Effects of oral exposure to bisphenol A on gene expression and global genomic DNA methylation in the prostate, female mammary gland, and uterus of NCTR Sprague-Dawley rats

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ABSTRACT

Bisphenol A (BPA), an industrial chemical used in the manufacture of polycarbonate and epoxy resins, binds to the nuclear estrogen receptor with an affinity 4-5 orders of magnitude lower than that of estradiol. We reported previously that “high BPA” (100,000 and 300,000 µg/kg body weight (bw)/day), but not “low BPA” [2.5-2,700 µg/kg bw/day], induced clear adverse effects in NCTR Sprague-Dawley rats gavaged daily from gestation day 6 through postnatal day 90. The “high BPA” effects partially overlapped those of ethinyl estradiol (EE₂, 0.5 and 5.0 µg/kg bw/day). To evaluate further the potential of “low BPA” to induce biological effects, here we assessed the global genomic DNA methylation and gene expression in the prostate and female mammary glands, tissues identified previously as potential targets of BPA, and uterus, a sensitive estrogen-responsive tissue. Both doses of EE₂ modulated gene expression, including of known estrogen-responsive genes, and PND 4 global gene expression data showed a partial overlap of the “high BPA” effects with those of EE₂. The “low BPA” doses modulated the expression of several genes; however, the absence of a dose response reduces the likelihood that these changes were causally linked to the treatment. These results are consistent with the toxicity outcomes.

Key Words: bisphenol A; ethinyl estradiol; global genomic DNA methylation; microarray; prostate; mammary gland

Abbreviations:

BPA, bisphenol A

bw, body weight

C3, complement component 3-coding gene

CMC, carboxymethylcellulose

Ct, threshold cycle

Dnmt, DNA methyltransferase-coding gene

EE₂, ethinyl estradiol

ER, estrogen receptor

Esr, estrogen receptor-coding gene

FDR, false discovery rate

GO, gene ontology

GP_{ER}, G-protein-coupled ER

LC-MS/MS, liquid chromatography tandem mass spectrometry

NCTR, National Center for Toxicological Research

NTP, National Toxicology Program

PCA, principal component analysis

PND, postnatal

Prg, progesterone receptor-coding gene

qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction

RIN, RNA integrity number

S100g, calbindin D9K-coding gene

Vegfa, vascular endothelial growth factor A-coding gene

1. INTRODUCTION

Bisphenol A (BPA), a high-production industrial chemical used in the manufacture of polycarbonate plastics and epoxy resins, can be found in many consumer products, including beverage and food containers, medical devices, dental sealants, and recycled and thermal paper. BPA is a ubiquitous environmental contaminant and the majority of the human population has some exposure to BPA (Calafat *et al.*, 2008), primarily orally (FAO/WHO, 2011; FDA, 2013). The estimated median daily intake for the overall US population is approximately 34 ng BPA/kg body weight (bw)/day (Lakind and Naiman, 2011).

BPA is known to exert estrogenic activity by interacting with the nuclear estrogen receptors alpha and beta (ER α and β) with a binding affinity that is 4-5 orders of magnitude lower than that of estradiol (Gould *et al.*, 1998; Kuiper *et al.*, 1998). Reported effects of BPA on well-characterized estrogenic endpoints in animal models include increased uterine weight and modulation of the expression of estrogen-sensitive genes in different tissues and cells (*e.g.*, An *et al.*, 2002; Bosquiazzi *et al.*, 2010; Diel *et al.*, 2000; Kim *et al.*, 2012). BPA was shown also to interact with the ER-related receptor gamma (ERR γ , Takayanagi *et al.*, 2006), with an affinity about 100-fold higher *versus* ERs, and G-protein-coupled ER (GPER, Thomas and Dong, 2006), with a relative binding affinity 2% that of estradiol. *In vitro* studies suggest further that BPA induces rapid, non-genomic responses via membrane-bound ERs, at similar levels as estradiol (Jeng and Watson, 2011; Prins *et al.*, 2014). In addition, effects of BPA on epigenetic mechanisms have been reported, including modulation of global and gene-specific DNA methylation, histone modifications, the expression level of chromatin-modifying genes,

including DNA methyltransferases, and microRNA expression (*e.g.*, Bromer *et al.*, 2010; Dhimolea *et al.*, 2014; Ho *et al.*, 2006; Kim *et al.*, 2014; Kovanecz *et al.*, 2014; Kundakovic *et al.*, 2013; Patel *et al.*, 2013; Tang *et al.*, 2012).

The current study incorporated elements of study design previously identified as critical for data interpretation of BPA studies (Goodman *et al.*, 2006; Shelby, 2008; Richter *et al.*, 2007), including the use of the oral route of exposure; use of a sufficient sample size; use of a wide range of BPA doses appropriately spaced to characterize the dose-response curve; inclusion of a concurrent reference estrogen control; use of the litter as the statistical unit; minimization and monitoring of exposure of the study animals to exogenous BPA and other xenoestrogens; and concurrent assessment of the internal dosimetry of BPA. In addition, neonates were dosed directly by gavage rather than via lactation, to augment the perinatal exposure to BPA (Doerge *et al.*, 2010b). The effects of BPA and ethinyl estradiol (EE₂) on body and organ weights, markers of puberty, histopathology, serum hormone levels, clinical chemistry, levels of ER in the brain, and life-stage internal dosimetry of BPA and EE₂ in the same cohort of animals used in the present study were reported previously (Delclos *et al.*, 2014; Rebuli *et al.*, 2014; Churchwell *et al.*, 2014). Seven “low BPA” doses (2.5-2,700 µg/kg bw/day, spaced half-log) were evaluated to cover a range where effects of BPA have been reported in various research studies (Shelby, 2008), but not in the guideline-compliant studies (Tyl *et al.*, 2002, 2008). No adverse effects were detected in the “low BPA” dose range (Delclos *et al.*, 2014), although statistically significant changes were observed in the expression level of ERα- and ERβ-coding genes in the hypothalamus (Rebuli *et al.*, 2014).

To assess further the potential of the “low BPA” doses to induce changes at the molecular level and characterize the dose response of such changes, here we evaluated the effects of this BPA dose range on epigenetic, estrogenic, and other molecular pathways in the prostate and female mammary gland. Prostate and mammary gland were selected based on the conclusions of the NTP Center for the Evaluation of Risks to Human Reproduction and the joint Food and Agriculture Organization/World Health Organization expert panels, which identified these tissues as potential targets of BPA (Shelby, 2008; FAO/WHO, 2010). Tissues were evaluated at postnatal day (PND) 4, the time when the circulating levels of aglycone (bioactive) BPA was highest (Churchwell *et al.*, 2014), and at PND 90, the time of longest exposure in the study. Evaluation of the PND 90 uterus was also included because the response of this organ to estrogenic compounds is well characterized in adult rats. We also evaluated the effects of two high doses of BPA (100,000 and 300,000 µg/kg bw/day), which were found to induce adverse effects in multiple toxicity endpoints in this animal cohort (Delclos *et al.*, 2014). In addition, two doses of the reference estrogen EE₂ (0.5 and 5 µg/kg bw/day) were included, to characterize further the sensitivity of the animal model to this estrogenic compound and compare its effects with those of BPA. Finally, comparison of a naïve (not gavaged) and vehicle control allowed the evaluation of the effects of the daily gavage. The status of global genomic DNA methylation and the gene expression level of DNA methyltransferases and of several receptors to which BPA has been shown to have binding affinity (ERα and β, ERRγ, and GPER) were evaluated. Since limited effects were detected in these targeted molecular endpoints, we further assessed the genome-wide gene expression levels in prostate and female mammary

gland at PND 4, an age when the levels of bioactive aglycone BPA available to reach molecular targets were highest, due to the immature phase II metabolic capability of the pups (Doerge *et al.*, 2010a; Churchwell *et al.*, 2014).

2. MATERIALS AND METHODS

2.1 Animal Handling Procedures

Procedures involving care and handling of rats were reviewed and approved by the NCTR Institutional Animal Care and Use Committee and are thoroughly described in Delclos *et al.* (2014). Briefly, Sprague-Dawley rats from the NCTR breeding colony were maintained in polysulfone cages with glass water bottles and hardwood chip bedding, and provided water and a soy- and alfalfa-free feed (Purina 5K96) *ad libitum*. F₀ rats were placed in study housing conditions at wean (PND 21) and mated when they were 10-15 weeks-of-age. When a female was determined to be pregnant, the dam was housed individually. Starting at gestation day 6, the pregnant rats were weighed and orally gavaged daily until the beginning of parturition; naïve control dams were weighed daily, but were not gavaged. On the day after birth (day of birth = PND 0), the F₁ litters were culled randomly up to 5 pups/sex/litter. Each pup was weighed and orally gavaged daily with the same dose that its dam had received during gestation; naïve control pups were weighed daily, but were not gavaged. The gavage needle did not enter the esophagus for pups younger than PND 5. There were thirteen treatment groups: naïve (untreated control); vehicle control [0.3% carboxymethylcellulose (CMC) in water]; seven “low BPA” (2.5, 8, 25, 80, 260, 840, and 2,700 µg/kg bw/day); two “high BPA”

(100,000 and 300,000 $\mu\text{g/kg bw/day}$); and two reference estrogen controls (EE_2 , 0.5 and 5.0 $\mu\text{g/kg bw/day}$). For the molecular endpoints reported here, ten animals per dose group (1/sex/litter, randomly assigned) were euthanized at PND 4 or 90 ± 5 ; these animals are littermates of animals used in the study reported by Delclos *et al.* (2014). One pup/sex/litter was sacrificed at PND 4, if there were 5 live same-sex littermates on the day of sacrifice, and at PND 90 ± 5 , if there were 2 live same-sex littermates on the day of sacrifice. The use of a single pup/sex/litter per time point obviated the need to control for litter effects in the statistical analyses. Daily vaginal smears were collected starting on PND 85 in an attempt to necropsy the cycling females at estrus, to minimize biological variability due to the estrous cycle. A terminal vaginal smear confirmed all cycling females used in this study were sacrificed in estrus, except one in the 8 $\mu\text{g BPA/kg bw/day}$ dose, which was euthanized in diestrus, and two in the 300,000 $\mu\text{g BPA/kg bw/day}$ dose and each of the two EE_2 doses, which were euthanized in an intermediate stage between diestrus and estrus. PND 4 and PND 90 animals were exsanguinated by cardiac puncture and retro-orbital bleeding, respectively, after CO_2 asphyxiation. The PND 4 and 90 prostate (ventral, dorsal, and lateral lobes combined), PND 4 and 90 fifth inguinal mammary gland, and PND 90 uteri (both horns combined) were used. Tissues were immediately flash-frozen in an isopentane/dry ice slurry upon dissection and were used without pooling. Frozen PND 90 tissues were macerated to a fine powder using a mortar and pestle, while being kept frozen with liquid nitrogen. Samples were stored at -80°C until further processing; extreme care was taken not to thaw the samples prior to addition of a lysis buffer.

2.2. Genomic DNA Isolation and Global Genomic DNA Methylation Status Analysis by Cytosine Extension Assay

Genomic DNA was isolated from the PND 90 prostate, female mammary gland, and uterus using a DNeasy Blood & Tissue Mini kit, following the manufacturer's instructions (Qiagen, Valencia, CA). Frozen tissue powder (20-30 mg) was homogenized in lysis buffer using a pellet pestle grinder (Kimble Chase Kontes, Vineland, NJ). Approximately 2 µg of genomic DNA digested with HpaII restriction endonuclease (New England Biolabs, Beverly, MA) were evaluated using the [³H]dCTP extension assay described in Pogribny *et al.* (1999).

2.3 Quantification of Cytosine and 5-Methylcytosine by Liquid Chromatography Tandem Mass Spectrometry

The quantification of cytosine and 5-methylcytosine was determined using 2 µg of genomic DNA dissolved in 20 µL of Tris-EDTA buffer, pH 8.0, as described in Pogribny *et al.* (2013). The percentage of methylation was calculated as the quotient between the number of moles of 5-methylcytosine and the sum of the number of moles of cytosine and 5-methylcytosine in each sample.

2.4 Total RNA Isolation and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from the prostate and uterus using a RNeasy Mini kit and from the female mammary gland using a RNeasy Lipid Tissue Mini kit, following the manufacturer's instructions (both Qiagen). The entire frozen PND 4 tissues or 10-20 mg of frozen PND 90 tissue

powder were individually homogenized using a 5 mm stainless steel bead and a TissueLyser II (Qiagen) for 2x 2 min at 20 Hz. The uterus homogenate was further digested with proteinase K prior to RNA isolation. RNA was treated with DNase I in-column. The RNA purity and concentration of all samples were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and the RNA integrity was assessed in randomly selected samples using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA samples had a 260/280 nm absorbance ratio greater than 2.0 and the RNA integrity number (RIN) was 9.63 ± 0.46 [mean $\pm$ standard deviation (SD); $n = 288$], confirming that the RNA samples were of high quality (Schroeder *et al.*, 2006). One microgram of total RNA was reverse-transcribed using random hexamer primers and an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA), following the manufacturer's protocol. The cDNA was diluted 1:40 with nuclease-free water and used as a template in the qRT-PCR assays, as described in Camacho *et al.* (2011). Supplementary Table 1 lists the TaqMan assays (Applied Biosystems, Foster City, CA) used and the range of threshold cycle (Ct) values. Each cDNA sample was run in duplicate. No-template controls, in which the cDNA sample was replaced by an equal volume of nuclease-free water, were included in duplicate in each plate and found to be negative in all cases. *Gapdh* (NM_017008) was used as the endogenous control gene for all tissues, except PND 4 mammary gland because its expression was not stable with these samples (data not shown); instead, *Mrlp19* (NM_001029898) was used. At least two independent qRT-PCRs were performed to quantify the expression level of the endogenous control genes and the average expression was used to normalize the expression of each gene of interest. Data were analyzed using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

2.5 Gene Expression Analysis Using Microarray Technology

An Agilent rat whole genome 4x44k (v3) microarray platform (Agilent Technologies, Santa Clara, CA) was used to determine the gene expression profiles in the PND 4 prostate and female mammary glands. Seven RNA samples per dose group were used, which were randomly selected from the 10/sex/dose group that were available; the RIN range was 9.7-10 and 9.2-10 for the PND 4 prostate and female mammary gland RNA samples, respectively. The sample size was chosen based on *a priori* power calculations that showed that a sample size of 7/dose group would provide greater than 80% statistical power to detect a 1.5-fold change in gene expression level *versus* vehicle control under the current experimental error. The sample labeling and microarray process were performed according to Agilent "One-Color Microarray-Based Gene Expression Analysis" protocol (Version 5.5). Briefly, 500 ng of total RNA were reverse-transcribed and amplified to Cy3-labeled cRNA. A total of 1.6 µg of labeled cRNA from each sample was used to hybridize to Agilent microarray for 17 hrs at 65 °C. The hybridized slides were washed in Washing buffer I at room temperature and Washing buffer II at 37 °C before being scanned in an Agilent G2505C DNA Microarray scanner (Agilent Technologies) at 5 µm resolution. The resulting images were analyzed by using the Agilent Feature Extraction Software (version 10.7) to determine the Cy3 fluorescence intensity of all gene spots (features) on each array. The raw data were uploaded into the ArrayTrack database (Fang *et al.*, 2009) and normalized using quantile scaling normalization before further statistical analysis.

2.6 Statistical Analyses

The qRT-PCR and global genomic DNA methylation data are presented as mean $\pm$ standard error of the mean (SEM). Samples from male and female rats and from different tissues and PNDs were analyzed separately. Outliers in these assays were identified as observations that fell outside of a pre-defined boundary (25% quantile minus 2 times the range between 25%-75% quantiles and 75% quantile plus 2 times that range) for log2 transformed data. Five and fifteen samples (out of 390) were identified as outliers in qRT-PCR and global genomic DNA methylation assays, respectively; since their exclusion did not change the statistical outcome, the data presented include all samples analyzed ($n = 10/\text{dose/sex/PND}$). One-way ANOVA followed by a Dunnett's post-hoc test was used to detect statistically significant differences between each dose group, including the naïve, and the vehicle control. A two-sided p -value < 0.05 was considered statistically significant.

The microarray data were transformed to transcript level and the technical replicates were averaged before quantile normalization. The correlation between technical replicates was 0.985, indicating a high consistency and good experimental quality. A one-way ANOVA was used to identify the overall statistically significant differentially expressed genes. A p -value < 0.005 was used to filter the genes with no overall changes. False discovery rate (FDR) adjustment (Benjamini and Hochberg, 1995; Storey, 2003) was applied to identify the genes with a FDR under 10%. The genes identified to be significantly modulated *versus* vehicle control by one-way ANOVA were further analyzed by a Dunnett's post-hoc test to identify the statistically significant differences between each dose group and the vehicle control; a p -value < 0.05 was considered statistically significant. Principal component analysis (PCA) and

hierarchical clustering were performed. Categorization of the gene ontology (GO) biological process of genes modulated significantly *versus* vehicle control was performed using the GOFFA (Gene Ontology for Functional Analysis) tool of ArrayTrack (Sun *et al.*, 2006) and further confirmed using the PANTHER (protein annotation through evolutionary relationship) classification system (Mi *et al.*, 2013). In addition, the Gene Ontology enRIchment anaLysis and visuaLizAtion tool (Eron *et al.*, 2009) was used for enrichment analysis. The *p*-value and the corresponding FDR *q*-value of each mapped GO term were calculated; enriched GO terms with a *p*-value < 0.001 and more than three representative genes in the dataset were considered to be significant. One sample from the 300,000 µg BPA/kg bw/day dose group (mammary gland # 8110; arrow in Figure 2) was consistently identified as an outlier, but its exclusion did not change the results; hence, the data presented include all samples analyzed (*n* = 7/dose/tissue).

3. RESULTS

3.1 Global Genomic DNA Methylation Status at PND 90 and Expression of DNA

Methyltransferase Coding Genes at PND 4 and 90

The effect of BPA and EE₂ on the global genomic DNA methylation status in the PND 90 prostate, female mammary gland, and uterus was determined by a cytosine extension assay (Table 1). The one-way ANOVA suggested that there was a global genomic DNA methylation effect in the PND 90 uterus (*p*-value = 0.021); however, pairwise comparisons using the Dunnett's test did not indicate a significant effect for any individual dose group *versus* the tissue-matched vehicle control. No other statistically significant differences were found. Similarly, direct quantification of 5-methylcytosine by HPLC-ES-MS/MS DNA did not indicate a

difference between dose groups (Table 1). We assessed further the effect of BPA and EE₂ on the expression of the DNA methyltransferase-coding genes (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) in PND 4 prostate and female mammary gland and in PND 90 prostate, female mammary gland, and uterus (Supplementary Tables 2-4). The only significant effect observed was on the expression of *Dnmt1* in the PND 90 female mammary gland (one-way ANOVA *p*-value = 0.028); however, pairwise comparisons to the vehicle control using the Dunnett's test failed to identify a significant effect on any individual dose group.

3.2 Expression of Estrogen Receptor and Estrogen Receptor-Related Receptor Genes at PND 4 and 90

The effect of BPA and EE₂ on the expression level of the genes encoding the nuclear ERs (*Esr1* and *Esr2*), G-protein-coupled ER (*Gper*), and members of the ERR family (*Esrra*, *Essrb*, and *Esrrg*) was assessed in the prostate and female mammary glands at PND 4 and PND 90, and in the uterus at PND 90. Supplementary Tables 5 and 6 show the prostate and female mammary gland data, respectively, and Figure 1 shows the uterus data. There were no differences between the naïve and vehicle controls in the expression level of any of the genes analyzed, at either PND or in any tissue. One-way ANOVA identified a significant effect in the expression of *Esrrg* in PND 4 female mammary gland (*p*-value = 0.015) and in the expression of *Esrra* in PND 4 prostate (*p*-value = 0.045), but pairwise comparisons to the vehicle control using the Dunnett's test did not identify any significant differences in individual dose groups. In the PND 90 prostate, the expression of *Esr1* was down-regulated 1.4-fold (*p*-value = 0.048) by 840 µg BPA/kg bw/day *versus* the vehicle control, but not by any other dose. In the PND 90 female

mammary gland, *Esrrb* was up-regulated 3-fold by the low dose of EE₂ (p -value = 0.013) and *Esr2* was up-regulated about 2.5-fold by both doses of EE₂ *versus* the vehicle control (p -value = 0.001, Supplementary Table 6). *Esrrg* was up-regulated about 2.5-fold by both doses of EE₂ in the PND 90 uterus (p -value = 0.003 and 0.006 for 0.5 and 5 µg EE₂/kg bw/day, respectively, Figure 1).

3.3 Expression of Estrogen-Responsive Genes in PND 90 Uterus

The expression of four estrogen-responsive genes, coding complement component 3 (*C3*), progesterone receptor (*Pgr*), calbindin D9K (*S100g*), and vascular endothelial growth factor A (*Vegfa*), was assessed in the adult uterus (Figure 1). The expression of *C3* was not affected by any dose of BPA or EE₂. Both EE₂ doses up-regulated *Pgr* and *S100g*, while *Vegfa* was significantly up-regulated by the high EE₂. *Pgr* was up-regulated 4.5-fold (p -value = 0.010) and 3.9-fold (p -value = 0.025) by 0.5 and 5 µg EE₂ /kg bw/day, respectively, while *S100g* was up-regulated 2.5-fold (p -value = 0.001) and 2.8-fold (p -value = 0.001) by 0.5 and 5 µg EE₂ /kg bw/day, respectively. *Vegfa* was up-regulated 1.5-fold (p -value = 0.016) by 5 µg EE₂ /kg bw/day *versus* vehicle control. No significant differences were detected between the naïve or BPA dose groups and the vehicle control.

3.4 Genome-Wide Gene Expression in PND 4 Prostate and Female Mammary Glands

Since limited effects were detected at the targeted assays described above, to characterize further the effect of BPA (and EE₂) on the gene expression of prostate and female mammary glands, genome-wide gene expression was analyzed by DNA microarray technology. Tissues

from PND 4 animals were used, since the internal concentration of aglycone (bioactive) BPA was highest due to the immature phase II metabolic capability of the pups (Doerge *et al.*, 2010a; Churchwell *et al.*, 2014) and due to the potential for developmental disruption at this more sensitive age.

Seven samples per dose group were used, based on *a priori* statistical power analysis. To reduce the number of samples, but still allow the characterization of the dose-response curve in the “low BPA” dose range, four “low BPA” doses were used (2.5, 25, 260, and 2,700 µg BPA/kg bw/day), along with the vehicle control, the highest dose of BPA (300,000 µg BPA/kg bw/day), and the two doses of EE₂ (0.5 and 5.0 µg EE₂/kg bw/day). To verify the reproducibility of the microarray data and characterize further the dose-response curve of the gene expression changes observed in the “low BPA” dose range, qRT-PCRs were performed; ten samples per dose group from all thirteen dose groups available in the study were used in these assays. As shown in Figures 3 and 4 (columns A and C), there was good correlation between the microarray and qRT-PCR data.

One-way ANOVA (p -value < 0.005) identified 218 genes differentially modulated in the prostate, 8 of which had a FDR below 10%, *versus* tissue-matched vehicle control (Supplementary Table 7). In the female mammary gland, the expression of 257 genes was differentially modulated, 18 of which had a FDR below 10%, *versus* tissue-matched vehicle control (Supplementary Table 8). Regardless of the statistical method used, the high EE₂ dose modulated the largest number of genes both in the prostate and female mammary gland, followed by the low EE₂ dose. Consistently, PCA using the significantly modulated genes

showed a clear separation of the high EE₂ from the vehicle control, as did the low EE₂ samples (Figure 2 and Supplementary Figure 1). In addition, the “high BPA” samples tended to segregate from the vehicle control in the female mammary gland. No obvious separation was observed among the “low BPA” dose groups and the vehicle control.

Tables 2 and 3 list the genes significantly modulated at least 1.5-fold by BPA or EE₂ *versus* vehicle control in PND 4 prostate and female mammary gland, respectively, and their gene ontology process; the complete list of genes is shown in Supplementary Tables 9 (prostate) and 10 (female mammary gland). The majority of these genes were involved in cell proliferation, cell death, cell adhesion, cell signaling, and metabolic processes in both tissues. In addition, genes involved in inflammation and immune response and in oxidation-reduction processes were modulated in the mammary gland. GO term enrichment analysis in the female mammary gland confirmed the over-representation of genes associated with immune system process and cytosolic calcium ion homeostasis in the low EE₂ dose group and showed an over-representation of genes involved in cellular response to estrogen stimulus in the high EE₂ dose group (Supplementary Table 11). In the prostate, the high and low EE₂ doses had an over-representation of genes involved in axis specification and in the regulation of the non-canonical Wnt signaling pathway, respectively. The only BPA doses that yielded significant GO term enrichments were the 2.5 µg BPA/kg bw/day and 25 µg BPA/kg bw/day dose groups. In the female mammary gland, these related to cellular response to stimuli and the metabolic process, respectively. In the prostate, 25 µg BPA/kg bw/day modulated more than expected genes involved in the oxidation-reduction process, while the 2.5 µg BPA/kg bw/day dose group

modulated genes involved in the regulation of kinase activity and assembly of cell projection (Supplementary Table 11) .

Although significant gene expression changes with at least a 1.5-fold change were observed in all “low BPA” doses *versus* the tissue-matched control and significant GO term enrichments were observed in some “low BPA” dose groups, it should be noted that nine of the 10 (90%) prostate genes and 34 of the 40 (85%) female mammary gland genes were modulated by a single “low BPA” dose level (Tables 2 and 3). Analysis of all genes significantly modulated in the “low BPA” dose range *versus* vehicle control, regardless of the fold-change of the modulation, yielded a similar result, with twenty-nine of the 33 (88%) genes and 43 of the 50 (86%) genes found to be modulated by a single “low BPA” dose level in the PND 4 prostate and female mammary gland, respectively (Supplementary Tables 7 and 8). The remaining genes (*Bbs7*, *Bco2*, *Mdh1b*, *Mtfr1*, and *Ube2* in the prostate, and *Clec2d2*, *LOC100302465*, *LOC685171*, *LOC690402*, *Ndufa10l1*, *Rasa4*, *RGD1562720*, *Slitrk6*, and *Vom2r5* in the female mammary gland) were modulated by multiple “low BPA” dose levels, but the gene expression fold-change was not correlated with the BPA dose nor was it further amplified by the high BPA and/or EE₂ doses (Tables 2 and 3 and Supplementary Tables 7 and 8). The majority of the genes modulated by multiple “low BPA” dose levels had a very low expression level; in fact, only *Ube2a*, *Clec2d2*, and *Rasa4* were expressed at sufficient level to be detected by qRT-PCR. qRT-PCR failed to confirm the modulation of these three genes by the “low BPA” dose levels (Figures 3 and 4). The difference in outcome between microarray and qRT-PCR assays may be due to the existence of multiple *Ube2a* transcripts (a second microarray probe did not indicate

modulation of *Ube2a* gene, Supplementary Table 7) or the sub-optimal design of the *Clec2d2* Taqman assay (which targets not only *Clec2d2*, but also *Clec2d1* and *LOC689757*, Supplementary Table 1). qRT-PCR confirmed the statistically significant up-regulation of *Rasa4* by 300,000 µg BPA/kg bw/day (1.51-fold, *p*-value = 0.04) and 0.5 µg EE₂/kg bw/day (1.58-fold, *p*-value = 0.01), but not by 2.5 or 2,700 µg BPA/kg bw/day (Figure 4).

4. DISCUSSION

The current study assessed the effects of a wide range of oral BPA doses (2.5-300,000 µg/kg bw/day) at the molecular level in rat prostate and female mammary gland. These two tissues were selected because important data gaps regarding BPA toxicity were identified previously, including poor characterization of the molecular pathways modulated by BPA at doses below those needed to activate the ERs (Shelby, 2008; FAO/OWH, 2010; FDA, 2013). The adult rat uterus, a well-characterized estrogen-sensitive tissue, was also included. The tissues analyzed were harvested from littermates of animals used in the study reported by Delclos *et al.* (2014), which summarized the effects observed at the body and organ weight, clinical chemistry, estrous cyclicity, sperm parameters, and histopathology level, and Churchwell *et al.* (2014), which summarized the findings at the internal dosimetry level. In addition to the nine BPA dose groups analyzed, two negative controls (naïve and vehicle), and two reference estrogen controls (EE₂, 0.5 and 5.0 µg/kg bw/day) were also included. All together, these aspects of study design resulted in a very robust and well-controlled study.

To circumvent the poor lactational transfer of BPA due to its efficient metabolism and excretion by the dam (Doerge *et al.*, 2010b), which would result in an attenuated exposure during the critical neonatal period, BPA was administered directly to the pups by gavage from the day after birth. The effect of this dosing procedure and of the vehicle solution was assessed by comparing a naïve (not gavaged) control with the vehicle control. No statistically significant differences were detected between the two negative controls in any molecular assay, regardless of tissue or PND. Together with the lack of differences between naïve and vehicle controls reported in this animal cohort by Delclos *et al.* (2014), our data support further the lack of effect of the daily gavage on the endpoints analyzed in this study.

We first sought to determine the effects of BPA (and EE₂) on targeted molecular endpoints, selected based on previous reports. These included global genomic DNA methylation and expression levels of DNA methyltransferases and BPA-binding receptors coding genes. We analyzed further the effects of BPA on the genome-wide gene expression level of PND 4 prostate and mammary gland by microarray technology. As reported in Churchwell *et al.* (2014), despite the meticulous care taken to minimize exposure of the study animals to exogenous sources of BPA during housing and dosing, both naïve and vehicle controls had detectable levels of circulating BPA glucuronide, a metabolite of BPA produced *in vivo*. The mean serum levels of BPA glucuronide in the naïve and vehicle controls and in animals dosed with 2.5 µg BPA/kg bw/day were undistinguishable, thus precluding the assessment of treatment-related effects of the lowest BPA dose in the study *versus* the negative controls; however, the margin of exposure between animals treated with > 8 µg BPA/kg bw/day and

vehicle controls was sufficient to interpret any effect in the context of the BPA treatment (Delclos *et al.*, 2014; Rebuli *et al.*, 2014).

We assessed the effect of BPA and EE₂ on the PND 90 global genomic DNA methylation using both an enzymatic and a non-enzymatic method. The non-enzymatic LC-MS/MS method measures the absolute DNA methylation status and is considered as a “gold” standard in global DNA methylation analyses. The enzymatic cytosine extension method detects the extent of the methylation status of the cytosine residues within the recognition sequence of the HpaII restriction endonuclease, which are randomly distributed across the genome (Rocha *et al.*, 2010). Regardless of the method, we did not detect a statistically significant PND 90 global genomic DNA methylation change in either BPA- or EE₂-treated animals *versus* the vehicle control. To the best of our knowledge, this is the first study examining the effect of BPA on the global genomic DNA methylation in the prostate; however, we cannot exclude the possibility of local region-specific DNA methylation changes induced by BPA- or EE₂-treatment. Ho *et al.* (2006) and Tang *et al.* (2012) reported that the subcutaneous injection of Zivic-Miller Sprague-Dawley rats with 10 µg BPA/kg bw/day in the perinatal period induced changes in the methylation status of specific gene promoters in the dorsal prostate. These authors further reported the modulation of the gene expression level of *Dnmt3a/b* in the dorsal prostate by BPA, an effect not observed in the current study. The effects of BPA in the ventral and lateral prostate lobes were not reported by Ho *et al.* (2006) or Tang *et al.* (2012). The use of the combined ventral and dorsolateral lobes in the current study *versus* the use of the dorsal lobe by Tang *et al.* (2012) may explain the differences observed, since the prostatic lobes exhibit

different sensitivity to different estrogenic compounds (Ofner *et al.*, 1992, Tam *et al.*, 2008) and the use of combined lobes would dilute a lobe-specific effect. In addition, the use of a younger age in our study, when the rat prostate is still undergoing arborization and the different lobes are becoming anatomically distinct (Hayashi *et al.*, 1991) could contribute to the difference in findings. Recently, the effect of BPA on the epigenome of the Harlan Wistar-Furth rat female whole mammary gland was reported by Dhimolea *et al.* (2014). These authors demonstrated transient region-specific DNA methylation changes induced by BPA treatment; however, similar to our results, they did not identify any significant hypo- or hypermethylation trends in genomic DNA methylation.

Several treatment-related effects were observed in the EE₂ dose groups at the gene expression level. The expression of previously proposed molecular biomarkers of estrogenic exposure, including *Vegfa*, *Pgr*, and *S100g* (An *et al.*, 2002; Diel *et al.*, 2002; Jung *et al.*, 2012; Schmidt *et al.*, 2006) were up-regulated by one or both EE₂ doses in the PND 90 uterus. In addition, *S100g* was up-regulated by EE₂ at PND 4 in both the prostate and female mammary glands. One or both doses of EE₂ further modulated the expression of ER or ERR-coding genes in the PND 90 uterus and female mammary gland. PCA of the statistically significant microarray data showed a segregation of the high and low EE₂ from the vehicle control at PND 4 in both prostate and female mammary glands, and GO term enrichment analysis showed an over-representation of genes involved in response to estrogen stimulus in the genes modulated by the high EE₂ in the female mammary gland. Of the genes modulated by one or both doses of EE₂ in the PND 4 tissues analyzed, several are known estrogen-responsive genes. Genes up-regulated by EE₂ in

the current study included *Angpt2* in the prostate and *Akr1c13*, *Il4ra*, *Ramp3*, *Serpinb9*, *Spon1*, and *Trhr2* in the female mammary gland. These genes were previously shown to be up-regulated by estrogen (Aenlle *et al.*, 2009; Hong *et al.*, 2004; Jiang *et al.*, 2008; Kimura *et al.*, 1994; Rincón-Rodríguez *et al.*, 2013; Ye *et al.*, 2002). Conversely, *Apln* and *Clock* were found to be down-regulated by EE₂ in the female mammary gland in the current study, in agreement with previous reports (Heneweer *et al.*, 2007; Damdimopoulou *et al.*, 2011). In all cases, the direction of the gene expression change was consistent with those reported previously. Taken together, our data confirmed the sensitivity of the NCTR Sprague-Dawley rat to the reference estrogen EE₂, consistent with our previous studies (NTP, 2010a, b; Delclos *et al.*, 2014; Rebuli *et al.*, 2014).

Both 300,000 µg BPA/kg bw/day and EE₂ modulated the expression level of *Dclk3*, *Gnai2*, *Gzmc*, *LOC687090*, *Map4k5*, and *Ppic* in the PND 4 prostate and *Coro1c*, *Ctrc*, *Lgals4*, *LOC685106*, *Ntng1*, and *RGD1311378* in the PND 4 female mammary gland, and the direction of the change *versus* the vehicle control was the same for both compounds in all cases. In addition, PCA of the statistically significant microarray data suggested that the effect of the high BPA dose level at the genome-wide gene expression overlapped to some extent with that of the low dose EE₂, in particular in the female mammary gland. Our data thus suggest that the high BPA dose induced estrogenic effects that were more pronounced in the female than in the male tissue. These data are consistent with the overall conclusion of the 90-day subchronic toxicology study, in which 300,000 µg BPA/kg bw/day had a clear effect in multiple endpoints in female rats, some of which overlapped with the EE₂ dose groups, while the effects of this high BPA dose

level in male rats were much more restricted (Deldos *et al.*, 2014). The internal dosimetry data collected in littermates of animals used in the current study further support that treatment with the 300,000 µg BPA/kg bw/day results in levels of circulating bioactive, aglycone BPA sufficient to activate the ER and mediate estrogenic effects (Churchwell *et al.*, 2014).

Several rodent in vivo studies reported the modulation of the gene expression level by BPA doses within or below the “low BPA” dose range as defined in the present study, including in the rat prostate and female mammary gland. These include the above mentioned Ho *et al.* (2006) and Tang *et al.* (2012) studies that report gene expression modulation in the prostate of Sprague-Dawley rats treated subcutaneously with 10 µg BPA/kg bw/day on PND 1, 3, and 5. The modulation of female mammary gland gene expression in rodent models was reported by Moral *et al.* (2008; Sprague-Dawley rats, 25 and 250 µg BPA/kg bw/day, daily gavage, from GD 10 to 21), Wadia *et al.* (2013; ERα(+/-) and ERα (-/-) C57BL/6 mice, 250 ng BPA/kg BW/day, subcutaneous osmotic pump, from GD 8 to 19), and Dhimolea *et al.* (2014; Wistar-Furth rats, 250 µg BPA/kg bw/day, subcutaneous osmotic pump, from GD 9 to PND 1). Similarly, in our study, several genes were found to be modulated both in PND 4 prostate and female mammary glands in the “low BPA” dose range *versus* the vehicle control. Of these, over 85% were affected only by a single BPA dose level, despite the close spacing between consecutive “low BPA” dose levels (~10-fold in microarray and ~3.3-fold in the qRT-PCR assays). This was observed for both the prostate and female mammary gland. Only five genes in the prostate and eight genes in the female mammary gland were modulated by multiple BPA dose levels; however, the fold-change of these genes was similar across the wide range of doses tested,

despite the 120,000-fold difference between the lowest and the highest dose of BPA tested, reducing our confidence that these observations were related to the BPA treatment. The sample size used in the current study was selected to provide greater than 80% statistical power to detect a 1.5-fold change of a treated group *versus* the vehicle control, as determined by *a priori* power calculation analysis, although it is possible that a larger sample size may have resulted in the detection of additional statistically significant findings, including of dose-related changes in the “low BPA” dose range. Due to the gaps between the 2,700, 100,000, and 300,000 µg BPA/kg bw/day dose groups in the present study, we cannot exclude the possibility that BPA induced further effects in this dose range that could have gone undetected; however, these doses represent approximately 5,000 to 500,000 times the mean human dose of BPA (Lakind and Naiman, 2011), and thus would bear little significance in the context of human exposure. Our observation that, although genes were found to be statistically modulated in all “low BPA” dose groups *versus* vehicle control, none showed a clear dose-response emphasizes the importance of incorporating multiple and appropriately spaced dose levels in the study design to better interpret the biological significance of the statistical findings.

PND 4 tissues were used to assess the effects of BPA at the genome-wide gene expression level because the perinatal period is usually recognized as a susceptible window to disruption (Macon and Fenton, 2013; Prins and Ho, 2010) and the levels of circulating bioactive aglycone BPA were highest at this age (Churchwell *et al.*, 2014). Indeed, the PND 4 gene expression profiles were modulated in the prostate and female mammary gland by the EE₂ and high BPA dose groups, but no treatment-related changes were observed in the “low BPA” dose range. It

is possible that longer exposures to “low BPA” could result in such changes. This hypothesis is being assessed currently in the NCTR Sprague-Dawley rat by other investigators within the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA). These ongoing studies will provide further insight into the molecular, functional, and morphological effects of a wide-range of oral BPA upon longer exposure periods, including at PND 21, PND 90, 6 months, and 1 year of age (Schug *et al.*, 2013).

5. CONCLUSIONS

Our study reports that both 0.5 and 5.0 µg EE₂/kg bw/day modulated the expression level of several genes. In addition, our microarray data showed that 300,000 µg BPA/kg bw/day induced effects that partially overlapped with the lowest dose of EE₂, confirming the estrogenic potential of this high BPA dose level. Microarray data showed further the modulation of several genes in the “low BPA” dose range, but the lack of a dose-response reduces the likelihood that these effects were causally linked to the treatment with “low BPA”. These results are consistent with the toxicity outcomes reported previously for this animal cohort (Delclos *et al.*, 2014). Endpoints complementary to the ones reported here are being assessed currently in NCTR Sprague-Dawley rats exposed to BPA for longer exposure periods by other investigators participating in the CLARITY-BPA study. Those data will help to elucidate further the effects of a wide-range of oral BPA at the epigenetic and gene expression level in this rat model and integrate the molecular findings in the context of structural and functional effects.

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FIGURE LEGENDS

Figure 1. Fold-change *versus* vehicle control of the expression level of ER-, GPER-, and ERR-coding or estrogen-responsive genes in uterus of Sprague-Dawley rats untreated (naïve control, NC) or treated by oral gavage daily with vehicle (0.3% CMC in water), BPA (2.5, 8, 25, 80, 260, 840, 2,700, 100,000, or 300,000 µg/kg bw/day), or EE₂ (0.5 or 5.0 µg/kg bw/day) from gestation day 6 until PND 90 ± 5. Data shown as mean ± SEM, n = 10/dose group, except *Esr2* assay, where n = 6-9 due to undetermined Ct values.

Figure 2. Principal component analysis of the genes significantly (one-way ANOVA *p*-value < 0.005) modulated by BPA or EE₂ *versus* vehicle control, in the prostate and female mammary gland of Sprague-Dawley rats treated by oral gavage daily with vehicle (0.3% CMC in water), BPA (2.5, 8, 25, 80, 260, 840, 2,700, 100,000, or 300,000 µg/kg bw/day), or EE₂ (0.5 or 5.0 µg/kg bw/day) from gestation day 6 until PND 4. n = 7/dose group/tissue. The arrow indicates a statistical outlier.

Figure 3. Comparison of the fold-change *versus* vehicle control of the expression level of selected genes assessed by genome-wide microarray and qRT-PCR in the prostate Sprague-Dawley rats untreated (naïve control, NC) or treated by oral gavage daily with vehicle (0.3%

CMC in water), BPA (2.5, 8, 25, 80, 260, 840, 2,700, 100,000, or 300,000 µg/kg bw/day), or EE₂ (0.5 or 5.0 µg/kg bw/day) from gestation day 6 until PND 4. Columns A and C compare microarray (grey bars) and qRT-PCR (black bars) data in the same subset of samples (n = 7/dose group). Columns B and D show qRT-PCR data using all samples (n = 10/dose group, except *Gzm* where n = 5-7, *Lefty2* and *Slpil2* where n = 6-7, and *S100g* where n = 4-7 due to undetermined Ct values). Data shown as mean ± SEM.

Figure 4. Comparison of the fold-change *versus* vehicle control of the expression level of selected genes assessed by genome-wide microarray and qRT-PCR in the female mammary gland of Sprague-Dawley rats untreated (naïve control, NC) or treated by oral gavage daily with vehicle (0.3% CMC in water), BPA (2.5, 8, 25, 80, 260, 840, 2,700, 100,000, or 300,000 µg/kg bw/day), or EE₂ (0.5 or 5.0 µg/kg bw/day) from gestation day 6 until PND 4. Columns A and C compare microarray (grey bars) and qRT-PCR (black bars) data in the same subset of samples (n = 7/dose group). Columns B and D show qRT-PCR data using all samples (n = 10/dose group, except *Ccl5* where n = 6-7/dose group due to a computer failure). Data shown as mean ± SEM.

Table 1. Status of global genomic DNA in the prostate, female mammary gland, and uterus of PND 90 Sprague-Dawley rats untreated (naïve control) or treated by oral gavage daily with vehicle (0.3% CMC in water), BPA (2.5, 8, 25, 80, 260, 840, 2,700, 100,000, or 300,000 µg/kg bw/day), or EE₂ (0.5 or 5.0 µg/kg bw/day) from gestation day 6 until PND 90 ± 5. The decays per minute (cytosine extension method) or the % 5-methylcytosine (LC-MS/MS method) are shown. Data shown as mean ± SEM, n = 10/dose group/tissue.

Assay and Tissue	Dose (µg/kg bw/day)												
	Naïve	Vehicle	BPA 2.5	BPA 8	BPA 25	BPA 80	BPA 260	BPA 840	BPA 2,700	BPA 100K	BPA 300K	EE ₂ 0.5	EE ₂ 5.0
Cytosine extension method (decays per minute)													
Prostate	31965 ± 1020	33158 ± 1090	36505 ± 2903	32163 ± 1095	30885 ± 1429	31667 ± 1872	33030 ± 996	34656 ± 897	38494 ± 2266	35226 ± 1385	37137 ± 1848	35414 ± 1445	33638 ± 1715
Female mammary gland	564 ± 122	5441 ± 92	5106 ± 157	5343 ± 135	5397 ± 228	5461 ± 220	5522 ± 113	5256 ± 72	5398 ± 155	5498 ± 147	5367 ± 102	5399 ± 124	5428 ± 161
Uterus	42260 ± 1528	41791 ± 2196	44626 ± 2772	39373 ± 1762	43961 ± 1849	47972 ± 3740	42294 ± 1859	47482 ± 1677	44290 ± 1813	43384 ± 2009	44575 ± 2297	39596 ± 1827	36620 ± 1879
LC-MS/MS method (% 5-methylcytosine)													
Uterus	2.50 ± 0.12	2.51 ± 0.08	2.50 ± 0.13	2.53 ± 0.08	2.48 ± 0.05	2.54 ± 0.07	2.53 ± 0.08	2.54 ± 0.09	2.49 ± 0.11	2.52 ± 0.12	2.50 ± 0.08	2.49 ± 0.09	2.50 ± 0.09

Table 2: List of genes significantly modulated ≥ 1.5 -fold by BPA or EE₂ *versus* vehicle control in the prostate of PND 4 Sprague-Dawley rats treated by oral gavage daily with vehicle (0.3% CMC in water), BPA (2.5, 25, 260, 2,700, or 300,000 $\mu\text{g/kg}$ bw/day), or EE₂ (0.5 or 5.0 $\mu\text{g/kg}$ bw/day) from gestation day 6 until PND 4. N = 7/dose group. Genes are grouped according to their gene ontology biological process. The fold-change *versus* vehicle control is shown; the *p*-value of the pairwise comparison is shown between parentheses if gene modulation was significant *versus* vehicle control (one-way ANOVA *p*-value < 0.005 and Dunnett's test *p*-value < 0.05). (0.00) indicates a *p*-value < 0.01. Genes with a false discovery rate below 10% and ≥ 1.5 -fold change *versus* vehicle control are marked with an asterisk.

Gene Symbol	GenBank Accession Number	BPA 2.5	BPA 25	BPA 260	BPA 2,700	BPA 300,000	EE ₂ 0.5	EE ₂ 5.0
Cell adhesion								
<i>Hapln2</i>	NM_022285	1.06	1.04	0.98	1.02	1.02	1.16	1.60 (0.00)
<i>Rxfp1</i>	NM_201417	1.35	1.06	0.91	1.13	1.11	1.56	2.58 (0.00)
Cell proliferation and cell death								
<i>Axin2</i>	NM_024355	1.17	0.92	0.80	0.89	1.06	0.91	0.61 (0.01)
<i>Barhl1</i>	NM_057109	1.19	0.83	1.50 (0.04)	1.01	1.09	0.97	0.86
<i>Cp110</i>	NM_001108501	0.99	1.04	1.15	0.84	0.90	0.67 (0.03)	1.21
<i>Tnfsf15</i>	NM_145765	1.64 (0.03)	1.25	1.27	0.93	1.54	1.6 (0.05)	0.98
Cell signaling and cytoskeleton								
<i>Angpt2</i>	NM_134454	0.90	0.95	0.92	0.98	0.90	1.13	1.55 (0.01)
<i>Kb23*</i>	NM_001008813	1.09	0.98	1.08	1.02	1.09	1.10	1.63 (0.00)
<i>Lefty1</i>	NM_001109080	1.02	0.98	1.02	1.08	0.99	1.00	2.05 (0.00)
<i>Lefty2</i>	NM_001007556	1.00	1.07	1.18	1.03	0.97	1.23	2.52 (0.00)
<i>Sfrp5</i>	NM_001107591	0.84	1.05	1.11	0.93	0.71	0.63 (0.04)	1.14
<i>V1rf4</i>	NM_001009522	0.99	1.00	1.01	1.01	1.01	1.62 (0.00)	1.02
Ion transport								
<i>RGD1563815*</i>	NM_001127556	0.96	0.92	1.00	1.15	1.07	2.09 (0.00)	3.33 (0.00)
<i>Scn11a</i>	NM_019265	0.45	0.95	1.04	0.69	0.43	0.31 (0.01)	1.11

Metabolism

<i>Bco2</i>	NM_001127712	2.70 (0.00)	1.79	2.55 (0.01)	2.55 (0.01)	2.57 (0.00)	2.71 (0.00)	2.46 (0.01)
<i>Cyp4x1</i>	NM_145675	0.93	1.62 (0.01)	1.28	1.05	1.07	0.93	1.44
<i>Dnase2b</i>	NM_021664	0.57	0.83	0.93	0.87	0.41	0.34 (0.01)	0.21 (0.00)
<i>Elovl4</i>	NM_001191796	0.87	1.08	1.18	0.83	0.8	0.43 (0.02)	1.41
<i>Gzmb</i>	NM_138517	1.02	1.03	0.85	1.46	0.44 (0.02)	0.49	0.73
<i>Gzmc</i>	NM_134332	0.87	0.75	0.66	0.68	0.38 (0.01)	0.36 (0.01)	0.42 (0.02)
<i>Lbx2</i>	NM_001109244	1.15	1.05	1.02	0.93	1.03	1.98 (0.00)	1.21
<i>Mgat4c</i>	NM_001135814	1.19	1.05	1.11	1.27	1.11	1.19	1.72 (0.00)
<i>Pnliprp1</i>	NM_032081	0.72	0.97	0.67	0.63	1.18	0.74	3.01 (0.03)
<i>S100g*</i>	NM_012521	0.85	0.82	2.83	1.06	7.11	8.73	49.09 (0.00)
<i>Slpil2</i>	NM_001008872	0.71	0.90	0.94	0.89	0.62	0.59	0.38 (0.00)

Other (including uncharacterized)

<i>Ano4</i>	NM_001106778	0.92	1.22	1.09	1.04	1.04	1.25	1.80 (0.00)
<i>Ctxn3</i>	NM_001134696	0.94	1.01	0.94	1.00	0.94	1.04	2.28 (0.00)
<i>Hist1h2aa</i>	NM_021839	1.04	0.76	0.50 (0.03)	0.72	0.80	1.27	0.58
<i>LOC362795</i>	BC098733	1.11	1.15	1.77 (0.04)	1.09	1.67	2.10 (0.00)	1.45
<i>Nlgn3</i>	NM_134336	0.46 (0.03)	0.95	1.35	0.88	0.72	0.37 (0.00)	1.19
<i>Olr1491</i>	NM_001000717	0.92	0.95	0.98	1.25	0.98	0.94	2.68 (0.00)
<i>Plcxd2</i>	NM_001134481	0.93	1.16	1.03	0.86	0.67	0.45 (0.01)	1.00
<i>Tmem154</i>	NM_001108553	1.17	0.67 (0.04)	1.32	0.83	0.91	0.92	1.09

Table 3: List of genes significantly modulated ≥ 1.5 -fold by BPA or EE₂ *versus* vehicle control in the female mammary gland of PND 4 Sprague-Dawley rats treated by oral gavage daily with vehicle (0.3% CMC in water), BPA (2.5, 25, 260, 2,700, or 300,000 $\mu\text{g}/\text{kg}$ bw/day), or EE₂ (0.5 or 5.0 $\mu\text{g}/\text{kg}$ bw/day) from gestation day 6 until PND 4. N = 7/dose group. Genes are grouped according to their gene ontology biological process. The fold-change *versus* vehicle control is shown; the *p*-value of the pairwise comparison is shown between parentheses if gene modulation was significant *versus* vehicle control (one-way ANOVA *p*-value < 0.005 and Dunnett's test *p*-value < 0.05). (0.00) indicates a *p*-value < 0.01. Genes with a false discovery rate below 10% and ≥ 1.5 -fold change *versus* vehicle control are marked with an asterisk.

Gene Symbol	GenBank Accession Number	BPA 2.5	BPA 25	BPA 260	BPA 2,700	BPA 300,000	EE ₂ 0.5	EE ₂ 5.0
<i>Cell adhesion and cell growth</i>								
<i>Cbln1</i> *	NM_001109127	1.43	1.24	1.18	1.17	0.91	2.32 (0.01)	3.67 (0.00)
<i>Lgals4</i>	NM_012975	0.88	1.58	1.56	1.68	2.44 (0.01)	2.09 (0.03)	2.06 (0.03)
<i>Lppr4</i>	NM_001001508	0.82	1.91 (0.04)	0.88	0.97	0.89	0.69	0.63
<i>Matn4</i>	NM_001106539	0.96	0.61 (0.03)	0.79	0.71	0.93	0.76	0.51 (0.00)
<i>Nov</i>	NM_030868	0.86	0.89	0.91	0.79	0.84	0.89	0.60 (0.00)
<i>Ntng1</i>	NM_001106465	1.64	0.95	1.28	1.62	1.83 (0.04)	2.01 (0.01)	1.78 (0.05)
<i>Omd</i> *	NM_031817	0.90	0.75	0.83	0.93	1.10	1.12	1.87 (0.00)
<i>Snip</i>	NM_019378	0.86	1.08	1.03	1.13	1.35	1.32	2.17 (0.00)
<i>Spon1</i> *	NM_172067	0.96	0.92	0.96	0.95	1.11	1.35 (0.03)	1.94 (0.00)
<i>Cell proliferation and cell death</i>								
<i>Apln</i>	NM_031612	0.91	0.86	1.03	0.84	0.76	0.62	0.47 (0.00)
<i>Esm1</i>	NM_022604	1.09	0.74	1.08	0.96	0.74	0.86	0.59 (0.01)
<i>Fas</i>	NM_139194	2.35 (0.00)	1.13	1.05	1.44	1.31	1.80 (0.04)	2.08 (0.01)
<i>Ppm1f</i>	NM_175755	1.14	1.16	1.06	1.05	0.90	0.92	0.67 (0.00)
<i>Cell signaling and cytoskeleton</i>								
<i>Kalrn</i>	NM_032062	1.30	1.42	1.23	1.08	1.20	1.88 (0.00)	1.02
<i>Kctd16</i>	NM_001172155	0.74	0.84	1.12	0.85	1.52	1.20	1.96 (0.03)
<i>Necab2</i>	NM_133415	0.90	0.85	0.95	1.27	1.14	1.23	1.74 (0.00)

<i>Nfe2</i>	NM_001012224	1.08	0.99	0.97	0.93	0.92	0.97	0.65 (0.00)
<i>Olr499</i>	NM_001000931	0.91	0.91	0.90	0.91	1.56 (0.01)	1.02	1.01
<i>Rab39</i>	NM_001108148	0.78	1.05	2.36 (0.00)	0.90	0.89	0.93	0.83
<i>Ramp3*</i>	NM_020100	1.01	0.84	0.95	0.91	1.12	1.17	1.50 (0.00)
<i>Rasa4</i>	XM_002724808	1.44 (0.01)	1.21	1.13	1.38 (0.04)	1.57 (0.00)	1.47 (0.01)	1.31
<i>Spta1</i>	NM_001011908	1.33	1.16	1.04	1.13	0.78	0.87	0.57 (0.02)
<i>Trhr2</i>	NM_181364	0.80	1.13	1.02	1.00	1.12	1.95	2.06 (0.04)
<i>Vom2r5</i>	NM_001099462	0.73 (0.04)	0.66 (0.00)	0.66 (0.00)	0.65 (0.00)	0.65 (0.00)	0.69 (0.01)	0.65 (0.00)
Inflammation and immune response								
<i>Ccl11*</i>	NM_019205	0.77	0.61 (0.00)	0.82	0.84	0.95	0.8	0.52 (0.00)
<i>Ccl5</i>	NM_031116	1.83 (0.04)	0.82	1.19	0.92	1.42	1.67	1.08
<i>Cd163l1</i>	NM_001106312	1.55	0.93	1.14	1.27	2.00 (0.00)	1.33	0.93
<i>Clec2d2</i>	NM_001085402	1.33 (0.04)	1.44 (0.01)	1.26	1.41 (0.01)	1.34 (0.04)	1.61 (0.00)	1.41 (0.01)
<i>Il4ra*</i>	NM_133380	1.14	0.98	1.02	1.03	1.12	1.48 (0.01)	1.71 (0.00)
<i>Mca32</i>	NM_021585	0.86	0.63 (0.00)	0.79	0.77	0.8	0.89	0.60 (0.00)
<i>Orm1</i>	NM_053288	1.00	2.83 (0.02)	1.10	2.07	0.78	1.19	0.84
<i>Prtg</i>	NM_001037651	1.13	0.95	1.10	1.20	1.21	1.41	1.77 (0.00)
<i>Serpinb9</i>	NM_001007732	1.27	1.04	1.30	1.06	1.30	1.54 (0.00)	1.40 (0.02)
Ion transport								
<i>Atp2b2</i>	NM_012508	0.52	0.43	1.25	0.40	0.72	0.26 (0.01)	0.36
<i>Naglt1</i>	NM_176080	1.56 (0.00)	0.99	0.98	0.98	0.99	1.05	1.00
<i>Scn1a</i>	NM_030875	1.07	1.32	1.65	2.91 (0.00)	2.05 (0.04)	1.25	1.41
<i>Slc26a1</i>	NM_022287	1.04	1.08	1.06	0.98	0.79	0.99	0.65 (0.01)
<i>Slc4a1</i>	NM_012651	1.10	1.20	1.04	1.08	0.92	0.85	0.62 (0.01)
Metabolism								
<i>Ak7</i>	NM_001108055	1.26	2.13 (0.00)	0.94	1.54	0.98	1.37	0.82
<i>B3gnt3</i>	NM_001106068	0.65	2.81 (0.01)	1.07	1.21	0.91	1.10	1.54
<i>Ces1a</i>	NM_001190375	1.01	0.82	1.08	1.05	1.46	1.83 (0.01)	1.49
<i>Clock*</i>	NM_021856	1.11	1.25	0.96	1.15	1.06	1.22	0.57 (0.00)
<i>Cpeb1</i>	NM_001106276	1.01	1.31	0.92	1.90 (0.01)	0.97	1.17	0.89
<i>Ctrc</i>	NM_001077649	1.25	1.05	1.11	0.98	1.36 (0.04)	1.55 (0.00)	1.26
<i>Elov12</i>	NM_001109118	1.54	1.13	1.21	1.05	1.24	1.68 (0.01)	1.79 (0.00)
<i>Gck</i>	NM_012565	1.69 (0.00)	1.31	1.05	1.34	0.90	1.46	1.06
<i>Ghr</i>	NM_017094	0.99	1.84 (0.02)	1.01	1.39	0.85	1.04	0.89

<i>Inmt</i>	NM_001109022	0.95	0.73	0.94	0.63	2.31 (0.04)	1.28	1.46
<i>Kel</i>	NM_001191611	1.03	1.15	1.10	1.06	0.82	0.89	0.54 (0.00)
<i>Nt5e*</i>	NM_021576	0.92	0.99	1.01	0.94	1.08	1.51 (0.00)	1.73 (0.00)
<i>RGD1565002</i>	XM_001080634	1.11	1.12	1.01	1.13	1.04	1.29	1.53 (0.00)
<i>S100g</i>	NM_012521	0.67	0.72	1.33	1.41	0.73	1.63	4.30 (0.01)
Oxidation-reduction process								
<i>Akr1c14</i>	NM_138547	1.51	0.76	0.79	0.81	1.04	1.69	2.61 (0.02)
<i>Akr1c1*</i>	NM_001109900	0.90	1.38	0.76	1.07	1.14	2.49 (0.00)	4.79 (0.00)
<i>Cyp2r1</i>	NM_001108499	0.86	1.69 (0.04)	0.96	1.04	0.77	0.97	0.73
<i>Dio3</i>	NM_017210	1.05	1.10	0.92	0.85	1.71	1.91	3.64 (0.00)
<i>LOC685171</i>	NM_001109459	0.56 (0.01)	0.73	0.54 (0.00)	0.53 (0.00)	0.52 (0.00)	0.68	0.83
Other (including uncharacterized)								
<i>LOC302576</i>	NM_001013959	3.84 (0.01)	1.19	0.90	0.83	1.10	0.99	2.71
<i>LOC304027</i>	XR_005615	0.92	1.14	0.99	1.24	0.85	0.87	0.60 (0.02)
<i>LOC500265</i>	XR_007267	3.74 (0.00)	1.24	1.18	1.23	1.45	1.25	1.42
<i>LOC679566</i>	NM_001109372	0.99	0.91	1.03	0.89	1.46	1.13	1.58 (0.02)
<i>LOC680214</i>	XM_001056138	1.04	1.65 (0.00)	1.03	1.04	1.05	1.06	1.00
<i>LOC680967</i>	XM_002730137	0.26 (0.00)	0.81	0.67	0.75	0.73	0.61	1.18
<i>LOC685106*</i>	XM_001062312	0.39	0.91	1.48	1.52	0.07 (0.02)	0.03 (0.00)	2.06
<i>LOC690402</i>	NM_001109588	0.32 (0.01)	0.34 (0.01)	0.29 (0.00)	0.38 (0.03)	0.26 (0.00)	0.25 (0.00)	0.25 (0.00)
<i>Mansc1</i>	NM_001109603	0.84	1.00	0.88	0.97	1.04	1.81 (0.01)	0.85
<i>RGD1311378</i>	NM_001106547	0.92	1.33	0.96	0.97	0.61 (0.04)	0.98	0.61 (0.04)
<i>RGD1562378</i>	XM_575704	1.00	1.61 (0.04)	1.07	1.07	0.77	0.95	0.82
<i>RGD1562720</i>	NM_001109240	0.24 (0.00)	0.30 (0.01)	0.61	0.26 (0.00)	0.26 (0.00)	0.49	0.34 (0.03)
<i>RGD1564859</i>	XM_347061	0.97	1.30	1.02	0.88	0.81	0.98	0.56 (0.00)
<i>Scube1*</i>	NM_001134884	0.92	0.90	0.93	1.00	1.07	1.43 (0.01)	2.03 (0.00)
<i>Thsd7b</i>	NM_001191669	0.84	1.03	0.81	1.00	0.54	0.91	0.35 (0.00)
<i>Tmcc2</i>	XM_223107	0.64	1.06	1.04	0.89	0.70	0.71	0.39 (0.00)
<i>Tmem144</i>	NM_001108551	1.03	0.92	0.91	0.91	0.97	1.54	2.88 (0.00)

Figure 1 – PND 90 uterus

● Vehicle
● BPA 2.5
● BPA 25
● BPA 260

● BPA 2,700
● BPA 300,000
● EE₂ 0.5
● EE₂ 5.0

Figure 2 – PCA, PND 4 tissues, one-way ANOVA

Figure 3 – PND 4 prostate

Figure 4 – PND 4 female mammary gland