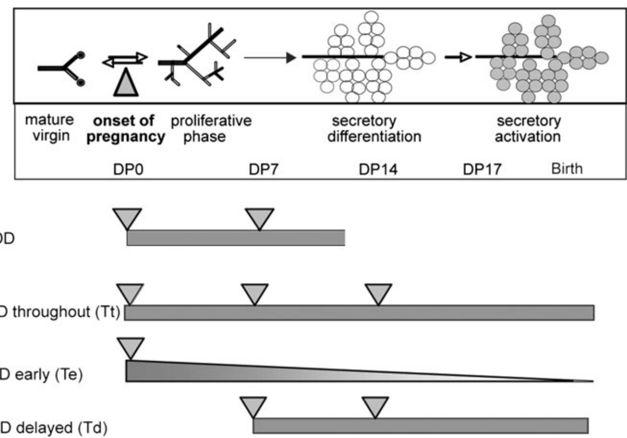


**FIG. 1.** Schematic representation of mammary development and experimental design. The cartoon represents the development of the mammary gland during pregnancy. From DP0 until DP7 the mammary gland undergoes a highly proliferative phase, followed by differentiation of the epithelial cells into alveolar structures, which ultimately will produce milk. (A) To test the effects of exposure to TCDD on early epithelial cell proliferation, apoptosis and early mammary differentiation, impregnated females were exposed to TCDD (5 μg/kg) or vehicle (veh.) on DP0 and 7 and sacrificed on DP6, 9, and 12. (B) The effects of administering TCDD during three different windows of time were evaluated using three exposure paradigms. **TCDD early (Te)**: TCDD (5 μg/kg) was administered one time, on DP0; **TCDD delayed (Td)**: The first dose of TCDD was delayed to the end of the highly proliferative phase (DP7). Animals sacrificed after DP12 received a second dose of TCDD on DP14; **TCDD throughout (Tt)**: animals were treated throughout pregnancy (DP0, DP7, and DP14). A fourth group of animals received peanut oil vehicle control. Animals were sacrificed on DP6, DP9, DP12, and DP17. The number of animals in each time in point varied from 6 to 9 for each treatment group.

## MATERIAL AND METHODS

***Animals and treatments.*** C57BL/6J mice (age 6 weeks) were obtained from the Jackson Laboratory (Bar Harbor, ME) or NCI (Frederick, MD). C57Bl/129J outbred p21 (−/−) mice (B6;129S2-Cdkn1a$^{tm1Tyj}$/J; −p21KO) were backcrossed 10 generations to C57BL/6J mice (O'Reilly *et al*., 2001) obtained from Jackson laboratories (Bar Harbor, ME).

Female mice were housed with males, and checked daily for presence of vaginal plugs. The day the vaginal plug was found was designated day 0 of pregnancy (DP0). Pregnant mice were then individually housed in micro-isolator units for the remainder of the study. Age-matched virgin mice included as controls were not housed with males. Animals were given food and water *ad libitum,* and were maintained on a 12:12-h light cycle. All animal treatments were conducted with approval of the Institutional Animal Care and Use Committee.

Female mice were treated with 5 μg TCDD/kg body weight or vehicle control by gavage. Stock TCDD—20 μg/ml (Cambridge Isotopes Laboratory, Andover, MA) was dissolved in anisole (2%) and diluted in peanut oil, to a final concentration of 0.5 μg/ml, 0.1% anisole. Vehicle control consisted of peanut oil containing an equivalent concentration of anisole (0.1%). A single dose of 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU—Sigma-Aldrich, St Louis, MO) was injected (i.p.) 3 h before sacrifice for cell proliferation studies. Mice were sacrificed by injection (i.p.) of avertin: (2% 2,2,2-tribromoethanol, 2% tert-amyl alcohol), and blood was collected immediately by cardiac puncture. Thoracic mammary glands were removed, immediately frozen in liquid nitrogen and stored at -80°C; these glands were then used for protein, enzyme (right side) and RNA analysis (left side). Abdominal glands were removed and used for whole mounts (left side) and immunohistochemical assays (right side).

***Experimental groups.*** Treatment of animals with TCDD was conducted in the context of several different exposure paradigms, which are described here. Paradigm A: impregnated females were exposed to TCDD or vehicle on DP0 and 7 and sacrificed on DP6, 9 and 12 (Fig. 1A, *n* = 6). This was used to examine effects of sustained AhR activation on epithelial cell proliferation, apoptosis, milk gene expression and early differentiation of the mammary glands (Figs. 2–5). Paradigm B: Impregnated mice were exposed to TCDD or peanut oil vehicle (Veh) in one of three different windows of time during

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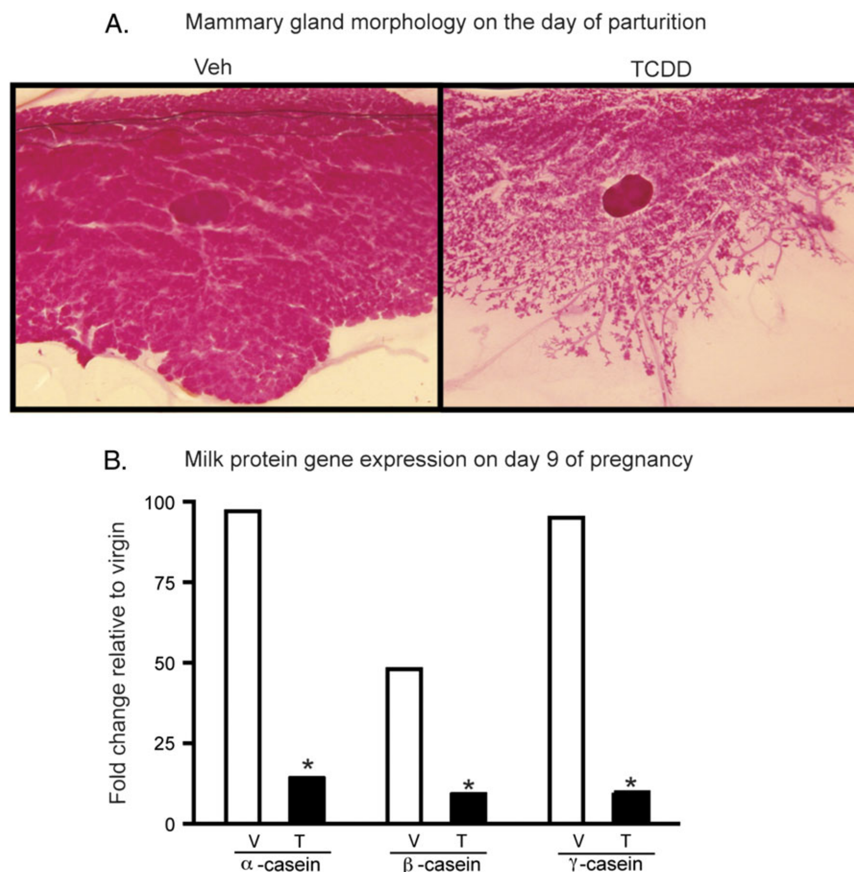


**FIG. 2.** AhR activation during pregnancy impairs mammary gland development and lactogenesis. (A) Mammary glands were collected on the day of parturition from animals treated with vehicle (Veh) or 5 μg/kg TCDD on DP0, 7, and 14. Whole mounts were fixed in Carnoy's fixative and stained with carmine alum, as detailed in the ''Material and Methods'' section. Representative images were obtained using a Zeiss dissecting microscope (1.25× magnification) with a Nikon Coolpix E995 camera. (B) Impregnated mice were treated with vehicle or TCDD (5 μg/kg body weight) on DP0 and DP7 and sacrificed on DP9. Age-matched virgin (AMV) mice were treated with two doses of vehicle or TCDD administered 9 and 2 days prior to sacrifice. The bar graph shows the fold change in expression of milk protein genes in pregnant animals compared with AMV. Statistical analyses were performed by comparing mean gene expression levels between pregnant vehicle- and TCDD-treated animals. Asterisks denote statistically significant differences between the vehicle and TCDD treatments ($p < 0.05$, $n = 3$).

pregnancy (Fig. 1B, and Figs. 6–8). One group was treated with TCDD on DP0, DP7, and DP14, and is referred as "TCDD throughout" (this is the same exposure paradigm used in our previous report (Vorderstrasse *et al*., 2004) and in our experiment with p21KO mice (Supplementary Data) Another group was treated with a single dose of TCDD on DP0 and is referred to as "TCDD early." A third group of animals was treated with TCDD on DP7 and DP14, and is referred to as "TCDD delayed." As a control, age-matched pregnant mice received the vehicle control on equivalent days.

***Morphological development analyses.*** Mammary gland whole mounts were prepared as described previously (Vorderstrasse *et al*., 2004). Briefly, glands were removed, fixed in Carnoy's fixative, stained with carmine alum, gradually dehydrated, cleared in xylenes, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Whole mounts were evaluated without knowledge of treatment by at least two different scientists, using a Zeiss Stemi 2000C microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Glands were given a developmental score based on a four-point scale (1 = poor development/ differentiation to 4 = excellent growth and development). The subjective scoring scales were specific to the stage of development at each time point examined, and considered ductal branching, development of lobulo-alveolar units, and the size of the structures. For quantitative morphometric analysis on DP6, four sections randomly selected from different regions of mammary gland whole mounts were photographed. Digital micrographs were taken using a Zeiss Stemi 2000C Microscope with a Nikon KoolPix 995 digital camera. Images were evaluated in a blinded fashion. The number of lobuloalveolar units was determined using printed images representing 16 $mm^2$ of tissue. The number of branches in a total length of about 35 mm of duct was evaluated using a PlanWheel SA2 (Scalex Corporation, Carlsbad, CA). Mean scores for each group were computed and analyzed for differences due to treatment.

***Immunohistochemistry.*** Glands were removed, fixed overnight in 10% buffered formalin, transferred to 70% ethanol, embedded in paraffin and sectioned (4 μm). Deparaffinized sections were subjected to antigen retrieval, which involved boiling in 10mM citrate buffer for 20 min. For BrdU incorporation analysis, nonspecific staining was blocked using normal rabbit serum prior to incubation for 1 h at room temperature with a mouse anti-BrdU monoclonal antibody (1:100 dilution) (DakoCytomation, Denmark A/S). Next, sections were incubated for 30 min at room temperature with biotinylated anti-mouse IgG (1:500 dilution) (Vector Laboratories, CA) and StrepABComplex/ HRP (DakoCytomation). Antibody complexes were visualized using DAB (Vector Laboratories, CA). Apoptotic cells were identified in separate sections by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL, Apoptag In Situ Apoptosis Detection Kit, Chemicon International, Temecula, CA). Mammary glands obtained during involution and lactation were used as positive and negative controls for apoptosis, respectively. Slides were counterstained with hematoxylin (Vector Laboratories) and analyzed

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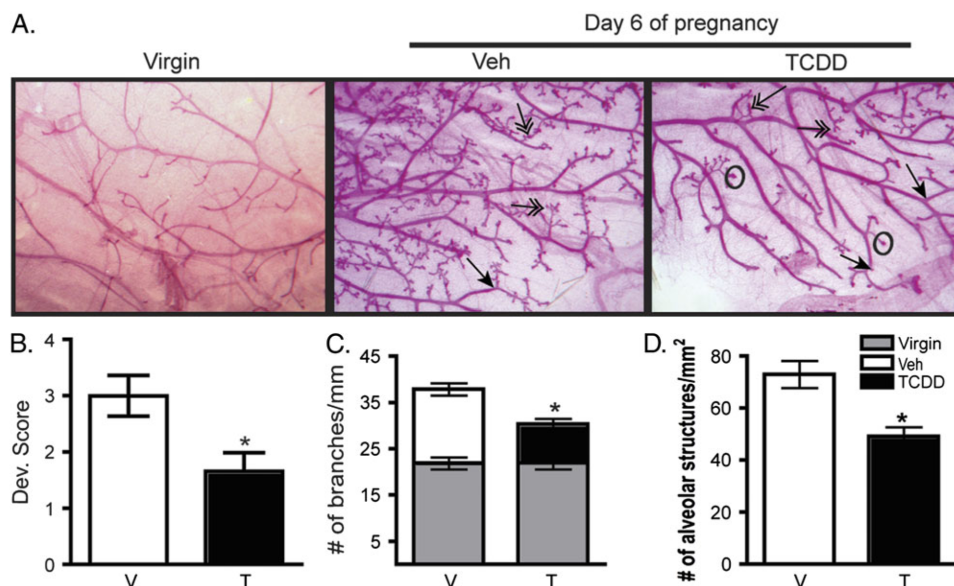


**FIG. 3.** Activation of AhR impairs mammary gland development as early as the DP6. (A) Mammary glands from mice treated with TCDD (T, 5 μg/kg) or vehicle (V) were collected on DP6 or from age matched virgin animals ($n = 6$–$9$). Whole mounts were fixed in Carnoy's fixative and stained in carmine alum. A. Representative images were obtained using a Zeiss dissecting microscope (5× magnification) with Nikon Coolpix E995 camera. Branches are denoted by a solid head arrow, and double-headed arrows point to lobulo-alveolar structures. The circle indicates a TEB. (B) The graph shows the average developmental scores on DP6, determined as described in the Material and Methods section. (C) The average number of branches per linear mm was determined in mammary glands from virgin and pregnant mice that were treated with vehicle or TCDD. Two areas of each gland (3.2× magnification) were photographed. The number of branches in an average of 35 linear mm of duct was counted. (D) The average number of lobules was determined on DP6 in mammary glands from TCDD and vehicle treated animals. Lobules were counted in four different areas of 4 $mm^2$ in each gland. Asterisks indicate statistically significant differences between TCDD and vehicle treated animals ($p < 0.05$). Data are representative of two separate experiments.

using an Olympus BX51 microscope with a RT-Color diagnostic digital camera. To determine the number of BrdU-positive cells and TUNEL positive cells, 10 different areas of each gland were randomly photographed (20× magnification) and the average number of BrdU or TUNEL positive cells was determined.

***Caspase-3 assay.*** Caspase-3 activity of nuclear extracts prepared from frozen thoracic left mammary glands was measured using a fluorometric assay (CaspACE Assay System, Fluorimetric, Promega, Madison, WI). The assay was performed following the manufacturer's information. Briefly, nuclear extracts (100 μg protein) were mixed with caspase buffer, dimethyl sulfoxide, 1M dithiothreitol, and a synthetic 7-amino-4-methylcoumarin, N-acetyl-L-aspartyl-Lglutamyl-L-valyl-l-aspartic acid amide caspase-3 substrate. The emitted fluorescence was kinetically measured at 30°C for 50 min with a Spectramax M5 Gemini Fluorometer (Molecular Devices, Sunnyvale, CA). The relative cleavage was determined by calculating the slope of the accumulation of 7-amino-4-methylcoumarin fluorochrome during the assay. For statistical analyses, caspase activity in nuclear extracts from involuting glands was set to 100% and the other values were calculated relative to this measurement (Marti *et al.*, 2001).

***Milk protein gene expression.*** Mammary glands were removed on DP9 and from age matched virgin animals, and RNA was isolated using Trizol reagent. Purity, integrity, and concentration of the RNA in each sample was determined using a spectrophotometer and gel electrophoresis. Nondegraded RNA (10 μg/sample) was amplified, biotinylated and fragmented for hybridization at the Washington State University Genomics Core Facility. Samples from each mouse were hybridized separately to Affymetrix Murine Genome chips (Set 430A) using an Affymetrix instrument system (scanner, fluidics station, and hybridization oven). Raw data were examined using Microarray Suite software (Affymetrix, Santa Clara, CA) and the overall quality of each chip was visually examined and found to be within normal parameters. Quality control using spiked genes and housekeeping genes was conducted to evaluate each chip. Four-way comparisons of the data were analyzed using Genesifter.net (VizXlabs, Seattle, WA).

***Western blots.*** Thoracic mammary glands were homogenized in the following buffer: 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1mM ethylenediaminetetraacetic acid, 150mM NaCl, 0.6% NP-40, with 0.1M phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin. Protein concentration was measured by Pierce BCA assay (Pierce, Rockford, IL) and 25 μg of protein from each sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970), and transferred to nitrocellulose membranes. β-Casein protein was visualized by probing with a goat polyclonal anti-mouse β-casein antibody, diluted 1:1000 and incubated overnight at 4°C (sc-17971, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by horseradish-peroxidase–conjugated donkey-anti-goat antibody, diluted 1:10,000 and incubated for 2 h at room temperature (Santa Cruz Biotechnology Inc). Antibody complexes were visualized using chemiluminescent enhanced chemiluminescence reagents (Amersham Pharmacia, Piscataway, NJ).

***Statistical analyses.*** Data were analyzed using StatView software (SAS Software, Cary, NC). Using a two-way ANOVA followed by *post hoc* tests (Bonferroni/Dunn test), differences between independent variables were compared over time and between treatment groups. Differences between two groups at a single point in time were evaluated using a Students $t$-test. Differences were considered significant when $p$ values were $< 0.05$.

## RESULTS

### *Exposure to TCDD Impairs Mammary Development, with Noticeable Effects as Early as DP6*

We had previously discovered that TCDD treatment disrupts mammary gland development, with morphological changes

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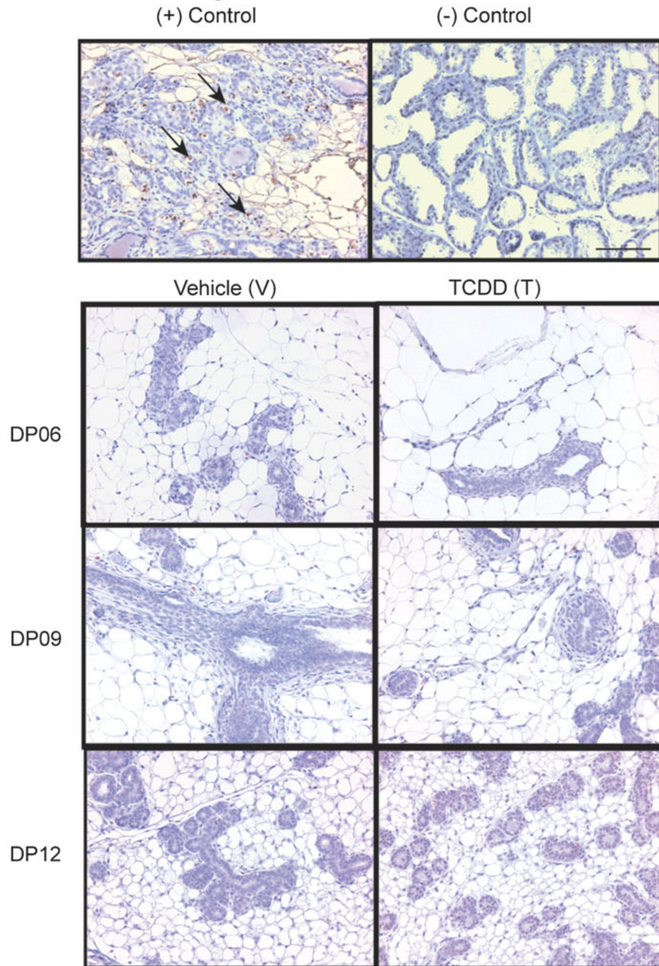


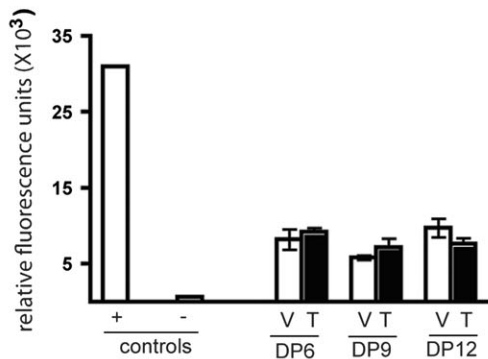


**FIG. 4.** Exposure to TCDD does not induce early involution in the mammary gland. Mammary glands from pregnant mice treated with TCDD (T) or vehicle control (V) were collected on DP6, DP9, and DP12 ($n = 6$/group/day). (A) Representative micrographs show TUNEL staining in mammary glands during involution (+), lactation (−), and pregnancy. The positive control (+) depicts apoptotic cells (arrow) in an involuting mammary gland. Lactating glands served as the negative control (−) and had no TUNEL staining. (B) The average caspase-3 levels (±SEM) in mammary tissue was determined by colorimetric and fluorimetric caspase-3 activity assays.

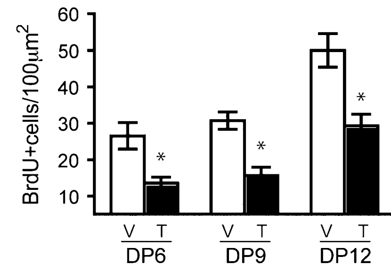


**FIG. 5.** Exposure to TCDD in early pregnancy decreases BrdU incorporation by mammary epithelial cells. Mice were treated as in Figure 1, except that 5-bromo-2′-deoxyuridine (BrdU) was administered (i.p.) 3 h prior to sacrifice. Details about the experimental groups and immunohistochemistry can be found in the ''Material and Methods'' section. The graph represents the average number of $BrdU^+$ cells from 10 different photographed areas of glands on DP6, DP9, and DP12. The average number of $BrdU^+$ cells in 100 $\mu m^2$ tissue was calculated. Asterisks represent statistically significant differences between the vehicle and TCDD treatment groups ($p < 0.01$, $n = 6$–9).

observed between the DP9 and DP17 (Vorderstrasse *et al.*, 2004). Here, we extend these observation to include earlier and later points in time. On the day of parturition, mammary glands collected from vehicle-treated animals were completely populated with alveoli, to such extent that they covered the adipose tissue. As a result of the dense alveoli present, ductal structures of the parenchymal tissue are difficult to appreciate at this stage (Fig. 2A). Moreover, during excision, the presence of milk could be noticed in the tissue. In contrast, severe defects were visible in glands collected from animals treated with TCDD. For instance, the adipose tissue was apparent, with parenchymal tissue containing fewer numbers of alveoli, and when present they appeared unfilled and underdeveloped (Fig. 2A). In addition to the morphological changes observed, TCDD altered the coordinated induction of milk protein genes, which occurs around the DP9. The alpha-, beta-, and gamma-casein genes encode common milk proteins and are markers of mammary gland differentiation. Examination of their expression on DP9 revealed a severe decrease in the induction of these genes in mammary glands from dams exposed to TCDD when compared with glands collected from control animals (Fig. 2B). These results suggest that the effects of exposure to TCDD on mammary gland development are noticeable at the beginning of the secretory differentiation phase of the development.

We next determined whether defects in pregnancy-induced differentiation occur prior to DP9; during the proliferative phase of early pregnancy. In addition to extensive mammary epithelial cell proliferation, ductal branching morphogenesis begins in early pregnancy. During this time, secondary and tertiary branches fill the stromal tissue, forming a complex web of branches. In addition, mature lobules start populating the tissue and these structures eventually become alveoli, the milk producing units of the gland. The initial stage of glandular differentiation occurs during the first third of pregnancy, and alterations during this phase could lead to impairment in milk

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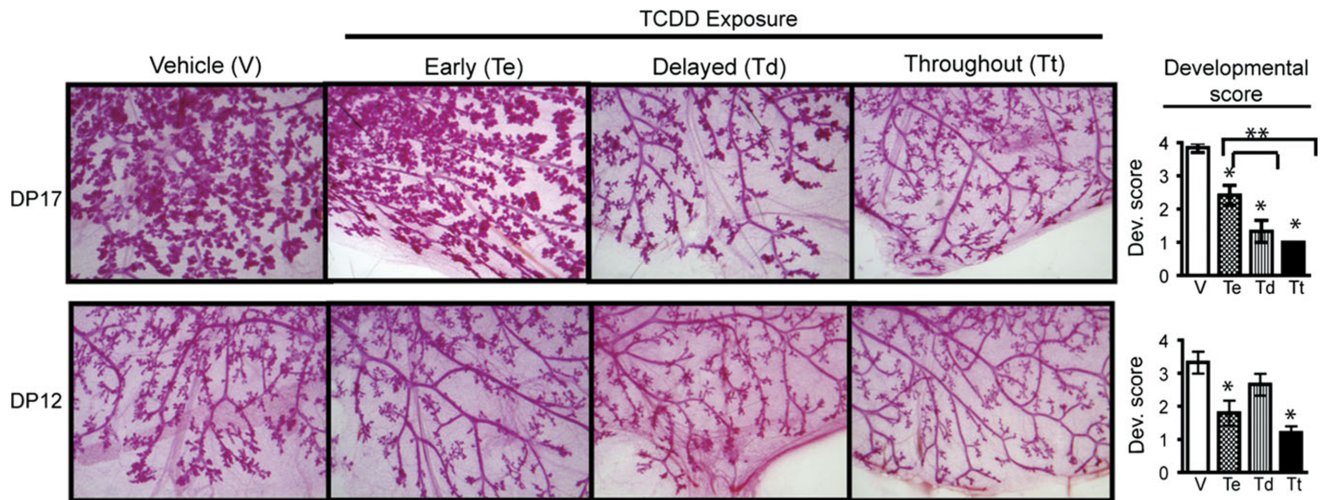


**FIG. 6.** TCDD exposure during different windows of time alters mammary development. Separate groups of nulliparous mice were treated with TCDD early (DP0 only), delayed (DP7 and DP14), or throughout pregnancy (DP0, DP7, and DP14). Control mice received vehicle treatment on equivalent days. Information about the experimental groups can be found in Figure 5. Left abdominal mammary glands were collected, fixed in Carnoy's fixative and stained with carmine alum, as detailed in the ''Material and Methods.'' Representative images were obtained using a Zeiss dissecting microscope (5× magnification), with a Nikon Coolpix E995 camera. Three different researchers scored the glands without knowledge of treatment group. A single asterisk represents a statistically significant difference between vehicle TCDD treated groups ($p < 0.05$, $n = 6$–9). Double asterisks indicate a significant difference ($p < 0.05$) from the group that received TCDD just one time, on DP0.

production later in pregnancy. Therefore, we examined the effects of exposure to TCDD on branching and lobule formation during early pregnancy. As shown in Figure 3, a single dose of TCDD on DP0 reduced glandular differentiation on DP6. At this stage the best-developed glands collected from control animals were scored as 4. Those glands showed visible secondary branches throughout, with most areas being populated with tertiary branches ending in mature lobules. In contrast, the least developed glands, which were collected from TCDD-treated animals scored as 1, had very few complex structures, and retained some terminal end buds (TEBs; Fig. 3A, circle). On average, mammary glands collected from vehicle control animals were scored 50% higher than glands collected from TCDD-treated animals (Fig. 3B).

Impairment in mammary development observed on DP6 could be due to decreased ductal branching, reduced formation of lobules, or a combination of both. Therefore, we investigated whether TCDD administration decreases ductal

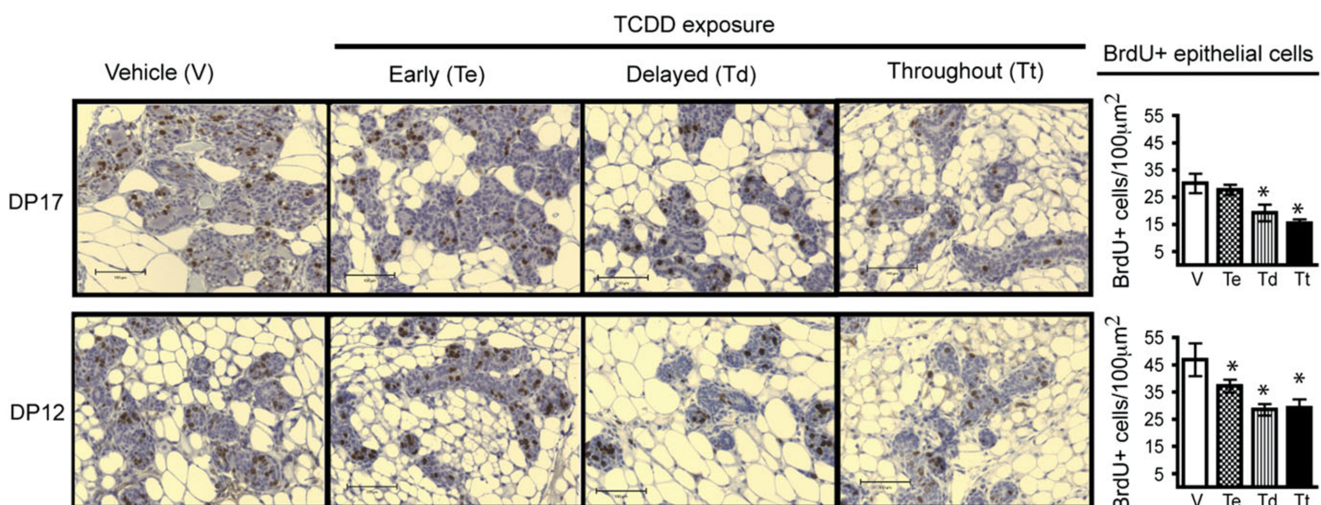


**FIG. 7.** Exposure to TCDD during different windows of time decreases mammary epithelial cell proliferation. Mice were treated as Figure 5 and administered (i.p.) BrdU 3 h prior to sacrifice. Representative images were obtained using a Spot Pursuit camera (20× magnification). The number of $BrdU^+$ cells was determined by quantitative morphometry, as described in the Materials and Methods. The average number of $BrdU^+$ cells in 100 $\mu m^2$ was calculated. Asterisks represent statistically significant differences between the vehicle and indicated TCDD exposure groups ($p < 0.05$, $n = 6$–9).

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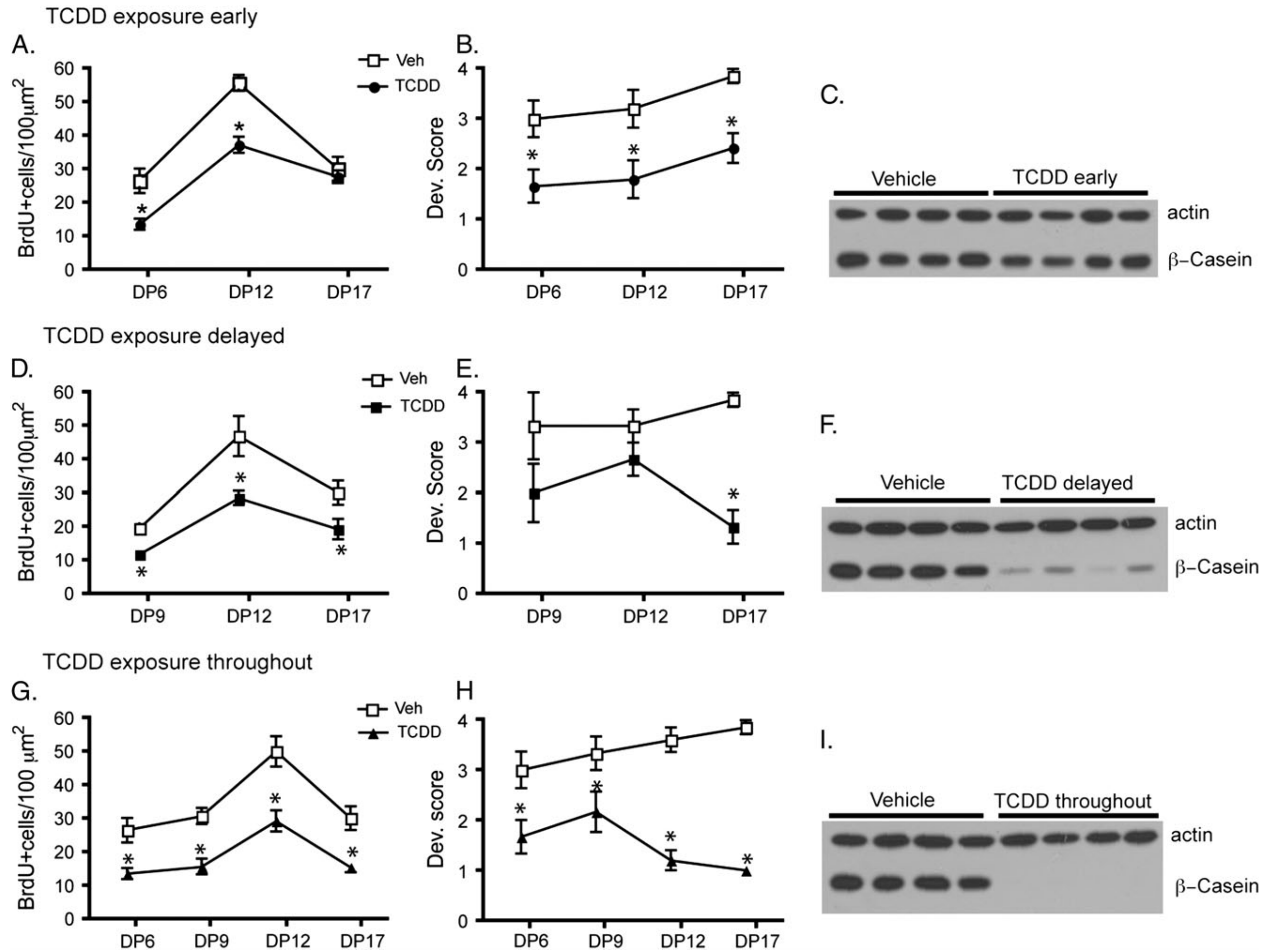


**FIG. 8.** Exposure to TCDD during different windows of time in pregnancy has distinct outcomes in the development of mammary gland. The different patterns of BrdU incorporation by mammary epithelial cell and developmental scoring following exposure to TCDD early (A, B), delayed (D, E), or throughout pregnancy (G, H). The line graphs represent the average number of $BrdU^+$ cells (A, D, G) and developmental scoring (C, F, I) on the indicated days of pregnancy. Asterisks indicate statistically significant differences between the vehicle and TCDD treatment group ($p < 0.05$, $n = 6$–9). Representative immunoblots show β-casein protein levels in mammary glands collected on DP17 from animals exposed to vehicle control and TCDD early, (C), delayed (F), or throughout (I) pregnancy ($n = 6$–9). β-Actin was used as loading control.

branching and/or alveolarization. Ductal morphogenesis starts at puberty and continues during pregnancy (Hovey *et al.*, 2002); thus we measured ductal branching in glands collected on DP6 and compared it with glands from vehicle- and TCDD-treated virgin animals. Both vehicle- and TCDD-treated pregnant animals had enhanced secondary and tertiary branching when compared with virgin animals; however, in vehicle-treated animals the increase was much more robust (70% higher than virgin) than in TCDD treated animals (about 40% higher than virgin; Fig. 3C). In contrast to ductal branching, alveolarization starts during pregnancy. We compared glands from TCDD- and vehicle-treated dams at DP6 and found a 30% decrease in number of lobules following exposure to TCDD (Fig. 3D). There was no difference in the total surface area of whole-mounted glands from different treatment groups (data not shown). The mammary gland achieves its full surface area prior to pregnancy; therefore an effect in total surface area was not expected because TCDD exposure in virgin animals did not alter mammary development (Vorderstrasse *et al.*, 2004). In summary, exposure to TCDD decreased number of branches and mature lobules on DP6.

### *Exposure to TCDD during Pregnancy Decreases Proliferation but Does Not Increase Apoptosis of Mammary Epithelial Cells*

The observed impairment in mammary development during pregnancy followed by TCDD exposure could be a result of an increase in apoptosis or decrease in proliferation of epithelial cells. In contrast to involuting glands, which have a high index of apoptotic cells, there was little to no TUNEL staining in glands collected from pregnant mice regardless of TCDD exposure (Fig. 4A). Apoptosis was also examined by caspase-3

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activity assay. Involuting glands were used as positive control and had at about 70% higher caspase-3 activity than mammary glands collected from pregnant animals. Importantly, there were no statistically significant differences between vehicle- and TCDD-treated pregnant animals (Fig. 4B). These results suggest TCDD-induced impairment of mammary gland development during pregnancy is not caused by premature involution.

Because the development of the mammary gland in pregnancy is dependant on epithelial cell proliferation, the defects in mammary development observed following exposure to TCDD could be due to a decrease in epithelial cell proliferation. In contrast to apoptosis, which occurs primarily in involuting glands, mammary glands have a high rate of proliferation, especially during early and mid-pregnancy. Therefore, we next examined the effects of exposure to TCDD on epithelial cell proliferation. We analyzed proliferation through quantification of BrdU incorporation by mammary epithelial cells in tissue collected on DP6, DP9, and DP12. As early as DP6, there was a 50% decrease in the number of BrdU-positive epithelial cells in glands collected from TCDD-treated animals. Moreover, this decrease persisted on DP9 and DP12 indicating that exposure to TCDD decreases mammary epithelial cell proliferation during early and mid-pregnancy (Fig. 5).

### *Exposure to TCDD in Early Pregnancy is Neither Necessary Nor Sufficient to Impair Mammary Development and Lactogenesis*

On DP17 glands from all TCDD-treated groups had lower developmental scores than glands from mice in the vehicle group (Fig. 6). However, animals treated with TCDD only early in pregnancy had significantly higher scores than animals in which exposure to TCDD was delayed or maintained throughout pregnancy. In contrast, developmental scores did not differ between the TCDD delayed and TCDD throughout treatment groups. When glands were analyzed on DP12, the delayed TCDD treatment group did not show impaired development when compared with vehicle treated animals. However exposure to TCDD early and throughout pregnancy reduced mammary gland developmental scores when compared with vehicle-treated animals (Fig. 6).

In addition to examining development, we determined whether TCDD treatment in early pregnancy is necessary and sufficient to reduce cell proliferation. Compared with control animals, delayed exposure to TCDD or throughout pregnancy caused a 36 and 49% decrease in the number of BrdU-positive cells in glands collected on DP17, respectively. Interestingly, when animals were treated with TCDD on DP0 only, the number of BrdU-positive cells on DP17 did not differ from control. In contrast to differential effects observed on DP17, glands collected on DP12 a showed significantly BrdU-positive cells in all TCDD treated groups when compared with vehicle control glands (Fig. 7).

When we integrate these findings over time, some interesting observations standout. First, when animals were treated with TCDD only early in pregnancy there was a decrease in the number of BrdU-positive cells on DP6 and DP12 when compared with vehicle treated mice. However, exposure to TCDD did not prevent an increase in the number of BrdU-positive cells between DP6 and 12. In other words, even though there was a decrease in number of BrdU-positive cells compared with the vehicle group, the number of epithelial cells increased with progression of pregnancy. These findings suggest that there is a retardation of the developmental process, rather than a permanent injury to this process. In fact, on DP17 the number of BrdU-positive cells in the TCDD early group did not differ from control animals, suggesting a partial recovery of the glands (Fig. 8A). However, this partial recovery did not translate into normal development, as evidenced by the difference in developmental scoring between tissue from TCDD early and vehicle treated animals (Fig. 8B). Yet despite poorer development, the levels of β-casein protein in these glands were similar to that of the vehicle group, suggesting that these glands are potentially able to produce milk (Fig. 8C).

Another interesting observation stems from the group in which TCDD treatment was delayed. In this group, there was a statistically significant reduction in the number of BrdU-positive cells at every time point examined. When compared with glands from control animals, glands collected from animals sacrificed on DP9 had a 40% decrease in the number of BrdU-positive cells, and this decrease in proliferation persisted on DP12 and DP17 (Fig. 8D). In contrast, developmental impairment was only evident in tissues collected on DP17, but not at early points in time (Fig. 8E). Moreover, in this paradigm, developmental impairment correlated with decreased levels of β-casein on DP17, suggesting that glands collected from animals treated with TCDD later in pregnancy would not have the ability to produce milk (Fig. 8F). In contrast to differing outcomes between the TCDD early and TCDD delayed treatment groups, sustained activation of AhR throughout pregnancy significantly reduced the number of BrdU-positive cells (Fig. 8G) and the developmental score (Fig. 8H) of mammary glands at all points in time, and these effects correlated with a profound suppression in levels of β-casein protein on DP17 (Fig. 8I).

## DISCUSSION

Sustained AhR activation during pregnancy impairs mammary development and suppresses lactation (Vorderstrasse *et al.*, 2004). The present data demonstrate that AhR activation during different windows of time in pregnancy impairs mammary gland development, but that the timing of TCDD exposure influences the precise nature of the defect. Our findings suggest that AhR activation by TCDD adversely impacts both cell proliferation and glandular differentiation, but

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the mechanisms underlying these defects may be distinct and independent. Indeed, exposure to TCDD has been shown to decrease proliferation and impair differentiation in other model systems (Elferink, 2003; Huang and Elferink, 2005; Puga *et al.*, 2005) however the precise mechanism by which AhR ligands derail these processes is not fully understood.

When considering how AhR ligands could impact pregnancy-induced proliferation of mammary epithelial cells, several mechanisms are possible. Reduced epithelial cell number may be due to cell cycle arrest that results in fewer cells in the S-phase of the cell cycle. TCDD has been shown to cause fewer cells to progress from the G1 to S phase in an hepatocyte-derived cell line, and the cell cycle inhibitor $p21^{cip1}$ has been implicated the in this process (Barnes-Ellerbe *et al.*, 2004; Kolluri *et al.*, 1999). However, Mitchell et al. (2006), using liver regeneration as a model, found TCDD-mediated cell cycle arrest to be independent of $p21^{cip1}$. To determine the role of $p21^{Cip1}$ in the cell cycle effects observed in our *in vivo* model, we examined the effects of TCDD on the development of mammary glands in pregnant $p21^{Cip1}$ knock out (p21KO) mice and wild-type controls. Exposure to TCDD during pregnancy impaired mammary gland development in p21KO mice to the same extent as in wild type mice (Supplementary Data). Thus, similar to the previous study (Mitchell *et al.*, 2006), our data suggest that the effects of TCDD on mammary gland development during pregnancy are due to cell cycle arrest, but that this arrest is not dependent on the presence of the cell cycle inhibitor $p21^{Cip1}$.

In addition to impacting cell cycle progression, the impairment in mammary development caused by exposure to TCDD could be due to premature involution of the gland. Mammary gland involution is a process of apoptosis and remodeling that occurs naturally in the mammary tissue in response to weaning (Quarrie *et al.*, 1996; Richert *et al.*, 2000). TCDD and other AhR ligands have been reported to increase apoptosis in some experimental systems (Bock and Kohle, 2005; Puga *et al.*, 2009; Ray and Swanson, 2009). However, in our study, exposure to TCDD did not increase mammary epithelial cell apoptosis during pregnancy. This is consistent with several reports that TCDD treatment had either no effect or reduced apoptosis in some systems (Davis *et al.*, 2003; Mitchell and Lawrence, 2003; Mitchell *et al.*, 2006; Stinchcombe *et al.*, 1995; Teske *et al.*, 2005). AhR ligands impact apoptosis in a highly tissue and context-specific manner. In systems where the development rate is high, such as in differentiating mammary glands or regenerating liver, AhR activation seems to have no effect on apoptosis rate; whereas in tissues with an inherently high rate of apoptosis, such as the thymus, AhR ligands may further enhance this process. Regardless of differences in the impact of AhR activation on apoptosis among these different systems, our data indicate that exposure to TCDD did not alter apoptosis of mammary epithelial cells during pregnancy. Thus rather than induction of early involution, the impairment in pregnancy-associated mammary development after exposure to TCDD is due, at least in part, to a decrease in mammary epithelial cell proliferation.

An intriguing observation is the presence of TEBs in mammary glands collected from TCDD-treated animals on DP6. TEBs are sites at which cells rapidly divide to advance the elongation of ducts into the fat pad, and are typically seen in pubescent animals (Humphreys *et al.*, 1996). They disappear once the entire fat pad has been filled with ducts, which normally occurs in rodents at 12 weeks of age (Hennighausen and Robinson, 2005). The mice in this study were 6 weeks old when paired for breeding, and at this age TEBs are still present in virgin animals. However, TEBs should no longer be present by the DP6 (Brisken, 2002). The presence of these structures in glands collected from pregnant animals exposed to TCDD is of interest because it clearly denotes retardation in the process of mammary development. Moreover, TEBs have been shown to play a key role in mammary cancer development as they represent structures that are most immature, proliferative and thus susceptible to carcinogenesis (Jenkins *et al.*, 2007; Russo and Russo, 1978). In fact, the number of TEBs following prenatal exposure to TCDD was decreased in pre-pubertal young rats (Fenton *et al.*, 2002) and increased in more mature virgin animals (Brown *et al.*, 1998; Lewis *et al.*, 2001). Overall, the previous reports concluded that *in utero* exposure to TCDD results in mammary glands that are less differentiated and more susceptible to carcinogenic exposure (Brown *et al.*, 1998; Fenton *et al.*, 2002; Jenkins *et al.*, 2007; Lewis *et al.*, 2001). Likewise, in our study the mammary glands from mice exposed to TCDD early in pregnancy and collected on DP6 were immature and lacked substantial lobule development when compared with control animals. These data suggest that AhR activation during early pregnancy impairs mammary development by a decrease in epithelial cell proliferation with consequent abnormalities in ductal branching morphogenesis and formation of mature lobules.

With progression of pregnancy, these mature lobules populate the stromal tissue with alveolar-like units that eventually become the milk-producing units of the mammary gland (Hennighausen and Robinson, 2001). Thus we investigated if exposure to TCDD in early pregnancy is necessary and sufficient to impair functional development of the mammary gland. Using β-casein expression as a marker of differentiation (Desprez *et al.*, 1995), our data suggest that glands collected from animals exposed to TCDD only on DP0 were potentially able to produce milk, which would suggest that when AhR activation is not sustained throughout pregnancy the gland partially recovers. The partial impairment is likely due to decreased or abnormal ductal branching morphogenesis, rather than diminished lobule formation and alveolarization. The basis for this is that branching morphogenesis takes place in the beginning of pregnancy, which is when TCDD was administered. In contrast, alveolarization starts around DP7. In mice after DP14, alveoli are found all

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over the mammary gland, proliferation basically ceases and functional differentiation leads to lactogenesis (Brisken, 2002). Therefore, because the half-life of TCDD in rodents is about 8–10 days (Gasiewicz *et al.*, 1983) it is possible that animals only treated on DP0 did not maintain a sufficient level of TCDD in the system to impair later events of mammary development and lactogenesis. In contrast, in glands collected from animals that were exposed to TCDD starting later in pregnancy, the effects are likely due to a decrease in lobule formation and differentiation into alveoli. At the time of first exposure to TCDD (DP7) these glands were already populated in terms of branching, as this processes starts early in pregnancy (Hennighausen and Robinson, 1998). However, proliferation was still sensitive to TCDD treatment, resulting in fewer BrdU-positive cells and a reduction in the formation of lobulo-alevolar units. Taken together, our data suggest that an early exposure to TCDD is neither necessary nor sufficient to cause sustained and complete impairment in pregnancy-associated mammary development. Furthermore, AhR activation after DP7 appears to affect alvelolarization—by the time of first exposure to TCDD, the mammary gland was already populated with side branching.

The new findings reported herein suggest that exposure to TCDD during pregnancy influences proliferation in early pregnancy and differentiation in mid-pregnancy, and that these effects may occur through independent mechanisms. For example, the mechanisms that result in decreased proliferation appear to be independent of defects in secretory differentiation and lactogenesis. These findings explain, in part, the complex biological puzzle involving activation of AhR following exposure to TCDD during organogenesis and development. Our data suggest there are critical windows during pregnancy in which exposure to TCDD disrupts mammary gland development; depending on time of exposure, there are different outcomes in mammary gland development. Given that the mammary gland provides an excellent model for studying developmental processes because it develops mostly postpartum, our findings may be useful when considering mechanisms that underlie AhR-mediated alterations in proliferation and differentiation in other developing tissues.

In addition to providing further insight into how AhR regulates proliferation and differentiation, our findings address an area that is clinically relevant but receives very little attention. The American Association of Pediatrics recommends that all infants receive breast milk for at least the first six months after birth, and the consumption of breast-milk has been found to be highly beneficial, with demonstrated effects on brain development and metabolic status (Morley *et al.*, 2004; Mortaz *et al.*, 2001; Singhal *et al.*, 2004). Although the data are limited, it is estimated that that each year 3–6 million mothers of live infants worldwide are either unable to or have significant difficulty initiating breast-feeding. The cause of this problem is not clear, and numbers may be larger than this estimate because lactation success receives insufficient attention. However, it has been suggested that exposure to environmental contaminants, phytochemicals, and drugs may adversely impact pregnancy-associated mammary differentiation and milk production (Neville and Walsh, 1995). We show definitively that exposure to the pollutant dioxin has a profound and detrimental impact on mammary development during pregnancy, resulting in reduced induction of milk proteins and failure to nutritionally support offspring. Although it is unknown whether dioxin-like compounds have similar effects in humans, the regulatory processes in mice and humans are sufficiently similar to speculate that environmental contaminants negatively impact human lactation.

## SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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