MORPHOLOGICAL, CELLULAR, AND MOLECULAR EFFECTS OF BISPHENOL A (BPA) AND BISPHENOL ALTERNATIVES IN THE MOUSE MAMMARY GLAND

Deirdre K. Tucker

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Approved by:

William Coleman

Suzanne Fenton

Charles Perou

Lola Reid

John Rogers

ABSTRACT

Deirdre K. Tucker: Morphological, cellular, and molecular effects of bisphenol A (BPA) and bisphenol alternatives in the mouse mammary gland
(Under the direction of Suzanne E. Fenton)

Bisphenol A (BPA) is an industrial chemical used in manufacturing of epoxy resins and polycarbonate plastics that are in many consumer products, including canned foods, plastic bottles, and water supply pipes, thus increasing human exposure. The effects of early life exposure to BPA have been extensively documented in several tissues of multiple species. Animal studies have been critical in identifying morphological and transcriptional changes in the mammary gland, at human relevant BPA exposures (≤0.05 μg/kg bw/d). These studies have prompted increasing public concerns surrounding the use of BPA in consumer products and have warranted the implementation of alternative analogues, including the fluorinated and sulfonated derivatives, BPAF and BPS. Estrogenicity screenings of these analogues have revealed either enhanced or comparable properties to BPA, indicating their endocrine potential which may impact the mammary gland. This research investigates the potential effects of early life exposure to the bisphenol analogues, BPAF, BPS, and BPA on pubertal development and adult mammary gland alterations, as well as cellular and molecular pathways that are associated with these changes.

Disposition studies were performed in pregnant CD-1 dams exposed once to BPA (50 mg/kg), BPAF (5 mg/kg), or BPS (5 mg/kg) to confirm transplacental transfer and to determine serum and urinary pharmacokinetics. This information was used to establish a proper dosing

schedule for future developmental exposures. Dams had similar serum, urinary and fecal half-lives that were all ≤ 8 hr for all analogues. Urinary recovery was greatest for BPS whereas BPAF recovery was greater in the feces. All chemicals were confirmed to cross the placenta. Pregnant mice were then exposed to BPA (0.5-50 mg/kg) or BPAF and BPS (0.05-5 mg/kg) during the fetal period when rudimentary mammary placodes are forming (GD 10-17). Pubertal mammary glands from offspring exposed to every chemical exhibited accelerated development that included increased terminal end buds, branching density and longitudinal growth. The incidence of inflammation and proliferative epithelial lesions significantly increased in adult glands and produced adenocarcinomas at less than 12 mos. in chemically treated groups, with the highest occurrence in BPAF 5 mg/kg and BPS 0.5 mg/kg. Mammary transplants from digested FACS sorted mammary glands (PND 19-56) prenatally exposed to these two chemicals/concentrations revealed that neither BPAF nor BPS increased mammary repopulating unit frequency, changed expression of stem cell maintenance and differentiation genes, or altered total epithelial cell counts from pubertal mammary glands even though morphological changes at these time-points suggested that they would. Genome-wide analysis and RT-PCR validation of RNA from pubertal mammary glands revealed BPAF altered immune pathways, whereas BPS altered circadian rhythm and mitochondrial dysfunction pathways. Many gene changes occurred during early puberty (PND 20) and others continued into late puberty (PND 55). Neither chemical altered genes associated with apoptosis or from the nuclear receptor estrogen family except *Errg* (BPAF), but they did cause changes in *Evi5* and *Tsc22d1*, two genes that are influential in epithelial cancers and adenocarcinomas. Together these data suggest that prenatal exposure to BPAF and BPS can morphologically alter the pubertal mammary gland in ways that persist into adulthood and encourage neoplasia formation.

DEDICATION

To my family who has been very instrumental in my success as a woman, mother, and scientist.

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LIST OF ABBREVIATIONS AND SYMBOLS

Aco2	Aconitase
AREG	Amphiregulin
Arntl	Aryl hydrocarbon receptor nuclear translocator like
Ar	Androgen receptor
AUC	Area under the curve
Bax	Bcl-2 like protein 4
Bcl2	B-cell lymphoma 2
BPA	Bisphenol A
BPAF	Bisphenol AF
BPS	Bisphenol S
B3galt4	Beta-1,3-galactosyltransferase 5
BrDU	5-bromo-2'-deoxyuridine
CD	Cluster of differentiation
CFC	Colony forming cells
CK	Cytokeratin
C_{max}	Maximal concentration
c-jun	Jun protooncogene
CSCs	Cancer stem cells
DHEA	Dehydroepiandrosterone
DMBA	7, 12-Dimethylbenz(a)anthracene
ECM	Extracellular matrix
EDCs	Endocrine disrupting chemicals
EdU	5-ethynyl-2'-deoxyuridine
EpCAM	Epithelial cell adhesion molecule

ERα/Esr1	Estrogen receptor alpha
ERβ/Esr2	Estrogen receptor beta
Erbb2	Receptor tyrosine-protein kinase erB-2
Erra	Estrogen related receptor alpha
Errh	Estrogen related receptor gamma
ERKO	Estrogen receptor knockout
Evi5	Ecotropic viral integration site 5
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSC	Forward scatter
FSH	Follicle stimulating hormone
Gper1	G-protein coupled estrogen receptor 1
GD	Gestational day
GH	Growth hormones
GnRH	Gonadotrophin releasing hormone
GSH	Glutathione (reduced form)
H&E	Hematoxylin and eosin
HF	Hanks' balanced salt solution/HEPES/FBS
HGF	Hepatocyte growth factor
HPG	Hypothalamic pituitary gonadal
HPLC	High performance liquid chromatography
HLA-A	Human leukocyte antigen A, class I
HLA-DRB5	Human leukocyte antigen DRB5, class II
HLM	Human liver microsomes

HMEC	Human mammary epithelial cells
H2-Eb1	Histocompatibility 2, class II antigen E beta (mouse homolog of HLA-DRB5)
H2-Q4	Histocompatibility 2, Q region locus 4 (mouse homolog of HLA-A)
ICI	Estrogen receptor antagonist fulvestrant
IGF	Insulin growth factor
Il6	Interleukin 6
INHAND	International Harmonization of Nomenclature and Diagnostic Criteria
LH	Luteinizing hormone
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOQ	Limit of quantification
Lrp5	Low density lipoprotein receptor-related protein 5
Map2k7	Mitogen activated protein kinase 7
MaSC	Mammary stem cells
MEA	Mammary epithelial area
MMP	Matrix metalloproteins
MRU	Mammary repopulating unit
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NOAEL	No observed adverse effect level
Notch1	Notch homolog 1
Nr1d1	Nuclear receptor subfamily 1 group d member 1
NTP	National Toxicology Program
PBS	Phosphate buffer solution
Per3	Period 3

PPAR	Peroxisome proliferator activated receptor
Pr/Pgr	Progesterone receptor
PRKO	Progesterone receptor knockout
PIMEC	Parity-induced mammary epithelial cells
Pi3k	Phosphoinositide 3-kinase
PND	Postnatal day
RT-PCR	Reverse transcriptase polymerase chain reaction
SSC	Side scatter
SPE	Solid phase extraction
TEBs	Terminal end buds
TDLU	Terminal ductal lobular unit
T_{max}	Time at maximal concentration
TGF-β1	Transforming growth factor beta 1
TICs	Tumor initiating cells
Tsc22d1	Tscc2 domain family member 1
UDPGA	UDP-glucuronyltransferase
Uqcrc1	Ubiquinol cytochrome b-c1 complex subunit 1
VEGF	Vascular endothelial growth factor
VO	Vaginal opening
Wnt1	Wingless-type MMTV integration site family, member 1
Wnt5a	Wingless-type MMTV integration site family, member 5a

CHAPTER 1

Introduction

BACKGROUND

Breast cancer remains the most commonly diagnosed cancer amongst females in the United States and is estimated to affect 1 in 8 women over the course of their lifetime [1]. As a result of better screening and detection methods and better targeted therapies, the number of deaths attributed to this disease has significantly reduced. However, the number of breast cancer diagnoses remains the same or is increasing in premenopausal women [2]. Heritable risks account for approximately 15% of breast cancer cases, whereas the remaining cases are associated with risk factors that cannot be altered, such as sex, age, race, age at menarche and menopause and breast density as well as factors that may be controlled. These can include lifestyle and environmental factors that include but are not limited to parity, physical activity or the use of contraceptives [2]. Extensive research in rodents has shown that environmental chemicals can induce tumors in the mammary gland, but proving causation in human populations is difficult and only a small handful of examples exist (i.e. dioxin, DDT, DES and nuclear radiation) [3-7]. There is a need for understanding how chemicals modify mammary gland development and risk for later disease so that prevention of breast cancer may be increased through improved risk assessment on chemical classes and regulatory steps that reduce environmental exposures. Understanding the chemical classes that may modify breast cancer risk will also increase public awareness and lead to modified lifestyle choices, especially in the most susceptible populations.

Mammary gland growth occurs at a rate similar to other organs and tissues in early life. Once puberty begins, the gland undergoes exponential growth that includes increased terminal end buds (TEBs), ductal elongation and lateral branching. The TEBs contain highly mitotic cells that are vulnerable to chemical insults including endocrine disruptors. Endocrine disruptors (EDs) are chemicals that can alter hormonal homeostasis to produce adverse effects on development, metabolism, behavior, or reproduction. Rodent studies investigating various EDs and their effects on the mammary gland during critical windows of exposure (i.e. *in utero*, neonatally or perinatally) demonstrated their ability to alter normal tissue development and the epigenome and transcriptome that are responsible for mammary gland patterning [8, 9]. During each stage of development, the mammary gland relies on signaling from multiple hormones (Figure 1-1), therefore, it may be at an increased risk for altered growth and function following ED exposure, especially to those EDs whose mechanisms are facilitated through the estrogen receptor (ER) or happen during critical periods of development. The ER is abundant throughout the gland (i.e. epithelial and mesenchymal cells after weaning and mesenchymal cells in fetus/newborn) and can be directly and indirectly responsible for cellular proliferation in the gland as well as tumor promotion [10-12].

BPA is an established ED that is used in the production of polycarbonates and epoxy resins. Under normal and elevated temperatures this chemical has been shown to leach into food and beverage containers. Capable of binding to ERα, BPA is considered a weak estrogen in comparison to estradiol, but in several instances has been shown to elicit adverse effects on the developing mammary gland. Defining the mechanisms involved in these changes has not been an easy task, and the emergence of other bisphenol replacements that are structurally and estrogenically similar to BPA, such as the fluorinated and sulfated derivatives Bisphenol AF

(BPAF) and Bisphenol S (BPS), adds to this difficulty. Therefore, the overarching goal of this research is to evaluate the effects of BPA, BPAF, and BPS on the developing mammary gland and investigate potential modes of action for observed changes to determine how they may relate to human health. A detailed literature review on the mammary gland, BPA, BPAF and BPS follows and will provide the necessary background information to gain a better understanding of why this project was undertaken.

Life Stages of Normal Mammary Gland Development

Mice

Mammary gland morphogenesis is a unique and intricate process that requires extensive crosstalk between endocrine, paracrine, autocrine, and cellular networks. There are many documented similarities between human and rodent mammary development, however, due to the limitations of acquiring normal tissues from humans, similarity of some of the genes and pathways have yet to be determined. During embryogenesis in the mouse, rudimentary placodes arise from the ectoderm. Maternal hormones and epithelial-mesenchymal crosstalk then promote the formation of a primary ductal tree that is present before birth [13]. Following birth, the gland grows isometrically with the body until puberty when the hypothalamic-pituitary-gonadal (HPG) axis becomes activated. Both estrogen and growth hormone (GH) are released and signal to receptors located on epithelial and/or stromal cells. Binding of estrogen to the ER-α in the fetal mammary stroma induces hepatocyte growth factor (HGF), which is required for epithelial branching [14]. Secretion of GH from the pituitary gland stimulates insulin growth factor 1 (IGF1) production within the mammary stroma [15]. As a result a bi-layered duct forms, comprised of an inner luminal compartment and an outer monolayer of cap cells that eventually

develops into the myoepithelial layer [16]. At the time of birth, the mammary glands consist of rudimentary epithelial stalks. Growth remains constant with development until puberty.

During puberty, highly proliferative structures known as terminal end buds (TEBs) are prominent. The TEBs are tear-drop shaped structures located at the end of the extending ducts of the gland, and contain 4-6 layers of cuboidal epithelial cells, and cap cells on the basal surface, just below the basal lamina [17]. Cap cells exhibit poor apical-basal polarity, are incapable of forming cell junctions, and do not contain a highly organized cytoskeletal element [17]. These cells are thought to have pluripotent characteristics and will form into both luminal and myoepithelial compartments. Evidence suggests that TEBs are packed with luminal cells that undergo apoptosis to create the lumen space [18]. As lumen are being created, ductal elongation is also taking place as a result of end bud pressure through the stromal compartment [19]. As new cells are added to the TEB, the extracellular matrix (ECM) begins to constrict which allows for elongation. Matrix metalloproteinase 14 (MMP14) is particularly integral in this process and was elevated in the cells surrounding the TEBs [20]. While collagen has been shown to play an essential role in tubulogenesis, it can also bind to the ECM to help in creating mammary cell shape. The combination of fibroblast growth factor (FGF) and ECM remodeling by MMP2 and MMP3 causes TEBs to bifurcate, leading to epithelial branching [16, 21]. ECM remodeling also promotes angiogenesis and increased vascular endothelial growth factor (VEGF) expression during puberty. Adipocytes express an estrogen response element in their VEGF promoter that encourages communication between endothelial cells, epithelial cells, and adipocytes [22]. Binding of estrogen to ERα in the epithelium also induces amphiregulin (AREG), which becomes uncleaved from sheddase ADAM17. Uncleaved AREG then signals back to stromal cells by binding to the epidermal growth factor receptor (EGFR) to induce ductal branching [23].

Lateral duct branching may also be inhibited by stromal TGF-β1 that in turn inhibits the activity of HGF [24].

Similar to puberty, pregnancy is governed by many of the same endocrine and paracrine factors. Early in pregnancy secondary and tertiary branching are increased to promote alveolar development. During late pregnancy, prolactin, and progesterone work in concert to prepare the gland for lactation by promoting differentiation of alveoli, the structures responsible for synthesizing and secreting milk. Pathways downstream of progesterone signaling have implicated receptor activator of NFκB1 (RANK) and receptor activator of NFκB1 ligand (RANKL) in alveologenesis. Mammary involution is the final phase of lactation and occurs when milk-producing epithelial cells undergo apoptosis to return the gland to a simple ductal structure. Involution occurs in two distinct phases. In the first phase, apoptosis is triggered and plasmin helps by disrupting the cell-cell interactions with the ECM. During the second phase, remodeling of the basement membrane, collapse of alveoli, and differentiation of adipocytes occurs [25], while plasmin works in concert with MMP3 to disrupt the basal lamina and cause premature involution [26].

Humans

Human breast development begins around the 5th week of gestation, when a 2-4 cell layer 'milk streak' or 'mammary band' forms from the thickening of the ectoderm [27, 28]. Involution of the milk streak is involved in condensing the bud below the ectoderm into the mesenchyme where it will become the nipple primordium. The formation of early epithelial ducts appears shortly after week 12 of gestation. Ultrastructural assessment of the gland in the second trimester fetus shows two distinct epithelial populations, central and basal cells, which share a similar

morphology to the basal cells in the epidermis, where glycogen accumulation is greatest. Unlike the mouse where there is one primary duct that extends from the nipple into 5-10 secondary ducts, the human has many lactiferous ducts that will radially extend to form their own separate ductal system with surrounding stroma and are separate from neighboring ducts. Between 18-21 weeks of pregnancy, fetal epithelial outgrowths begin in the mesenchyme and quickly undergo formation of lumen that become lined with cuboidal cells. During this time the myoepithelium is fully developed. Ultrastructural images revealed epithelial cells in two stages, those that were larger and exhibited canonical apical protrusions and a smaller cell that was adjacent to the basement lamina, contained few organelles and had clear cytoplasm [29]. The breast epithelium is functional at birth, when hormones from the mother may cause secretory activity in the newborn.

Between birth and puberty, the mammary gland grows in proportion to the body. Of the few studies that report data from peri-pubertal tissues, the breast was described as having small bundles of primary and secondary ducts whose distal end develops into bulbous structures analogous to the rodent TEBs. New branches arise from the main ducts and lateral buds from these structures eventually evolve into alveolar buds that will form clusters around the duct that forms the lobule type 1. This lobule type 1 structure is termed the terminal ductal lobular unit (TDLU) [30]. TDLUs were apparent in some but not all tissues, which may be accounted for by the specimens stage within pubertal development [31]. However, these bulbous structures are also thought to contain an undifferentiated cell population, similar to the TEBs in rodents. Adult mammary glands are composed of 80% fibrous tissue in the form of intra- and extra-lobular connective tissue, where the fat is pushed to the periphery. Once breast development is complete, the TDLUs are hormonally influenced by the menstrual cycle. In the virgin post-pubertal gland

Lobule 1-type (Lob1-type) TDLUs are the predominant structures and are analogous to the TEBs found in rodents. These structures (in both rodents and humans) are the site at which ductal carcinomas arise. Eventually these structures develop into Lob2-type which have more lobules per duct [32]. Transition from Lob2 to Lob3 structures occurs over years with the development of new alveoli, and if pregnancy is not achieved this may not occur. During pregnancy, ducts containing the Lob2 and Lob3 eventually develop secretory acinar cells of Lob4 [31]. Following lactation and weaning, glands regress back to predominantly Lob2 structures, while the glands of nulliparous women contain more Lob1. When menopause occurs, all women regardless of childbearing status will regress back to Lob1 structures.

Mammary Stem and Progenitor Cells

Identification

The emergence of improved technologies and differentiating assays has advanced the field of mammary biology and the ability to isolate and characterize mammary stem cells (MaSC) in mice. The stem cell portion of the mammary gland is thought to play an essential role in development, differentiation, maintenance, pregnancy, and even carcinogenesis. Elucidating the mechanisms and dynamic changes that occur with these cell populations will be essential in understanding their role in normal and altered development.

The defining characteristics of a stem cell are its ability to self-renew and produce multi-lineage cell types. Studies performed over 50 years ago transplanted mammary tissue fragments into cleared endogenous fat pad, resulting in a gland that was capable of being fully re-constituted regardless of which portion of epithelium was used [33]. Serial transplants of mammary fragments revealed that mammary reconstitution could be achieved up to 4 transplants

and in some cases 7-8 successions before reaching senescence [34]. During this same time frame, pioneering studies uncovered that the reproductive age or developmental stage that mammary tissue was obtained from had little influence on its growth potential when transplanted into younger donors [35]. Smith et al. [36] also showed that, regardless of age and developmental stage, samples taken from any portion of the mammary gland were able to reconstitute outgrowths. Even following primary transplants, reconstituted tissues were able to produce secondary outgrowths using as few as a single cell [37]. While transplants remain the gold standard for demonstrating multipotency of isolated cells, this was only indirect evidence that a stem cell population existed. Similar to stem cells from other tissues, studies have shown MaSCs exclude Hoechst dye, retain their DNA label due to asymmetrical divisions, and actively cycle and divide [38]; however, early studies failed to isolate pure populations. Together these data show that the mammary gland stem cell population likely exists throughout the lifespan of the animal.

More recently, cytokeratin expression analysis revealed that the mouse mammary gland expressed lineage markers associated with basal and luminal phenotype during embryonic and early postnatal development and that throughout development they either retained these markers or switched [39]. Embryonic lineage tracing conducted in 129svXC57Bl/6 mice revealed that as early as E15.5 mammary buds co-expressed cytokeratin (CK) 5 and 14, but expression intensity often varied. Three days later and into infancy, CK8- positive cells localized in luminal-like cells and physically separated from CK14-staining outer layers. However, some CK14-positive cells were found in the inner layers co-expressing CK8. CK6 staining, which is thought of as a multipotent mammary epithelial progenitor marker due to its presence in the body cells of TEBs, was distinct from CK14+ cells and was abundant during late fetal development. By 2 weeks of

age, some double-positive CK6/14 cells were found in the inner core of the ductal termini and only one week later fewer double stained cells were evident. By weaning, CK6 was localized in TEB body cells and CK14 was dramatically decreased. Myoepithelial/basal cells co-expressed CK5/CK14 and in most cases CK5 expression was almost exclusively confined to the basal cells [39].

Other studies have confirmed some of these earlier findings of stage-specific cytokeratin expression and have provided information on cell and stage-specific protein expression. During placode formation (E11-12), basal markers, K14 and p63, and luminal marker, Gata3, were co-expressed [40]. By E15, p63 began to localize to the outer developing ducts. Gata3 was also expressed in the basal layer in the distal ductal tree and luminal compartment of the nipple region following birth. Double positive C14K/CK18 cells were found in the primary ducts close to the nipple area, but areas closer to the primary ducts became more distinctly separated. Pulse chase studies where CD-1 mice were exposed to 5-ethynyl-2'-deoxyuridine (EdU) between E14-18 followed by 5-bromo-2'-deoxyuridine (BrdU) treatment at PND 5 revealed that a small subset of these double stained cells were mitotically active indicating that a small population of embryonic stem cells may also be involved in postnatal mammary gland development. In contrast, CK6 expression was absent in the mammary gland during all developmental stages in a study conducted by another group and CK5 expression was not shown until postnatal day 21 (PND 21) [41]. Also, CK14 expression was in the apical sections of the basal layer as early as E13.5; however, expression was limited to luminal cells that were very distant from the epidermis indicating that these cells are not involved in the epidermal-luminal transition. Ultimately, various factors including differences in strain, antibody, and even antibody efficiency may have played a role in the discrepancy between protein expressions.

Isolation of MaSC

Prior to 2005, identification and isolation of mammary stem cells was a challenge due to insufficient cell markers and cell based assays that were incapable of preventing cell differentiation. Early studies using fluorescence activated cell sorting studies (FACS) identified stem cell antigen 1 (Sca1) as a potential marker because these epithelial cells showed label retaining capabilities, however, contaminating bipotent cells remained an issue [42]. Removal of endothelial and hematopoietic cells from freshly dissociated cells revealed that CD24 (heat stable antigen) and high expression of either CD29 (β_1-integrin) or CD49f (α_6-integrin) can form a heterodimer and serve as a potential marker of MaSCs. CD24 is also an antigen for cell surface glycoproteins. Similarly, CD29 is an integrin involved in cell adhesion and CD49f is a late activating antigen involved in cell adhesion and cell surface mediated signaling. $CD24^{high}/CD49f^{high}$ or $CD24^{high}/CD29f^{high}$ labelled cells were able to reconstitute an entire gland during primary and successive transplants and were able to undergo complete development during pregnancy [43, 44]. However, only small populations of these cells were considered stem cells while all others were basal/myoepithelial. Also, epithelial cells enriched in $CD24^{high}/CD49f^{high}$ were positive for CK5 and CK14 while $CD24^{high}/CD49f^{low}$ were found to be more mature and differentiated luminal cells. Prater el al. [45] found that enrichment of CD49f and EpCAM helps to separate the luminal cells much better from the myoepithelial/basal cells. Further sorting of the CD49f/EpCAM luminal population according to Sca1/CD49b status revealed three additional distinct luminal populations (Sca1+CD49b+, Sca1-CD49b+, and Sca1+CD49b-) that all have unique morphologies and cloning potentials [46]. Only the Sca1+/CD49b+ and Sca1-/CD49b+ populations were efficient in colony formation and were ER+. The Sca1+/CD49b+ were also observed to have a small proliferation capacity and increased expression of differentiation

luminal transcripts that included ER, FoxA1 and Gata3. Most stem cells are ERα- but there is evidence to suggest that a small population may be ERα+ and proliferate [47]. Expression of PR in mammary cells seems to be restricted to the luminal compartment [48]. Humans and rodents alike are constantly exposed to endogenous hormones when they undergo menstruation and an estrous cycle. Therefore, it is thought that both estrogen and progesterone elicit their effects on ER-/PR- cells through a paracrine mechanism. It is difficult to acquire normal human mammary tissue to test for self-renewal via transplants, therefore, *in vivo* and in *vitro* methods in mouse models have been used to identify potential human mammary markers. Similar to neuronal stem cells that form "neurospheres" under non-adherent and serum free conditions, the mammary gland has also been shown to produce "mammospheres" under similar conditions in the presence of bFGF and EGF. Mammospheres contained highly undifferentiated cells that were capable of forming multi-lineage colonies from single cells when grown under conditions containing serum [49]. With each successive passage the number of bi-potent progenitor cells increased. Sorting according to the surface markers Lin-CD49+EpCAM-/lo or CD10+ showed reconstitution in mouse mammary glands when transplanted with fibroblasts [50].

Stem Cell Maintenance and Differentiation

Similarities among the human and rodent mammary structure and mammary stem/progenitor populations have shown that there is great conservation of genes that are responsible for these populations [51]. Gene signatures from sorted FVB/N mice were identified and compared to the gene signatures from previously reported sorted mammary human populations and revealed that once normalization for species was accounted for, the samples within each species co-localized into three distinct cell types (luminal, stromal, and stem or basal

cells) [51]. When they compared transcriptional activity scores from each mouse subtype gene signature to that of the different human cell subtypes it revealed that genes within each subtype were highly conserved across species. Approximately 1200 signature mouse genes for all cell populations corresponded to their human orthologue, with the MaSC population having the highest number of signature genes and best conservation between species. Of these conserved genes, an array of upregulated genes included transcription factors (i.e. p63, *Egr2* and *Sox11*), cytokeratin's (CK5, 14, and 16), and genes associated with the Wnt/β-catenin and Notch pathways which have been shown to play a role in stem cell maintenance and differentiation [39, 52, 53]. Luminal progenitor and mature luminal cells were observed to have a lower degree of conservation. Luminal progenitor cells expressed *Kit, Elf5,* and *Cxcr4,* a gene involved in metastasis of breast cancer, and more mature luminal cells expressed ERα, PR, and the RANKL. qRT-PCR confirmed these findings [51]. A more recent study detailed the major pathways involved in MaSC maintenance and demonstrated that the Wnt pathway, which is involved in patterning during development, is required [54]. Signaling of the Wnt pathway leads to stabilization and accumulation of β-catenin. β-catenin then translocates into the nucleus binding to LEFT/TFC resulting in the transcription of various proliferation genes. MaSC experienced a 6.4-fold increase when *Wnt1* was overexpressed using a MMTV promoter [55]. Conversely, decreased Notch signaling in the Cbf-1 knockout mouse and p53-/- mice have resulted in increased MaSC activity, where activity was defined as the transplant repopulating frequency [56, 57]. The ΔN63 isoform of the transcription factor p63, which is associated with p53, can induce Wnt signaling and self-renewal capacity, whereas the ΔT-63 isoform is more involved in luminal commitment and the hedgehog pathway[54, 58].

The MaSC and Puberty

Several of the genes and signaling pathways identified during the discovery of MaSC are essential during pubertal mammary gland development, a period of extensive epithelial growth [59]. In the mouse, TEBs are prevalent during this period and are highly mitotic. Within the cap cells of the TEBs resides a pluripotent stem cell population that is capable of self-renewal and differentiation. Since these structures are increased during puberty it is also thought that the MaSC and luminal populations are increased and genes involved in these processes are also changed to coordinate with the changes in cellular morphology. Exposure to environmental toxicants may alter the MaSC and luminal epithelial cells. Advances in this research may provide details into the initiation, promotion and progression of cancer thought to be caused by environmental exposure to endocrine disrupting chemicals (EDCs).

The MaSC and Pregnancy

Emerging evidence has suggested that another type of MaSC may contribute to the alveolar expansion that occurs during pregnancy. Parity induced mammary epithelial cells (PI-MEC) are thought to originate from cells that have differentiated during pregnancy. Unlike more committed alveolar cells, this population is thought to either de-differentiate or not undergo apoptosis during involution or remodeling after lactation [60]. Use of the pregnant double transgenic Rosa-*lacZ*, which uses WAP-Cre and Rosa-lox-Stop-lox-lacZ and X-Gal staining, which identifies β-galactosidase activity in cells, revealed that Cre-activation was greatest in differentiated alveolar cells immediately following parturition. Whole mount preparations of glands undergoing involution had the greatest X-gal staining of positive cells located primarily in epithelial structures that were in close proximity to TDLUs. Interestingly, these cells resembled

terminal ducts seen in virgin mice; however, cell death did not occur in these cells. Subsequent pregnancies revealed that all developing alveoli were X-gal positive, but actively proliferating positive cells were restricted to the terminal ducts and developing alveoli. Transplantation of transgenic involuted whole tissue into the mammary fat pad of nulliparous recipients (9/22 glands, n=11 animals) produced outgrowths that were X-gal positive throughout the entire ductal tree. Further studies by this group showed following isolation and sorting that within the 12% PI-MEC population that 98% of GFP PI-MECs were CD24+, and a substantial number of them co-expressed CD49f. Notably, these cells were also able to form mammospheres in culture, a phenomenon that has been shown to occur with multipotent human cells. Isolated GFP+ single cells were also able to reconstitute an entire mammary gland [61]. In other studies, luciferase expressing MaSCs were transplanted into cleared fat pads of 3-week old mice, followed by induction of pregnancy, and the authors reported that MaSC populations expanded 200-fold followed by an immediate decrease following parturition. Baseline levels were found to be dependent on whether nursing of pups occurred [62]. Progenitor cells have also been shown to increase later in pregnancy and during lactation. Mammary cells that were separated according to CD29 and CD24 expression have previously revealed that $CD29^{lo}CD24+$ is representative of the luminal subtype. When this population was further sorted by CD61 expression, a marker of progenitor luminal cells, it was found that at GD 18, CD61+ cells fell by 15-fold but this decrease coincided with an increase in alveolar luminal differentiation [55].

Humans

Mammary stem cell identification in the human could not be obtained in the same fashion as rodents due to the ethical issues that surround human subjects. However, human mammary

epithelial cells (HMECs) isolated from mammary tissue from healthy individuals undergoing reduction mammoplasties were successfully found to survive, proliferate, and form spheroids in non-adhesive medium while most the cells experienced cell death [63]. These cells were termed 'mammospheres' and found to contain 8-fold more bi-lineage progenitor cells than freshly cultured human mammary cells. Successive passages produced colonies that could produce all three epithelial cell types. Secondary and later spheroid generations were generated from single cells. Secondary cells were found to express α6 integrin, CD10, CK5 with moderate expression of epithelial specific antigen (ESA) and CK14. Genotyping and transcription confirmation studies identified genes within the Wnt/Frizzled pathways, CXCR4 and transcription factors that are responsible for cell cycle arrest.

The Mammary Gland and Cancer Stem Cells (CSCs)

The breast cancer field was profoundly changed when Perou and colleagues used cDNA microarrays and hierarchal clustering to classify tumors based on specific cellular gene expression [64, 65]. For the first time, tumors were identified into 5 subtypes; ER+ or ER- status was determined, then ER+ tumors were described as either luminal subtype A (ER+, CK8/18, low grade, slow growing) or luminal B (ER+, CK8/18, low grade, quick growing tumors), and ER- tumors were ERBB2+ (increased expression of genes in the ERRB2 amplicon and GRB7) or basal-like (increased expression of CK5 and 17, laminin, and fatty acid binding protein 7) or normal breast like. More recently, a claudin-low population (low expression of genes involved in tight junctions and cell adhesions that include claudin 3, 4, 7, occluding and E-cadherin) was identified [66]. Clinical implications were also associated with these subtypes where ER-/PR- or ERRB2- tumors had a much poorer prognosis, because hormone therapies were not developed

for this specific gene signature. Thus, it has been postulated that these cancer subtypes may originate from a less differentiated or stem-like population of cells. Therefore, the level of differentiation that a cell undergoes prior to transformation may dictate the tumor type, prognosis, and potential therapies. However, because mammary tumors are complex and heterogeneous there may be a multitude of cancer stem cell (CSCs) populations.

MaSCs serve as good candidates for transformation due to their slow division rate, longer life spans, and increased potential for acquiring mutations due to long exposures to harmful agents. Similar to MaSC, mammary cancer stem cells or tumor initiating cells (TICs) are also capable of 1) self-renewal, 2) differentiation, 3) induction of apoptosis, 4) anchorage independence and migration and increased transporter activity [49]. Identification of their capabilities was illustrated by Al-Hajj et al. [47] when NOD/SCID mice were injected with primary or metastatic human tumor cells. Resulting tumors were identified by the cell surface markers CD44, CD24, and B38.1. CD44 is a cell surface marker for hyaluronic acid, but may also bind other extracellular matrix ligands that regulate cell to cell interactions, adhesion, and migration. Serial transplants of each sorted population showed that tumors were formed from CD44+CD24$^{-/low}$ populations requiring fewer cells compared to higher concentrations of unsorted cells [47]. When xenograft and unpassaged patient tumors were analyzed, they consisted of similar populations with diverse tumor cell types, and CD44+CD24$^{-/low}$ signatures. Serially passaging of this cell type produced phenotypically heterogeneous tumors that consisted of the CD44+CD24$^{-/low}$ and a diverse cell population containing non-tumorigenic cells. Recent evidence suggests that CD44+CD24- may also identify a mesenchymal phenotype that does not have regenerative capabilities [67]. Similar pathways associated with normal self-renewal/maintenance and differentiation has been implicated in tumor formation. Other methods,

including ALDH activity, showed that ALDH+CD44+CD24- populations were enriched with CSCs [68].

Molecular pathways similar to MaSCs have also been implicated in mammary tumorigenesis. Constitutive activation of the Wnt pathway has been shown to increase murine mammary tumors, potentially when β-catenin is signaled. MMTV-*Wnt* mice develop tumors that were capable of reconstituting glands similar to the original tumor with as few as 50 cells [69]. Other transgenic models including the MMTV-Her2-neu mice have been shown to develop focal mammary tumors that are capable of metastasizing after long latency [70]. These tumors were later found to overexpress CD49f, CD61, and ESA that were linked to a luminal progenitor cell type [71]. When cells were FACS sorted, $CD49f^{high}CD61^{high}$ cells included a TIC population capable of developing "tumorspheres" in culture. Since the MaSCs lacked ER, PR, and ERRB2 expression, it is thought they are the initiating cells of basal-like cancers. That the MaSCs resides within the TEB, a structure often targeted and altered by environmental toxicants and endocrine disruptors, suggests that they can induce the formation of other mammary tumor subtypes. The next section will explore several endocrine disruptors and the current state of knowledge of their effects on the mammary gland.

Bisphenol A (BPA)

Chemical Properties

BPA synthesis occurs with the condensation of two phenols and an acetone, in the presence of an acidic catalyst. The final product consists of the two phenol with hydroxyl groups oriented in the para position and bridged by a carbon with two methyl groups (Figure 1-2) [72].

Uses and Routes of Exposure

BPA is a high production volume chemical that is ubiquitous within the environment. BPA is used in the production of polycarbonate plastics, epoxy resins and as a developer in thermal paper. The main source of BPA exposure is through ingestion although transdermal and inhalation of contaminated dust are additional routes of exposure. The general population is thought to be exposed through contaminated food and beverages by leaching from the polycarbonates; BPA has successfully been identified in multiple food and beverage sources [73, 74]. Biomonitoring studies have detected BPA in urine samples from approximately 90% of the general population in the U.S., Canada, and several Asian countries [75-77]. Several studies have quantified BPA levels in maternal and cord sera, amniotic fluid, and umbilical cord [78-81]. BPA has also been measured in the urine of infants and small children [82, 83]. Fetuses, infants, and adolescent children are considered to be the most susceptible populations to the effects of BPA because BPA 1) can cross the placenta and influence organogenesis and neurogenesis, 2) can elicit more harmful effects in children compared to adults due to lower hepatic uridine-5'diphoso-gluruonosyltransferases (UGT) activity in children/infants, and 3) has a higher body burden and intake rate in children due to the products they drink from or put in their mouths [84]. Understanding the pharmacokinetics and disposition in younger populations will assist with building more robust physiological based pharmacokinetic models for an accurate risk assessment.

Absorption, Distribution, Metabolism, and Excretion

Following oral ingestion, BPA is readily absorbed and metabolized. In rodents, BPA is highly bound to plasma proteins and excreted in the bile, where it may be cleaved back to BPA

and reabsorbed from the intestine to the blood. Enterohepatic recirculation is common in rodents and can occur several times before being excreted into the feces. This repetitive process can prolong the presence of circulating free BPA in the body. Glucuronidation is the main metabolizing pathway for BPA in rodents, but urinary excretion is much lower compared to bile and fecal excretion. In contrast, humans undergo first pass metabolism; the parent compound is metabolized by the liver and gut UGTs, 2B15 and 1A9, and excreted into the urine, with approximately 80% chemical clearance in < 24 hr [85, 86]. The exact clearance rate varies based on route of administration, sex, dose, and age of exposure. The main metabolites of BPA are glucuronides, and a smaller percentage are sulfated conjugates that are metabolized by sulfotransferase 1A1 [87]. BPA is also capable of undergoing Phase I metabolism and this has been confirmed both *in vitro* and *in vivo* where the main metabolites were identified as ortho hydroxylated BPA and ortho-quinones [88-91]. There is new evidence to suggest that the BPA glucuronide (BPA-G) may not be as harmless as previously considered. Following the treatment of 3T3-L1 preadipocytes with BPA-G, lipid accumulation, adipogenesis, and significantly increased mRNA and protein levels of adipogenic and lipogenic markers were noted [92]. Although BPA-G was not estrogenically active, this finding suggests that adipose containing tissues such as the mammary gland may undergo changes that are driven by estrogen independent pathways in the stroma and estrogen-dependent mechanisms in the epithelium or vice versa.

Toxicity and Mutagenicity

BPA is not considered to be mutagenic, but there is clear evidence that it is genotoxic and aneuploidogenic [93-97]. Several epidemiological, animal, and *in vitro* studies have identified

health effects produced by BPA exposure. BPA affects sexual receptivity, maternal nurturing, and social behavior. Developmental exposures to BPA target endocrine regulated tissues such as the mammary glands, ovaries, testis, and thyroid, resulting in disorders linked to infertility, accelerated or delayed puberty, and altered mammary morphology or impaired lactation [98-101]. The burgeoning field of obesogens has also highlighted the potential for endocrine disruptors like BPA to cause changes in adipogenesis and lipogenesis and has been linked to obesity occurrence in the United States [102, 103]. A detailed assessment of BPAs effects on the mammary gland stroma will be discussed later in the chapter. Accumulation of fat in the liver has also been reported in addition to inducing oxidative stress, hepatic mitochondrial dysfunction, and dose dependent increases of liver tumors in adult rats [104-106].

Estrogenicity and Endocrine Activity

BPA is capable of binding to ERα (IC_{50}= 1,030 ± 70 nM) and ERβ (IC_{50}= 18.9 ± 0.84 nM) at higher concentrations compared to 17β-estradiol (ERα IC_{50}= 0.88 ± 0.04 nM and ERβ IC_{50}= 2.17 ± 0.12 nM) , but has a greater affinity for ERRγ compared to the traditional ERs [107]. Several studies have also shown BPA to be anti-androgenic [108, 109]. Many studies in the field have focused on the ERα classical pathway, but there is mounting evidence suggesting that BPA induces effects in a non-genomic and non-nuclear receptor-mediated mechanism [110-112]. BPA exposures *in vitro* antagonize T3-induced transcription by displacing T3 from the binding pocket [113].

Regulatory

For most of industry, and some government agencies and scientific communities, concerns about the safety of BPA in the marketplace has been minimized; it has been accepted that since BPA is readily metabolized in humans that the estrogenic effects do not pose a substantial risk to human health. As a result, the lowest observed adverse effect level (LOAEL) remains at 50 mg/kg/bw/day and the no observed adverse effect level (NOAEL) is set at 5 mg/kg/bw/day although studies using much lower and human relevant concentrations have reported numerous adverse effects in several target tissues. The NOAEL and LOAEL were established based on the findings of two multigenerational rodent studies. The authors found that systemic toxicity (i.e. changes in body weight and organ weights) was observed at 5 mg/kg/bw/d in Sprague Dawley rats and toxicity in CD-1 mouse offspring was observed at 50 mg/kg/bw/d [114, 115]. When the margins of safety (MOS) were calculated for infants and adults they were deemed adequate. Therefore, based on the LOAEL, the US EPA has set the reference dose at 0.05 mg/kg/body weight/day based on data from a small number of studies that were conducted at very high doses in adult animals and F1/F2/F3 generations. The US Food and Drug Administration (FDA) deems BPA safe at current levels occurring in food [116], but amendments passed in 2012 and 2013 now ban the use of BPA in baby bottles, sippy cups, and infant formula packaging, and many other countries including France, Turkey, and Sweden, have followed suit [116]. European countries have taken a more precautionary approach, and the European Union decreased the tolerable daily intake from 50 µg/kg down to 4 µg BPA/kg body weight; a level more than 10x lower than levels mandated by the U.S. EPA[117]. The EU has also passed a regulation stating that thermal paper containing BPA must be discontinued by 2020 [118]. In other countries, such as Germany and Japan, manufacturers have voluntarily taken BPA

out of certain products. Increased consumer and public awareness surrounding the potential effects of BPA have spurred industries to replace BPA in several consumer products with chemicals that are similar or enhanced in their estrogenic potency. Potential replacement chemicals such as those with fluorine and sulfate group substitutions have not been extensively studied and their potential for exposure warrants further investigations to assess any potential human risks and hazards.

Bisphenol AF (BPAF)

Chemical Properties

Similar to BPA, BPAF is composed of two phenolic groups that are joined by a central carbon, but the two methyl groups have been replaced by trifluoromethyl groups (Figure 1-2). In the presence of an organic sulfonic acid catalyst, hexafluoropropanone-2 and a phenol interact to synthesize BPAF [119]. The presence of the fluorinated groups increases its thermal and chemical stability to make it a durable component for many industrial and consumer applications, but as a result, may increase its persistence within the environment and potential human exposure.

Uses and Routes of Exposure

BPAF has been detected in indoor dust, domestic well water, soil, sediments, and foodstuffs in multiple countries [120-122]. Between 1986 and 2002, BPAF was moderately produced in the U.S. BPAF is commonly used as a crosslinking agent for fluoroelastomers, optical fibers, electronics, heat resistant adhesives, and as a monomer for polyimide, polyamides, polyester, and specialty polymers that include epoxy resins [123]. The manufacturing of rubber and plastic products and electrical/electronic equipment may have contributed to the potential

exposure of ~4400 employees [124]. Almost half of the exposed workers within the plastics industry during that period were women. Exposure to the U.S. general population is likely due to contact with resins and polycarbonates used in plastics. Neighborhoods downstream of a BPAF manufacturing plant in China measured BPAF in dust, soil, well water, and at low levels in tap water [120]. Due to increased soil and water contact and inhalation of dust, children are likely to have higher dermal and inhalation exposures. Higher incidences of inhalation exposures may occur in other countries as documented from collected indoor dust of Korean homes and businesses. BPAF was detected in 75.6% of Korean dust samples compared to 9% in Japan and no detection were observed in the U.S. samples [121]. However, exposure through foodstuffs may not be of high concern as it was detected at low levels in China and the U.S. [73, 122]. Urine from residents living near a BPAF manufacturing plant in China had total BPAF (adjusted for creatinine) that ranged from no detection to 0.217 ng/ml [125].

Serum bisphenol levels can provide details about exposure trends and establish human relevance in animal studies. Likely due to their rapid metabolism and excretion there are no U.S. studies that have reported serum BPAF levels from the general or occupational populations. One study measured urinary BPAF levels from convenience samples, and only detected it in < 3% of the samples and at concentrations ranging from < 0.1-0.12 µg/L [126]. An earlier study using HPLC extracted BPAF from human mammary and abdominal adipose tissue, validating the lipophilic properties and highlighting its potential target tissues [127].

Absorption, Metabolism, Disposition, and Excretion

The main routes of BPAF exposure are through oral or dermal sources. Only one pharmacokinetic disposition study exists for BPAF in which all tissues and excretion routes were

evaluated. Following a single i.v. or oral exposure to both male and female Harlan Sprague-Dawley rats and B6C3F1/N mice (3.4, 34, or 340 mg/kg) the majority of BPAF from all dose groups was recovered unchanged in the feces after 72 hr, and females tended to excrete more BPAF into the urine compared to males [128]. BPAF was highest in the male rat liver and GI tract following i.v. and oral administration of all doses, and highest in internal dose in the mouse gallbladder for both routes of administration. Glucuronide and sulfate metabolites were identified in the bile, and at lower levels in rat urine, but not in the feces. Earlier, more metabolic-specific studies in adult Sprague Dawley rats identified four urinary metabolites including BPAF-glucuronide, BPAF diglucuronide, BPAF glucuronide dehydrated, and BPAF sulfate [129]. Several UDP-glucuronosyltransferases (UGT) were identified which may mediate BPAF glucuronidation, and UGT 2B7 produced the highest metabolic efficiency. Bioactivation using human liver microsomes and recombinant CYP isozymes in the presence of NADPH and GSH either produced hydroxylated BPAF or 4-hexafluorohydroxyisopropylidene-phenol [130]. While the UGTs facilitate conjugation to decrease BPAF's activity in adult-derived tissues/cells, they may be less efficient at metabolizing BPAF in the fetus due to low expression and metabolic activity. One study showed that basal liver UGT 1A6 and 2B1 gene expression and glucuronidation activity following BPAF exposure was significantly lower in male and female offspring between GD 18.5 and PND 21when compared to their mother's liver UGT activity [131]. However, several other UGTs that were shown to have high glucuronidation efficiency in human liver microsomes [129] were not tested. Therefore, the activity of these important metabolic pathways remains unknown. Overall, BPAF metabolism is similar to BPA in that it is metabolized by conjugation and excreted in the urine and feces. This process is also thought to decrease the estrogenicity of the chemical. When BPAF and BPAF-G (10-1000 nM) were

assessed in the ER+ breast cancer cell lines MCF-7 and T47D, cell proliferation was increased in both cell lines by 100 nM and 1000 nM BPAF [129]. ER responsive genes (*GREB1*, *TFF1*, and *CTSD*) were also significantly increased at these concentrations. BPAF-G at any of the concentrations was unable to induce proliferation of gene expression changes in either cell line indicating that it had less estrogenic activity.

Toxicity and Mutagenicity

BPAF tested negative for mutagenicity in *S. typhimurium* TA98 and TA100 with and without the addition of S9 metabolic activation, and was not cytotoxic at concentrations ranging from 12.5-100 µmol/L [93]. In the same study, they showed that after 4 and 24 hr, BPAF concentrations ≤ 10 µmol/L were unable to induce DNA damage in HepG2 cells, however reduced cell viability in those cells was reported at concentrations ≥ 50 µmol/L [93]. BPAF arrested metaphase in a dose dependent manner in V79 lung fibroblasts cells and increased the number of micronucleated cells [95]. In Syrian Hamster Embryo cells (SHE), BPAF decreased cell growth at 50 µM and transformed Oua^r and TG^r mutations above control levels [96]. Genetic mutations were not observed at the Na+/K+ ATPase locus or the *hrprt* locus following 48 hr treatment, nor were chromosomal aberrations observed. Treatment of SHE cells with BPAF (12.5-50 µM) for 48 hr increased the percentage of aneuploid metaphases with chromosome losses and gains within the diploid range. Kanai et al. [132] showed that after 48 hr of BPAF treatment (12.5-50µM) SHE cells exhibited decreased colony transforming efficiencies in a dose dependent manner. Zebrafish studies conducted at concentrations ranging from 0.05-1 mg/L delayed hatch rates, decreased hatchling survival rates, and depressed egg fertilization in offspring [133-135]. Offspring experienced hepatotoxicity (male only), acellular testicular

development, delayed oocyte development, and pericardial edema. The LD50 in adult male rats following acute exposures is 3400 mg/kg [136]. Following a 28-day exposure in male and female rats, BPAF concentrations ≥ 30 mg/kg reduced body weights due to a decline in food consumption, reduced hematopoiesis, and dilated the lumen of the large intestines in both sexes [137]. Atrophy of the mammary gland, anterior pituitary, Leydig cells and adrenal zona fasciculata were evident, and irregularity of estrous cycles was also observed. In a 14-day study, male rats given ≥ 50 mg experienced a decrease in body weights without altering absolute testis weight and only increased the relative testis weight at the highest concentration [138].

Estrogenicity and Endocrine Activity

MCF-7 cell-based estrogenicity tests (E-SCREEN) revealed that BPAF was able to induce proliferation and PR and pS2 expression at concentrations comparable to BPA [108, 139-141]. Co-treatment with ICI 182,780 (an estrogen receptor antagonist) and BPAF in MCF-7 cells diminished the previously observed proliferative effects [132]. SHE cells (ER- cell line) treated with increasing doses of BPAF decreased colony forming efficiency and induced morphological transformations at concentrations lower than those reported for BPA [132]. Changes that occur in this cell line following estrogenic exposure indicate a genotoxic effect (i.e. aneuploidy, gene mutation or chromosomal aberration). Other estrogenic assays using a yeast two hybrid system (+/- S9) found that BPAF was capable of increasing β-galactosidase in the presence and absence of S9 [141]. Luciferase and radio-ligand binding assays concluded that BPAF is a full agonist for ERα and an antagonist for ERβ. Also, BPAF had a greater affinity to the ERα compared to BPA, but had the greatest affinity for ERRγ [107]. Free triiodothyronine (1000 µg/L), testosterone (50-250 µg/L), and estradiol levels (50, 125 and 1000 µg/L) were increased in female zebrafish

offspring and decreased testosterone (25 and 125 µg/L) and increased estradiol (25 and 125 µg/L) in male offspring following exposure to BPAF for 28 days or 120 days [133, 135]. Vitellogenin, an egg yolk precursor protein expressed by female fish and other species may also be expressed in males following chemical exposures. Hepatic vitellogenin expression was increased in both sexes and significantly greater in males (25 and 125 µg/L) compared to females and serum vitellogenin levels were increased in adult males (50-1500 µg/L) [134, 135].

In uterotrophic assays conducted in the immature rat model, BPAF has been shown to increase absolute and relative uterine blotted weights (8, 40, and 100 mg/kg/bw/d), confirming estrogenicity reports in a hER/HeLa cell reporter gene assay [142]. BPAF (600 mg/kg) increased the glans penis weight in the Hershberger assay in male rats [143]. When BPAF was administered in conjunction with testosterone propionate (TP), the seminal vesicle (50 and 600 mg/kg), glans penis (50 and 600 mg/kg), and Cowper's gland (600 mg/kg) weights all increased compared to vehicle + TP. These high dose studies in adult male rats provided some animal evidence to support earlier findings from luciferase assays that BPAF had a greater androgen activity compared to BPA, however, the assay was too variable to make this conclusion [108]. Following 14 days of exposure to BPAF (0, 2, 10, 50, and 200 mg/kg/d) Sprague Dawley adult male rats experienced decreased cholesterol levels and serum testosterone, while it increased luteinizing hormone (LH) and follicle-stimulating hormone (FSH) at BPAF concentrations ≥ 50 mg/kg/d [138]. In this same study testicular mRNA and protein expression of genes involved in cholesterol biosynthesis (SREBP-1c), transport (SR-B1, StAR, P450cc), and steroidogenesis (17β-HSD, Inhibin B, LHR and ERα) were found to be reduced at the 200 mg BPAF/kg body weight dose.

Regulatory

The CDC's National Health Report has no human exposure data on BPAF, likely because it is not regarded as a high production volume chemical. Currently there are no regulations in place for BPAF at the US EPA or FDA. However, because of increasing studies and evidence that this chemical acts as an endocrine disruptor, the National Institutes of Environmental Health Sciences took the initiative in 2008 to nominate BPAF for future study within the National Toxicology Program (NTP). Only one study has come from this effort [128], but as more studies are performed they will help to determine BPAF's toxicological effects and provide information for health assessment in future regulatory and risk assessment decisions.

Bisphenol S (BPS)

Chemical Properties and Uses

BPS is composed of two phenolic groups bridged together by a sulfonyl group (Figure 1-2) and is synthesized with sulfonic acid or oleum and two phenols. During this process a phenol reacts with a sulphonating agent in an ortho-, meta or para chlorotoluene at temperatures between 100-200°C [144]. Its structure makes it more resistant to thermal challenges and light compared to BPA.

Routes of Exposure

Inhalation, dermal, and oral ingestion are the main routes of exposures for BPS due to its many applications. It is used as a curing agent and anticorrosive in epoxy glues and adhesives, polycarbonate in plastics, resins in canned foodstuffs, and a developer in thermal paper and dyes. BPS has been detected in various paper products (i.e. flyers, mailing envelopes, tickets, etc.) and

currency bills from 81 samples in the U.S. (Albany, NY), 10 samples in other U.S. cities, and in three Asian countries [145]. Foodstuffs were tested in nine cities in China and the highest levels were in meat and meat products, fish and other seafood, and cereals and cereal products. However, a sum of all measurable bisphenols revealed that the highest concentrations were measured in vegetables, beverages, fish and seafood, and condiments, all of which contact plastic packaging or resin lined cans [122]. Findings from the same group showed that BPS is found in indoor dust. BPS was measured in 100% of the tested samples, and was highest in Japan and the U.S.; it was most abundant in work offices and domestic households [121]. When daily intake levels by inhalation of indoor dust were estimated for the U.S. by age group, infants and toddlers were estimated to have higher levels than any other group. During development, children spend a great amount of time playing and sitting on the floor and have higher respiratory rates. Thus their rate of potential dust-borne exposure is greatly increased compared to adults.

The widespread use of BPS as a replacement for BPA has instigated several biomonitoring studies. BPS was measured in the majority of urine samples from cashiers prior to and post-shift but was only detectable in the serum of a small portion of those cashiers. Similar to indoor dust findings, urinary BPS concentrations from the U.S. and seven Asian countries were greatest in samples from Japan and the U.S. [77]. Urinary detection rates varied from 42-100% of samples; they were highest in males and in those participants ≤ 19 years old. The highest urinary concentrations were also measured in males, and in participants aged 40-49 years old. Urinary BPS levels in convenience samples collected between 2000-2014 revealed significant changes in detection rate and concentration over time; as time progressed, the detection rates increased as well as the geometric mean, from 0.18 to 0.25 μg/L [126]. BPA levels were the most often detected bisphenol in convenience samples during all collections,

compared to BPS, but the increases in BPS concentrations over time suggest a trend of increased exposure to humans. Ingestion by transfer from plastics or contaminated foods is the primary route of exposure in the general population. However, occupational settings may increase thermal paper exposures due to handling. This was evident in cashiers who had significantly increased post-shift urinary BPS levels compared to pre-shift [146].

Absorption, Distribution, Metabolism, and Excretion

Although studies involving BPS continue to increase in PubMed, there has yet to be published data that details the absorption, distribution, or preferential excretion route in any species. It is likely that BPS acts in a similar manner to BPA, but disposition studies in multiple species and route of excretion data are needed. A handful of studies have shown that BPS metabolism may be similar to that of BPAF and BPA. Testing of human urine revealed that BPS was detected in 78% of samples and the mean amount in the conjugated form was 97%. These data imply that BPS is highly conjugated by glucuronidation and readily excreted into the urine [147]. In later studies using human liver microsomes (HLM), it was determined that UGT 1A9 enzyme had the highest efficiency for metabolizing BPS [148]. Incubations with human intestinal microsomes (HIM) showed that BPS was minimally glucuronidated, suggesting that most BPS conjugation occurs in the liver. Further studies revealed that BPS can be bioactivated to an ortho hydroxylated metabolite when HLMs and HIMs were incubated in the presence of NADPH and cytochrome P450s (CYP450); the CYP most active towards BPS was CYP3A4 [149]. BPS was assessed for reactive metabolite formation with HLM, NADPH, and glutathione and no reactive metabolites were formed, a stark contrast from BPA. Lastly, incubations with NADPH and UDPGA were completed to assess metabolic competitiveness and glucuronide

production was heavily favored and greatest in HLMs compared to HIMs. Similarly, when BPS was incubated with HLMs in the presence or absence of UDPGA, BPSM1, the hydroxylated metabolite, formation was reduced when UDPGA was present.

Toxicity, Mutagenicity, and Genotoxicity

In the Ames test using TA98 and TA100 and the *umu* test (T1A135), which induces β-galactosidase activity in the absence and presence of S9, BPS was unable to increase reversion or β-galactosidase activity at concentrations ranging from 4-500 µg/plate [93, 94]. Cytotoxicity was not evident after 24 hr in HepG2 cells (12.5-100 µmol/L), nor was acute toxicity achieved in *Daphnia magna*. BPS (0.1µmol/L and 10 µmol/L) induced DNA damage after 24 hr in HepG2 cells but the effects were not dose dependent. In wild type and mutant DT40 chicken embryo cultures, RAD54$^{-/-}$, a mutant deficient in the homologous recombination pathway, was sensitive to bisphenol A and several other bisphenol alternatives [150]. Specifically, BPS caused a decrease in cell proliferation and increased chromosomal anomalies. Recently, Zhang et al. [151] reported that BPS exposure in hepatic and renal cells did not induce toxicity, apoptosis, or catalase (CAT) activity at concentrations ranging from 1 µM-0.1 mM. Only at high doses (0.1-1 mM), during the same time frame, did both cell types exhibit BPS dose dependent increases in toxicity, apoptosis, and CAT activity [151]. Using multiple fluorescence spectral analyses, they also determined that at high doses, BPS changed the structure and activity of CAT by binding to Gly 117 residue where the substrate binds to the enzyme.

Estrogenicity and Endocrine Activity

In vitro and *in vivo* studies have clearly shown BPS to be estrogenic, although to a lesser extent compared to same doses of BPA. Although BPS has been shown to induce proliferative effects in MCF-7 cells, (10^{-9}-10^{-5}M), the effects were only found to be greater than BPA at very high concentrations ($\geq 10^{-5}$M) [141]. At the highest concentration tested, β-galactosidase was increased when incubated with S9 and 50% inhibition was achieved in a fluorescence polarization test. Kitamura et al. [108] confirmed similar findings in MCF-7 cells (EC50 BPA >BPS, estrogenicity test) and that NIH-3T3 transfected cells (adrogenicity test) treated with BPS had an IC50 that was 10-fold lower than BPA [108]. BPS was positive for anti-adrogenicity, negative for androgenic activity, and produced no activity in the GH3 cells. The lack of GH3 activity implies that BPS does not induce thyroid-hormone like activity. BPS can also produce non-genomic estrogenic effects [152]. In the $GH_3/B_6/F_{10}$ rat pituitary cell line BPS decreased ERK response in the presence of E_2 and caused photoactivation in a non-monotonic dose response. By itself, BPS increased cell proliferation but decreased cell numbers when E_2 was present by activating apoptosis via caspase 8 and 9.

Regulatory

Currently, there are no regulatory guidelines that have been put forth for BPS, however, the continued use in products, monitoring in humans and increasing number of scientific studies will likely prompt changes in the near future.

BPA and the Mammary Gland

Mammary Morphology and Carcinogenesis

Only a handful of chemicals besides BPA have been extensively studied for effects on the mammary gland (i.e.diethylstilbesterol, perfluorooctanoic acid, genistein, DDT, and atrazine). In utero exposure by daily subcutaneous injections to 0.5 or 10 mg/kg BPA accelerated mammary gland development in 4-week old female CD-1 mice when corpora lutea were present [153]. The same route of exposure given during a pre-pubertal exposure (PND 15) did not produce any effects in the mammary gland at 4, 8, or 24 weeks of age [154]. Prenatal exposures to human relevant BPA doses (25-250 µg/kg; peristaltic pump) in CD-1 mice caused altered ductal migration in the mammary stroma (1 mo). By 6 mo., TEBs, ducts, and alveolar buds were increased [155]. BrdU incorporation in the epithelium was decreased at 10 d, but increased within the stroma by 6 mo. Prepubertal BPA administration to ovariectomized CD-1 and C57Bl/6 weanling mice increased the glandular sensitivity to estradiol during puberty, and as a result increased TEBs, ductal extension, and decreased apoptotic activity [99]. Progesterone receptor (PR) expression was increased in the epithelium, but Wnt4, which is downstream of PR and plays a role in lateral branching, was unchanged. Wadia et al. [156] showed that when perinatally BPA (250 ng/kg/bw/d) exposed CD-1 and C57Bl/6 mice were ovariectomized (PND 25) and administered estradiol (0, 0.5 and 1 µgE_2/kg/bw/d) for 10 days, they experienced similar sensitivity to E_2 in the mammary gland. In both strains, the number of TEBs increased with increased E_2 doses and was significantly greater with 0.5 µg E2 + 250 ng BPA compared to 0 ng E2 +250 ng BPA. Together these studies revealed that the timing of exposure and amount of BPA exposure are critical to determining 1) the effects observed in the mammary gland, 2) when these effects can be detected, and 3) how they will influence later life development.

Early life exposure to BPA in rodents has also been linked to a shift in susceptibility to carcinogenesis. Following prenatal exposure of Wistar-Furth rats to 25 µg BPA/kg/body weight, an increased BrdU:apoptosis index in the epithelial and stroma during puberty were reported [157]. The percentage of hyperplastic lesions was also significantly increased above control levels at PND 110 and 180 (at 25 mg/kg/bw). Administration of the carcinogen, N-nitroso-N-methyl urea (NMU, 25 mg/kg/bw, i.p.), at PND 50 increased the incidence of hyperplastic ducts at PND 110 and 180 and produced malignant tumors. As early as 3 mos, a beaded duct was observed but only at the lowest concentration. By 9 mos., beaded ducts were apparent in all BPA-exposed groups. When Sprague Dawley rats perinatally exposed (25 or 250 µg BPA/kg body weight; oral gavage) were administered the mammary carcinogen dimethylbenzanthracene (DMBA; single dose 30 mg/kg/bw) at PND 50, they experienced increased tumor formation in a dose dependent manner and decreased tumor latency with significance achieved at the highest dose. At PND 50, the time of carcinogen exposure, mammary glands from the highest dose exhibited increased cell proliferation and decreased apoptosis. Protein expression of Akt, pAkt, PR-A, and SRC1-3 were all increased in this group [158]. FVB/N mice exposed to BPA in utero and given DMBA also had increased mammary tumor frequency, in a dose dependent manner [159]. Prenatal exposure to 0.25-25 µg/kg BPA in CD-1 mice caused an increased fraction of alveolar buds at 3 (0.25 µg/kg) and 9 mo. (2.5 µg/kg) and a decreased volume in ducts at 9 mo. (0.25 µg/kg) [160]. In yet another study, Sprague Dawley female rats perinatally exposed to 25 - 250 µg BPA/kg body weight (s.c. via Alzet osmotic pump) had preneoplastic lesions in the gestational and gestational/lactational exposed groups as early as PND 50 and progressed into neoplastic lesions as early as PND 90 [161]. Delclos et al. [162] observed minimal grade ductal hyperplasia in female rats at PND 21 from three dose groups following oral exposure to BPA

(2.5-2700 µg/kg/bw/d) between GD 6 and PND 90. Hyperplasia was significantly increased at PND 90 in the 2700 µg/kg group. When lesion severity was considered, 2/3 groups presenting with hyperplasia at PND 21 were also significantly increased at PND 90. By PND 90 mammary glands from females in the 2.5 µg/kg group had developed adenocarcinoma. Males all experienced ductal hyperplasia at PND 90 when severity was evaluated.

The male mammary gland has rarely been assessed for effects of EDCs due to limited amount of epithelial structure in male mice and limited information available on male mammary development over the life stages. Male offspring perinatally exposed to BPA developed mammary glands that had increased branching points and ductal area at 3-4 mo., but in a non-monotonic dose response [163]. Between 7-9 mos., significantly increased branching points was only observed in 2.5 and 25 µg/kg, and in male mice 12-15 mos. of age, the response shifted and was only present at the two highest concentrations. In 7-9 mo. old males, cell proliferation was increased (25 µg/kg), but apoptosis was unchanged. Interestingly, following prenatal subcutaneous injections, male rat offspring experienced increased ductal growth at PND 5 (250 µg/kg) followed by significantly decreased epithelial growth when measured as the distance between the lymph node and the mammary gland final edge at PND 30 (250 µg/kg) [164]. This group also exposed rats to BPA (64 µg/kg/bw/d) between GD 9 and PND 21 via drinking water. Mammary gland changes in BPA-exposed male offspring were not observed at PND 5, but at PND 15 and 30 the number of TEBs was reduced in BPA-exposed glands. Only at PND 30 was the distance between the lymph node and the mammary gland final edge significantly decreased. At PND 30 AR expression in the ducts, TEBs and TDs were all reduced whereas ER expression and the proliferation index were unchanged and PR was not expressed in any of the mammary structures. Taken together this data reinforces the concept that the mammary gland is sensitive to

endocrine disrupting chemicals and that the same chemical can exert opposing effects in the male and female mammary glands.

Mechanisms of Action

Due to the mammary gland's structural and signaling complexity, the introduction of an endocrine disruptor like BPA during critical windows of development may initiate several different mechanisms of action.

BPA did not promote the differentiation of human embryonic stem cells into 'induced' differentiated mammary epithelial cells, but increased mammosphere area in a dose-dependent manner ($p<0.05$, 10^{-9}M) [165]. Pluripotent embryonic stem cell markers (*Oct4*, *Sox2* and *Nanog*) were increased in a dose dependent manner for BPA and at low E2 (10^{-9}M) levels, but decreased E-Cadherin was decreased following low-dose BPA exposure alone. This suggests that BPA may be able to drive pluripotency in an estrogen-dependent manner, while promoting an estrogen-independent pathway for E-cadherin expression. Perinatal BPA exposure has also been shown to increase mammary cell numbers, PR expression in luminal cells (6 and 12 mo.), and expression of the downstream regulator *Wnt4* without altering the individual number of FACS sorted basal, epithelial or stromal cells [48]. Wang et al. also discovered that Balb/C mice administered BPA during puberty demonstrated increased progenitor cell and transiently increased basal cell counts [166]. Following a single dose of DMBA (30 mg/kg) at 2 mo., mice treated with BPA and DMBA experienced increased hyperplastic lesions compared to control, BPA alone and DMBA alone. When solid 3D MaSC were transplanted to determine stem cell efficiency there was a significant increase in hyperplastic lesions in BPA MaSC and BPA+DMBA MaSC regenerated

glands at all time points. Deep sequencing profiles from the BPA exposed mammospheres revealed altered genes that were commonly seen in breast cancer patients.

Transcriptional and epigenetic profiles of the mammary gland have also been assessed to understand pathways associated with morphological changes. Fetal glands from $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice treated with EE2 or BPA (250 ng/kg) only had distinguishable transcriptional profiles when separated by stroma/epithelial compartments, and no significant differences were noted by treatment or ERα genotype [8]. Hierarchal clustering by tissue type revealed distinct changes with treatment in the stroma that were less evident in the epithelium, regardless of ER expression, suggesting that ER may not play a significant role in BPA-mediated fetal gland changes. BPA downregulated genes associated with the focal adhesion pathway in peri-ductal tissue from $ER\alpha^{+/+}$ mice compared to $ER\alpha^{+/+}$ vehicle mice. Genes involved in adipogenesis were also increased in peri-ductal tissue of both $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$. When the effects of BPA were assessed in the mammary epithelium from $ER\alpha^{+/+}$ tissues, it increased transcripts associated with apoptosis compared to vehicle. Transcripts analyzed for potential ER regulation in $ER\alpha^{+/+}$ showed a change in epithelial genes involved in branching, focal adhesion, and extracellular matrix, while the genes in the peri-ductal stroma were involved in breast cancer. These data demonstrate that not only can BPA affect transcriptional regulation in the mammary epithelium but also in the stroma, indicating that they are both important for BPA induced alterations. It also shows that BPA effects can be ER and non-ER mediated. Mammary glands from prenatally BPA exposed rats (25 and 250 µg/kg) were evaluated for gene expression changes during time points where morphological changes were observed (PND 21, 35, 50, and 100 days) [167]. Genes altered at PND 21 and PND 35 primarily involved cell differentiation and *Gad1*. The number of genes changed between PND 21 and 35 decreased in both dose groups (25 and 250 µg/kg), but

increased by PND 50 where most altered genes were associated with immune function, cell death and stress response (25 µg), differentiation, growth arrest, fatty acid binding protein 3, and whey acidic protein (250 µg). By PND 100, mammary glands from the highest dose had upregulation of genes involved in immune response and downregulation of differentiation genes. This may be an important mechanistic clue, as in addition to epithelial tissue, the mammary gland is comprised of immune cells which aid in normal development, as well as inhibition of tumor development and progression [168, 169].

Through microarray and RT-PCR validation, Fischer et al. [170] identified that CD-1 mice gestationally exposed to BPA (5 mg/kg) showed significant decreases in several genes from the chemokine family, interleukins, and interferons [170]. In this study the authors reported that important leukocyte cell markers (CD45, CD19, Ly6G, and FSP1) and ERα expression were decreased while ERβ was increased. The effect of BPA on the mammary epigenome has also been investigated [171]. Wistar-Furth rats prenatally exposed to BPA levels previously shown to induce ductal carcinomas *in situ* were evaluated for DNA methylation changes at several developmental time-points (PND 4, 21, and 50). These studies demonstrated that most methylation changes were evident at PND 21, but more transcriptional changes due to treatment were observed at PND 50. Hypo- and hypermethylation were noted across several chromosomal loci, but differences observed between BPA and vehicle varied over time and no consistent dose effect was noted. By PND 50, BPA increased *jun* expression and decreased *p57*, a gene involved in cell cycle regulation.

Research Goals

The NTP fact sheet on BPA suggests there is minimal concern for developmental toxicities in fetuses, infants and children pertaining to the mammary gland and early puberty in females [172]. However, there is an increasing body of evidence in animal studies using human relevant doses, as well as some epidemiological studies, that point to BPA's ability to change the breast architecture and increase risk of breast tumor development. In my studies, I will use a mouse model that has been prenatally exposed to BPA, BPAF, or BPS address several research gaps, such as 1) quantifying BP analogue internal dose following maternal exposure and confirm fetal transfer, 2) identifying altered mammary gland morphologies and the potential of these chemicals to induce abnormalities that may include neoplasia, and 3) examining early life signaling pathways that may play a role in the observed early and later life effects.

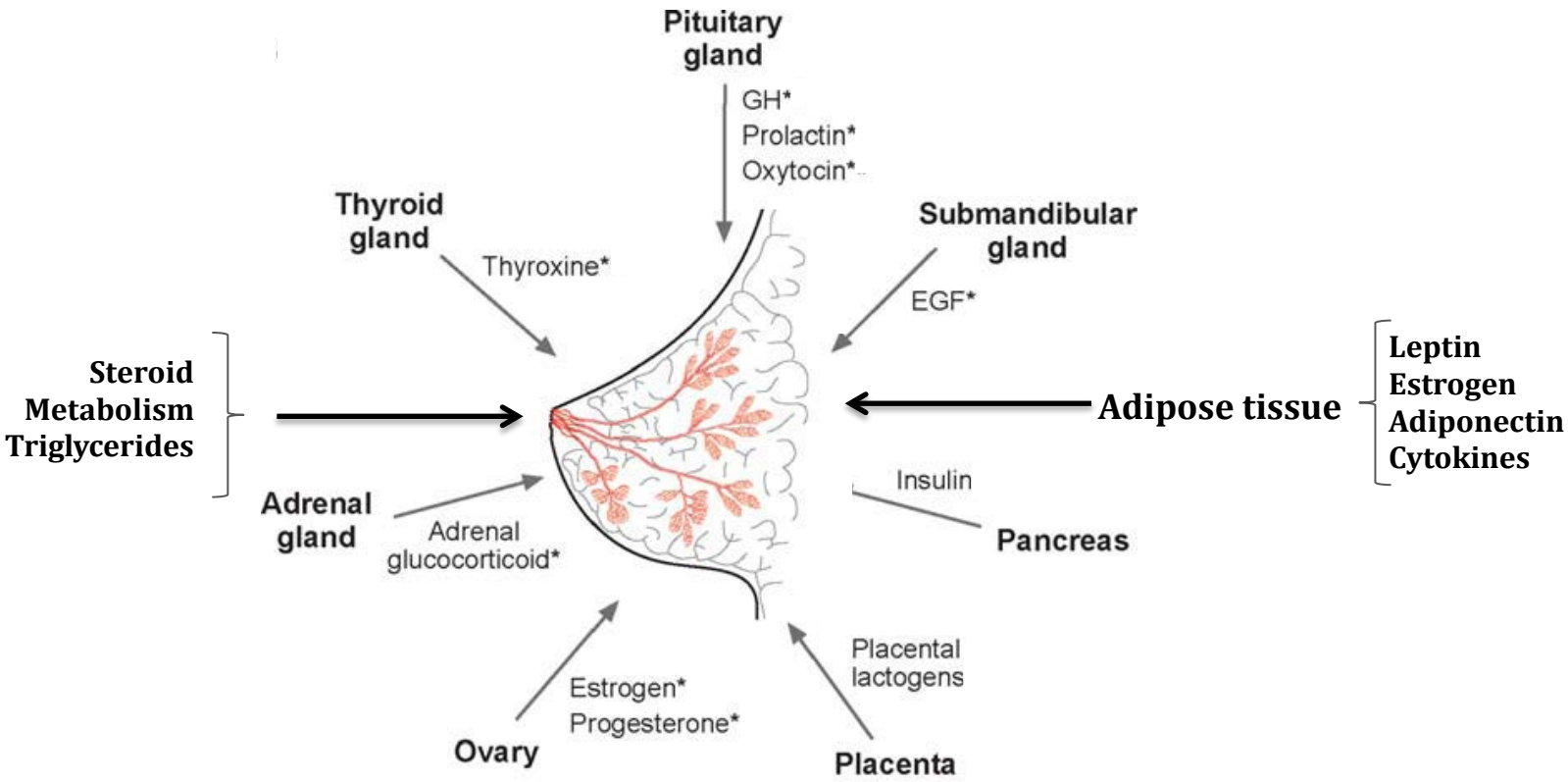

Figure 1-1: Endocrine disrupting compounds may interfere at multiple sites and times. As illustrated there are many tissues and endogenous chemicals including steroid hormones, metabolic hormones and cytokines that regulate breast development, structure, and function**.**

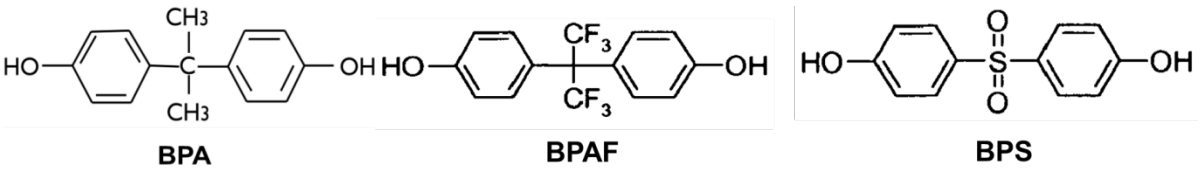

Figure 1-2: Chemical structures of BPA, BPAF, and BPS.

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CHAPTER 2

Disposition and early developmental effects of bisphenol analogues in CD-1 mice

OVERVIEW

Growing concern over the use of Bisphenol A in plastic consumables has resulted in the implementation of replacement analogues including the fluorinated and sulfonated derivatives, BPAF and BPS. Both chemicals have estrogenic activity that is comparable/enhanced vs BPA, which may cause detrimental changes to the developing offspring, often manifesting later in life. Current disposition data for these chemicals are either nonexistent or fail to address the uniqueness of maternal-fetal exposures. This study assesses the pharmacokinetic and disposition properties of BPA, BPAF, and BPS in the maternal-fetal unit. Timed pregnant CD-1 mice were given a single gavage dose of BPA (50 mg/kg), BPAF or BPS (5 mg/kg), or sesame oil at gestational day 16. At 2, 4, 8, 12, 18, 24, and 48 hr post-exposure, urine, feces, amniotic fluid, and serum were collected from dams. Similarly, on postnatal days (PND) 3, 4, and 5, serum and feces from dams and pooled serum from offspring were collected. Total concentrations (free plus conjugated species) of BPA, BPS, and BPAF in serum, urine, and amniotic fluid were quantified by online-solid phase extraction coupled to HPLC–isotope dilution–tandem MS. Concentrations of the reagent blanks suggested that external contamination did not occur during sample collection/analysis. Maximal maternal (C_{max}) serum and urinary concentrations were achieved within 2 hr for BPA and 4 hr for BPAF and BPS. Serum and urinary half-lives were similar between compartments and were shortest following BPAF exposure. BPAF fecal recovery was ~30-50% greater than BPS, but fecal half-life was like serum and urine. Each analogue was

recovered from the amniotic fluid as early as 2 hr post exposure but C_{max} was not reached for any chemical until 18 hr. Between PND 3 and 5, maternal serum concentrations decreased by 90-1000 fold or were below the limit of detection, suggesting that pup urine does not significantly contribute to chemical recirculation. Pup serum concentrations were similar between males and females. The slight differences in disposition across analogues were likely due to their chemical properties, but confirm placental transfer of BPAF and BPS. Recovery of each analogue in the amniotic fluid regardless of dose amount suggests the potential for fetal exposure and warrants additional studies to determine how early life exposure to these chemicals may impact early and long term offspring development.

INTRODUCTION

Bisphenols are a class of chemicals characterized by two hydroxphenyl groups often joined by a bridging carbon and one or more substitution groups. The most widely studied bisphenol is Bisphenol A (BPA), a synthetic component used in the production of plastics and epoxy resins. BPA has been shown to act as a weak estrogen and endocrine disruptor in rodent models. Within the last decade, several chemical analogues have gained notoriety due to their similar structure and estrogenic activity compared to BPA. Specifically, concerns involving the use of Bisphenol AF (BPAF), a fully fluorinated derivative, and Bisphenol S (BPS), which includes a sulfonyl group in place of the bridging carbon, continue to increase because of their potential to induce effects similar to BPA.

Bisphenol S was initially nominated in 1994 [1] to the National Toxicology Program (NTP) for toxicological assessment, but was later withdrawn. In 2014, it was once again

nominated due to its potential for human exposure and health effects [2]. Bisphenol S is also used as a component in plasticizers and resins and as a developer in thermal receipt paper. It is considered a high production volume chemical [3], with several routes of exposure including dermal, oral, and inhalation [4-7]. BPS was found to be estrogenic by studies conducted *in vitro* [8, 9] and *in vivo* [10]. Although increasing evidence of human exposure to BPS exists [7, 11, 12] there remains a lack of toxicity and pharmacokinetic data from any mammalian or laboratory species. Available data reported negative findings for BPS in the Ames test and only induced DNA strand breaks in HepG2 cells 24 hr post exposure [13]. Zhou et al. [14] identified conjugation as the main metabolic pathway (97-100%) for BPS in human urine samples. The main human hepatic enzyme responsible for glucuronidation was identified as UGT1A9 [15]. The same group later identified an orthohydroxylated BPS metabolite (BPSM1) that was formed following human liver, intestinal microsomal or cytochrome P450 incubation with (nicotinamide adenine dinucleotide phosphate) NADPH [16]. CYP3A4 and 2C9 produced the greatest enzymatic activity when human liver microsomes (HLM) were coupled with NADPH, and UDP-glucuronyltransferase (UDPGA) glucuronidation was favored [16].

In the United States, between 1986 and 2002, BPAF was moderately produced within the range of 10,000-500,000 pounds/annually [17]. Similar to BPS, BPAF was nominated to the NTP testing program in 2007 due to concerns of potential occupational and general population exposures [17]. Its primary uses are as a crosslinking agent in fluorelastomers and polycarbonate copolymers in several consumer products. Apart from a few laboratories, very little progress has been made in understanding the toxicity of this chemical. To date, studies have confirmed that BPAF is not mutagenic, nor able to induce DNA strand breaks 4 and 24 hr post exposure [13]. When assessed for aneuploidogenic potential, non-cytotoxic concentrations of BPAF induced

micronuclei and disrupted the cytoplasmic microtubule complex and mitotic spindle in V79 cells [18]. BPAF also inhibited cell growth (50 μM) in Syrian hamster embryo cells, but failed to alter gene mutations at the Na^+/K^+ ATPase locus. Similar studies confirmed the aneuploidy findings and revealed that they were induced within the near-diploid range [19]. BPAF has increased proliferation, pS2 secretion, and progesterone receptor expression in MCF-7 cells [20-22], and acted as an agonist for ERα and antagonist towards ERβ in transfected HeLA cells [23, 24]. Metabolism studies performed by Li and coworkers [25] identified glucuronidation as the major metabolizing pathway for BPAF and identified four metabolites in Sprague Dawley rats. They also determined that multiple recombinant human UGT enzymes were effective at glucuronidating BPAF, and identified UGT2B7 as the most efficient. A single disposition study involving both sexes of adult Harlan Sprague Dawley rats and $B6C3F_1/N$ mice showed that the main route of BPAF excretion was through feces compared to urine, following both oral and intravenous (i.v.) administration [26]. Incubations with [^{14}C] BPAF and human, rat, or mouse hepatocytes produced glucuronide and sulfate metabolites that were easily identified by liquid chromatography tandem mass spectrometry (LC/MS/MS) from parent BPAF. Rodent metabolite profiles also confirmed previous findings and showed that glucuronidation reduced BPAF's estrogenic effects [27]. Although to a lesser extent, BPAF bioactivation is also possible. Incubation of BPAF with HLM, NADPH with cytochrome P450's, or glutathione (GSH) produced three metabolites (OH-BPAF, 4-hexafluorohydroxy-isoprpylidene phenol, and GSH conjugate of 4-hexafluoroisopropylidene phenol) [28].

As concerns continue to mount over the potential human exposure to BPAF and BPS there remains limited disposition data and no pharmacokinetic data. The fact that BPA have been extensively assessed for toxicity and estrogenicity and that both BPAF and BPS share similar

structures, metabolism, and estrogenic properties with BPA, have prompted the need for more studies with these lesser characterized chemicals. It is especially important to understand how these chemicals may affect fetal and infant populations. With very little human disposition data to rely on and only limited BPA pregnant disposition studies in rodents [29-31], these studies were designed to determine the disposition and pharmacokinetics of BPAF, BPS, or BPA following a single oral exposure to pregnant CD-1 mice. Chemicals were measured in multiple matrices to determine the extent of offspring exposure. Once the excretory half-lives were established, time pregnant mice were orally exposed to these chemicals during pregnancy to determine early life effects on offspring. These studies will aid in understanding the implications that these chemicals may have on human health.

MATERIALS AND METHODS

Chemicals and Dosing Solutions

Bisphenol A (BPA) and Bisphenol S (BPS) (≥99.0 % and ≥97.5 % purity, respectively) were obtained from Sigma Aldrich (St. Louis, MO) and Bisphenol AF (BPAF) (98 % purity) was obtained from 3B Pharmachem International Co. Ltd. (Wuhan, P.R. China). Prior to use, each chemical's purity was confirmed by the NTP chemical contract lab. All dosing solutions were prepared daily by dissolving the test chemical in pure sesame oil (Jedwards, International, Inc., Braintree, MA) and stored in clear glass vials to avoid exogenous bisphenol (BP) contamination from plastics. $^{13}C_{12}$-BPA was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA) and $^{13}C_{12}$-BPS was purchased from CanSyn Chem Corp. (Toronto, Canada). $^{13}C_{12}$-BPA was used as an internal standard for BPA and BPAF [14].

Animals

Timed pregnant CD-1 mice were obtained from Charles River Laboratories (Raleigh, NC). Upon arrival at the NIEHS, dams were weighed and randomly assigned to the vehicle or treatment groups. Body weights were approximately equal in all groups prior to randomly assigning dose. All dams were acclimated for a minimum of 2-3 days prior to dosing. Pregnant dams were individually housed in polypropylene cages and received AIN-93G (Harlan Laboratories, Indianapolis, IN) feed. Tap water was provided *ad libitum* and supplied in polypropylene bottles that were tested for estrogenicity by E-SCREEN. Cages were lined with SaniChip Hardwood bedding (PJ Murphy Forest Products, Inc., Montville, NJ). Animal facilities were maintained on a 12:12h light-dark cycle, at a controlled temperature (20-24^{o}C) with 40-60% relative humidity. All animals were treated humanely and in accordance with the protocols of the National Institute of Environmental Health Sciences Animal Care and Use Committee. Dams were weighed daily prior to dosing and were administered each chemical by concealed allocation. Unless noted all dams carried to term and pups delivered to small litters (n<4 pups) were excluded from these studies.

Experimental Design

Disposition Studies. Pregnant dams arrived on gestational day 14 (GD 14), and on GD 16 dams were given a single oral gavage dose of either vehicle (n=10), 50 mg BPA/kg body weight (n=30), 5 mg BPAF/kg body weight (n=30), or 5 mg BPS/kg body weight (n=30). 50 mg BPA/kg is 1000x the Environmental Protection Agency recommended daily intake (EPA RDI) [32]. However, no human data for BPS/BPAF exist and therefore these concentrations were chosen to ensure that we could obtain an appreciable measurement in the collected tissue

samples. At 2, 4, 8, 12, 18, 24, and 48 hr post exposure, dams were sacrificed to obtain serum, urine, feces, amniotic fluid, and a randomly chosen fetus (1 pup/dam). A subset of animals from each dose group was randomly chosen to continue through pregnancy until full term. Parturition in CD-1 dams normally occurs on the eve of GD 18, where the next day is considered postnatal day 1 (PND 1). All pups remained with their respective dams, and litters were not equalized to maximize data collection from the offspring. On PND 3, 4, and 5, dams were sacrificed to obtain serum, urine, and fecal samples. Pups were sexed and sacrificed on those same days to obtain pooled male and female serum and urine, by litter.

Early Developmental Outcomes. In separate studies, the effects of BPA, BPS, and BPAF on early development and pubertal timing in mouse offspring were evaluated in two blocks (~3 weeks apart). Block 1 was received on GD 9 and Block 2 was received on GD 8. Dams were oral gavage dosed b.i.d (determined by disposition results) between GD 10-17 with either vehicle (n=7-8 dams/treatment/block), BPA 50, 5, or 0.5 mg/kg body weight (n=7 dams/treatment/block), BPAF 5, 0.5, or 0.05 mg/kg body weight (n=7 dams/treatment/block) or BPS 5, 0.5, or 0.05 mg/kg body weight (n=7 dams/treatment/block). 10-fold increments of dose were chosen to cover a wide range and to determine if any observed effects would occur in a dose responsive manner. Dams were closely monitored on GD18 (expected day of parturition) and all litters were sexed and weighed within one hour of birth (PND 1). On PND 3 (Block 1) or 4 (Block 2) each litter was equalized to 10 pups per dam, with a 6:4 (female: male) ratio when possible.

Disposition Studies

Matrix Collection, Preparation, and Analysis. Samples for each time-point were collected from a different dam within the same chemical dose group, as opposed to serial collections from the same dam, because the fetal tissue collections required euthanasia. Dam and pup/fetal trunk blood were obtained following swift decapitation. Dam blood was collected in polypropylene tubes and pup/fetal blood was collected in glass vials with deactivated glass inserts. Pup/fetal serum samples from the same sex within a litter were pooled to maximize volume/concentration yields. Blood samples settled at room temperature (26^{o}C) at least 30 min followed by centrifugation at 3000 rpm at 4^{o} C for 20 min. Dam serum was then transferred to glass vials and pup serum was transferred into new glass inserts and kept frozen at -80^{o}C until analyzed.

Dam urine was collected in microfuge tubes during elimination when animals were retrieved from their cage and it was transferred to glass vials with inserts. Ingestion of offspring urine by the dam during early lactation is a common maternal behavior and precluded the adequate collection of pup urine [33]. Fresh fecal excrement was obtained during natural elimination or by removing feces from the lower gastrointestinal tract at necropsy. Amniotic fluid was drawn from each fetal sac using a needle and syringe following removal of the entire litter from the uterus onto ice. Fetuses/pups were swiftly decapitated for blood collection; however, the fetus that was preserved for disposition was decapitated and then wholly contained in a tube (with blood) and frozen.

Metabolic cages were not used to collect excreta in order to minimize maternal stress, avoid fluctuations in food intake and weight, and to prevent the incidence of fetal skeletal malformations [34]. All samples were collected, weighed, and stored in glass vials followed by immediate transfer to ice, then storage at -80^{o}C.

A detailed method for urine, serum, and amniotic sample preparation for the detection of bisphenols can be found in Zhou et al. [14]. Briefly, samples were prepared with and without enzymatic treatment. Approximately 50 µl of $^{3}C_{12}$-BPA (Cambridge Isotope Laboratories, Inc, Andover, MA) or $^{13}C_{12}$-BPS (CanSyn Chem Corp, Toronto, Canada) were added to 100 µl of urine or amniotic fluid and diluted to 1 ml with 0.1 M formic acid to measure free bisphenol species. Total bisphenol concentrations were measured by mixing 100 µl of sample with 50 µl of deconjugation standard and 50 µl of glucuronidase/sulfatase. Samples were incubated with 750 µl of formic acid at 37^{o} C for 4 hr. All samples were vortexed, centrifuged and prepared for analysis on an automated online SPE-HPLC-MS-MS Agilent 1200 module (Santa Clara, CA) and an ABSciex 5500 QTRAP mass spectrometer (MS) (Sciex, Redwood City, CA). The limit of detection (LOD) for urine were BPA (0.4 ng/ml), BPAF (0.1 ng/ml), and BPS (0.1 ng/ml). Amniotic fluid and serum LOD were 0.1 ng/ml for all bisphenols.

Frozen fecal samples were lyophilized overnight. Approximately 25 mg of dry fecal material was hydrated in 800 ul of 1:1 acetonitrile-methanol containing 2 µg of heavy isotope labeled internal standard. Samples were sonicated for 30 min followed by centrifugation to remove particulates. Each sample was aliquoted (0.3 ml) into two tubes and either diluted with 1.2 ml of water for enzymatic deconjugation or prepared for solid phase extraction. Supel-Select SAX (Sigma Aldrich, St. Louis, MO) 30 mg (bed weight), 1 ml SPE tubes were conditioned with 3 ml 1:1 acetonitrile: methanol followed by 3 ml of water using a positive pressure manifold with a 1 ml/min flow rate. Samples were loaded into tubes and washed with 1 ml of 5% ammonia followed by a methanol wash and then washed with 1 ml of a 40% methanol-2% formic acid solution. The bed was completely dried with nitrogen and the bisphenols were eluted with 1:1 acetonitrile-methanol, containing 1% formic acid, solution followed by elution with nitrogen.

Half of the sample was prepared for liquid chromatography-electron spray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis. Samples prepared for enzymatic deconjugation were dissolved in 500 µl of 200 mM acetate buffer (pH 5.2). 200 µl of glucuronidase/sulfatase were added to each sample and incubated overnight at 37^{o} C. Samples were cooled to room temperature to perform solid phase extraction. LC-ESI-MS/MS analysis was performed on an Agilent 1200 capillary HPLC (Agilent Technologies, Santa Clara, CA) and an ABSciex API 3000 tandem MS (Sciex, Redwood City, CA). Separation was performed using a water: acetonitrile gradient with 0.1% acetic acid modifier on a 1.0 X 150 mm C18 column at a flow rate of 65 µL/min. The injection volume was 3 µL. Bisphenols were detected in negative ion mode and two MS/MS transitions were monitored for each analyte. Peak areas were used for quantitation with stable isotope labeled internal standard correction. Calibration curves (R^2 > 0.998, 1/X weighting) were constructed with matrix matched samples. LOD and LOQ were determined for BPS (0.3 ng and 2 ng), BPAF (0.6 ng and 2 ng), and BPA (1 ng and 15 ng).

Half-Life Calculations

Excretion half-life values are based on first order elimination and were calculated as follows, based on previous reports [35]

$$t_{1/2} = \frac{0.693}{m}$$

m=slope of the line from ln [BP Analog] vs. T'

T' = Time – Time at Peak Concentration

Area Under the Curve (AUC) Calculations

The AUC is a measurement of the body's exposure to a chemical after administration against time. AUC is usually calculated with plasma concentration following i.v. dosing and

assumed to distribute into the blood immediately. Since these animals were oral gavaged it takes a slightly longer time to reach systemic circulation and thus blood at time zero is not collected. In order to calculate AUC for an oral dose it was necessary to use back extrapolation to determine time zero [36]. Time zero was calculated using $Cp_0 = \ln (Cp_t + k_t)$ where:

Cp_0 = extrapolated plasma concentration at time zero

Cp_t = plasma concentration at any time

k_t = elimination rate constant

The linear trapezoidal method was used to calculate the area under the curve (AUC) from time zero to the last collection [37].

First segment: $\Delta AUC_{x-(x-1)} = \frac{(Cp_x + Cp_{x-1})}{2} \times (t_x - t_{x-1})$

Last segment: $\Delta AUC_{last-\infty} = Cp_{last}/k_{el}$

k_{el}= elimination constant

t_x = time at any segment

t_{x-1}= time at any segment minus one

Early Developmental Outcomes

On PND 1, male and female offspring from each litter were first sexed and then weighed as a sex-specific group. Mean pup weight was determined using the formula BW= sex-specific group weight/# of pups. The number of pups per litter was recorded and pup sex was used to calculate sex ratio for each dose group and vehicle control.

Statistical Analysis

In all studies, the dam is considered the unit of measurement and unless noted all data are represented as the mean ± standard error of the mean (SEM). Bisphenol content in excreta and bodily fluids is expressed as total bisphenol = (free bisphenol + conjugated bisphenol). A two-way ANOVA using Tukey's post hoc test was conducted to evaluate for block effects. No differences were observed between blocks and therefore they were combined and evaluated for outliers using GraphPad QuickCalcs Outlier test (http://graphpad.com/quickcalcs/Grubbs1.cfm). Statistical significance was determined using ANOVA, with a Dunnett's post-hoc test on SAS 9.3 Enterprise (Cary, NC). Bisphenol concentrations below the LOD/LOQ were represented as LOD/SQRT (2) and used in analyses. All graphs and tables were generated using Microsoft Excel 2010 and GraphPad Prism 7.

RESULTS

Half-life determination of bisphenols in the pregnant and lactating dam. The concentrations of BPA, BPAF, and BPS in maternal serum over two days during late gestation is illustrated in Figure 2-1. Within 12 hr of exposure, there was a rapid decline of maternal bisphenol serum concentration for each chemical and by 24 hr baseline levels were achieved for BPAF and BPS, while BPA achieved baseline levels at ~48 hr. BPAF and BPS serum levels peaked within 4 hr of exposure, compared to the earlier peak in BPA dams at 2 hr. Similarly, maximal urinary (Figure 2-2) and fecal (Figure 2-3) concentrations (C_{max}) for BPAF and BPS were achieved by 4 hr. Similar to the serum results, urinary BPA concentrations peaked at 2 hr. BPA, BPAF, and BPS all distributed into the amniotic fluid (Figure 2-4), but unlike the short

time to maximal concentration (T_{max}) in the excreta and serum, all chemicals demonstrated a T_{max} of approximately 18 hr in amniotic fluid.

The concentrations of each chemical were used to calculate the pharmacokinetic parameters found in Table 2-1. The mean serum $t_{1/2}$ of BPA, BPAF and BPS were 5.5, 4.5, and 7.0 hr., respectively and the extrapolated AUC_{0-1} and $AUC_{0-\infty}$ did not vary significantly by chemical. BPA administered levels were 10x greater than the other two phenols, and as expected, measured serum levels for both BPAF and BPS were approximately 6-10x lower in comparison to BPA serum levels. Urinary samples had similar $t_{1/2}$ as the serum for all three chemicals. Interestingly, BPS recovery was ~30x greater than BPAF, although both groups were administered the same dose (5 mg/kg). In contrast, BPAF recovery was ~3x greater in the feces compared to BPS although the administered dose, T_{max} and $t_{1/2}$ were very similar. Although the C_{max} for all three chemicals in the amniotic fluid was achieved ~18 hr post exposure, recovery levels of BPAF remained close to baseline within the final 30 hr of collection (2.1-24.7 ng/ml), compared to BPA (30.2-858 ng/ml) and BPS levels (2.9-357 ng/ml), and thus the $t_{1/2}$ was unable to be calculated. BPS was detected in the amniotic fluid at levels 10x greater than BPAF although the administered doses were equal.

Serum from dams was also collected between 3 and 5 days post-partum to assess whether internal dose was still measurable 5-7 d post-exposure. Figure 2-5 illustrates that dams had measurable BPA levels that decreased in a linear fashion from PND 3 to 5. A slight increase in serum BPA recovery was observed from GD 18 to PND 3, but the difference could have been due to maternal behaviors or variability between animals. Apart from PND 4, postnatal serum BPAF levels were ≤ LOD and although detectable, serum BPS levels were also significantly lower than GD 18 levels.

Serum concentrations of bisphenols from both male and female offspring were also measured on PND 3-5 to determine if chemical metabolizing differences existed due to sex (Table 2-2), which may result in higher measurable levels of one sex vs. the other. As previously noted, we attempted to collect pup urine, but it was difficult to obtain due to maternal grooming. Similar to dam serum BPA levels, offspring serum BPA levels were greater (≥ 10x) compared to BPAF and BPS likely due to the administered dose being 10x higher. The serum levels between BPAF and BPS offspring were very similar to one another. When dam serum levels from PND 3-5 were compared to pup serum levels (data not shown), regardless of sex, BPA and BPS dam serum levels ranged from 1.5-4.4-fold and 2.2-7.7-fold higher than pups, respectively. The range of BPAF serum fractions could not be calculated due to serum levels that were < LOD for either dam or offspring. Overall, dam serum levels exceeded pup levels by 2-8 fold and pup sex was not a significant modifier of neonatal serum concentrations for any of the three chemicals.

Early developmental outcomes. Because the determined serum and urinary half-life was short for the three bisphenols tested (Table 2-1), it was decided that twice daily dosing approximately 7 hours apart during daylight hours would prolong exposures. In addition to the single bisphenol concentrations administered in the disposition studies, concentrations that were 10 and100x lower were also assessed to determine potential health-related changes across a greater and more human relevant dose range. Following a late gestational exposure (GD 10-17) to vehicle, BPA, BPAF or BPS, maternal and offspring outcomes were measured. All treated dams gained a similar amount of weight during gestation, regardless of dose, when compared to vehicle (Figure 2-6). Approximately 50% of the vehicle dams gave birth on the expected day of parturition (GD18) compared to BPA (43-60%), BPAF (67-71%) and BPS (67-85%) (*data not shown*). On average, litter sizes for controls and all treatment groups ranged from 11-13 pups, a

size that is very common for CD-1 litters (Table 2-3). In the vehicle control group, the number of male offspring per litter was slightly greater (7.7 ± 0.8) compared to the treated groups, but statistical differences were not observed. Female body weights and counts per litter, and sex ratio were all unchanged with the exception of the BPS 0.5 mg/kg group that had significantly greater number of females compared to vehicle.

DISCUSSION

In this study, pregnant CD-1 dams were exposed to BPA, BPAF, or BPS to determine the disposition and pharmacokinetic half lives in a pregnant rodent model. As a result, we were able to establish the internal dosimetry within the dam and her offspring. All chemicals were rapidly absorbed and measurable in serum, excreta, and amniotic fluid within 2 hr post administration. The half-lives of BPA, BPAF, and BPS in serum, urine and feces were all < 24 hr and similar observations have been made in non-pregnant rodents and humans [38, 39]. This study is the first to confirm placental transfer of BPAF and BPS as both chemicals were recovered in the amniotic fluid, demonstrating the likelihood of fetal exposure. Measurement of the bisphenols in the offspring serum confirmed maternal to fetal transfer that was similar between male and females, suggesting that sex-specific differences in metabolism are negligible during this early time of development. Extended dosing studies using the same concentrations revealed that BPA, BPAF, and BPS were neither materno- nor fetotoxic as there were no changes observed in gestational weight gain for the dam, length of gestation, pup birth weights, postnatal mortality, or total litter size.

Following a late gestation single oral exposure to BPA, BPAF, or BPS, each chemical was quickly absorbed and detected at its peak concentration in the maternal serum within 2-4 hr. Previous studies that examined the disposition of BPA in pregnant mice (10 mg/kg) and rats (1g/kg) found that following a single administration of BPA maternal blood levels peaked at 15 and 20 min., respectively, continuously decreasing as time progressed [30, 40]. Therefore, it is quite possible that the C_{max} and T_{max} may have occurred within the first hour following BPA exposure in our study. BPA concentrations steadily declined after the 2 hr collection; however, both BPAF and BPS serum concentrations peaked at 4 hr and then began to decline. Therefore, we are confident that our BPAF and BPS $T_{max,}$ $C_{max\ and}$ $t_{1/2}$ findings are representative of the absorption/excretion of these chemicals. As expected, BPA concentration in the serum was greater than that of the other bisphenols tested due to the higher administered dose, and BPAF and BPS recovery differed by 6-10 fold in serum from BPA, suggesting that their structural differences had little influence on circulating blood levels.

A comparison of $t_{1/2}$ in the pregnant mouse model revealed that regardless of the chemical to which dams were exposed, or the matrix from which the $t_{1/2}$ was estimated, their $t_{1/2}$ was less than 24 hr; confirming a previous finding for BPA in non-pregnant rodents [38]. Bisphenols continued to decrease in the dam in a linear fashion into the postnatal period and were below the LOD in most BPAF samples at PND 3. Maternal BPS serum levels between PND 3 and 5 were all < 1 ng/ml, which is roughly 20-100 fold higher than pre-and post-shift serum levels (0.010-0.100 ng/ml) in cashiers exposed to receipts containing BPS [7].

The effects of BPA in humans remain a controversy because most BPA is thought to be rapidly metabolized and excreted, resulting in low levels of free circulating BPA that are not expected to cause any damage. Following absorption, BPA is rapidly glucuronidated, which

decreases its estrogenic activity and, depending on the species, is eliminated through feces via biliary excretion or the urine. It may also be sulfated and hydroxylated by cytochrome P450s, but to a lesser extent. Therefore, it is thought that the rapid metabolism and efficient removal of BPA negates its harmful effects in tissues. More recently, Li et al. [27] identified four BPAF urinary metabolites (BPAF-diglucuronide, -glucuronide, -glucuronide dehydrate, and -sulfate) following a single dose administration in non-pregnant Sprague Dawley rats. Similarly, BPS-glucuronide was found to be the predominant metabolite following co-incubation of the parent compound with NADPH and UDGPA [16]. These data indicate that all three analogues are likely eliminated following glucuronidation, although it is not known if that form is active or not. In fact, recent studies in mouse 3T3-L1 cells have shown that BPA-glucuronide is indeed active, stimulating adipogenesis of these cells in a dose responsive manner [41].

In our study, BPAF recovery in the urine was ~30 fold lower compared to BPS recovery, even though both groups were administered the same dose. Although overall fecal bisphenol recovery was lower compared to other maternal matrices, BPAF concentrations were 30-fold higher than BPS in this matrix. We hypothesize that low recovery in the feces may be a result of distribution to other tissues and will require additional testing. This data does not support the findings by Waidyanatha et al. [26], who demonstrated that cumulative excretion of BPAF regardless of sex, dose and administration was predominant in the feces of non-pregnant Sprague Dawley rats and B6C3F_1/N mice. However, it should be emphasized that because these animals were not placed in metabolic cages we were unable to perform cumulative collections of urine and feces and therefore were unable to calculate the cumulative excreta to determine the predominant route of excretion. If feces is the main route of excretion for BPAF this may be an indicator of why urine samples from a human population [12] collected between 2000 and 2014,

had BPAF levels that were below the limit of detection. We cannot simply assume that because samples from all collection points had non-detectable levels of BPAF, that those individuals were not exposed to BPAF, as the animals in our study certainly were exposed and we could not measure BPAF a few days later. It may simply suggest that BPAF has undergone rapid urinary elimination or that BPAF is predominantly excreted via feces and is being measured in an imperfect matrix. Since we measured lower total BPAF in the matrices collected compared to BPS, this may also suggest the alternative possibility that BPAF is being distributed to more lipophilic tissues such as the mammary gland or adipose tissues since it has been recovered from human adipose tissues [42].

This study is the first to our knowledge to confirm that BPAF and BPS undergo maternal-fetal transfer. In this study, each bisphenol chemical was detected in the amniotic fluid as early as 2 hr post exposure although it required an additional 16 hrs. to reach T_{max}. BPAF recovery was the lowest (20.2 ± 2.5 ng/ml) at its peak concentration, compared to BPS (277.3 ± 41.4 ng/ml) and BPA (735.3 ± 70.4 ng/ml). A potential explanation for this may be retention in the fetal tissues (and less excreted to amniotic fluid) or higher levels of BP analogues (free form) in the umbilical cord sera compared to amniotic fluid, since we did not observe a difference in dam: fetal serum between sexes [43]. BPA was also found to be more readily recovered in second trimester patients compared to third trimester, the latter of which is comparable to the gestational time point for this study [43]. Although fetal tissues were not assessed (no reliable analytical method), several studies involving pregnant rodents (mice and rats) and oral BPA exposure have shown that whole fetuses achieved maximal concentrations within the first 20 min (slower than maternal serum/urine) following maternal exposure and that their internal dose rapidly decreased in a manner similar to the maternal samples [29, 30]. This may not be the case for BPAF, as

suggested by our recovery data. Regardless of absolute levels of recovered bisphenols, these data demonstrate that the placenta does not act as a barrier to BPAF or BPS, similar to data for BPA.

Metabolic differences between males and females may play a role in the severity or lack of observed effects in some studies. In addition to an *in utero* exposure in our study, offspring were also exposed to any residual bisphenols through lactation. Serum from neonates of both sexes was measured between PND 3 and 5 to determine if sex differences existed. A single *in utero* exposure to the three chemicals did not result in any differences between male or female serum measurements. The lack of serum differences between sexes suggest that the CD-1 mouse model can serve as an appropriate model for determining the effects of BPAF and BPS for future studies involving additional time-points and for various collection types. Recent findings in C57Bl/6 neonates revealed low basal UGT expression between early gestation and PND 21 [44]. Based on these findings we anticipated that the metabolizing enzymes needed for bisphenol glucuronidation are not active in the CD-1 neonate, however, to confirm this it will be necessary to measure those enzymes in our model system. During gestation, compounds with varying chemical properties can traverse the placenta. In most cases an enzymatic conjugation reaction to make the compound more hydrophilic for rapid elimination occurs, however, the chemical may be deconjugated back to its parent compound or may accumulate within the fetus and neonate due to low metabolizing activity.

Yabusaki et al. [45] showed that UGT activity in perinatal liver microsomes following BPA and BPAF exposure were similar between adult males and females for each chemical, but overall activity was greater in BPA-exposed offspring compared to BPAF-exposed offspring. They also showed that the two predominant UGT isoforms (2B1 and 1A6) in C57Bl/6 offspring had relatively low expression and that was similar between males and females between GD 18.5

to PND 3. In our study, we attempted milk sample collections, but they were difficult to obtain in the volume needed for MS/MS. But, the fact remains that by PND 3 both BPAF and BPS in serum were below the LOD or close to baseline, so either any residual chemical that is being re-introduced by lactation is being rapidly eliminated or lactational transfer of bisphenols is negligible at this point. Total bisphenol A has been reported in the milk of lactating North Carolina women, and was typically within 3-fold of the LOD (0.3 ng/ml) for milk, and milk levels were 10-fold lower than urine concentrations [46].

Fetal or maternal toxicity were not observed with the doses chosen from the disposition study and were therefore used as the highest dose in our developmental study, along with the addition of several lower concentrations within a dose range of 100-fold. When these animals were orally dosed twice daily for 8 days, all dams exhibited a normal gestation period of 18 days and had similar weight gain during pregnancy. Altered weight gain in offspring has been reported in rats and mice following much higher levels of BPA exposure [47, 48]. No significant effects of bisphenol treatment were detected on litter size at birth or on PND 1 female and male body weights. However, sex ratio was statistically higher for females in the 0.5 mg/kg BPS group. Pre-weaning pup survival was also unchanged, like studies involving lower doses [48]. Taken together, these data imply that fetal and maternal toxicity are not occurring at the doses of bisphenols used in this study, however, it does not rule out the potential for other endocrine related effects to occur during later development.

Although BPA has been removed from certain consumer products, the use of BPAF or BPS as replacements, or in novel applications, may still pose an environmental and human health risk. BPA disposition studies have previously been performed for multiple species and strains as well as administration routes [38, 49-52]. The increased presence of other bisphenol analogues,

including BPAF and BPS also require an understanding of their dispositions to determine if they may pose many of the same risks that have been associated with exposure to BPA. This is especially concerning for populations that may be at greater risks for the potential effects, including infants and children during their formative years. Few studies have assessed the pharmacokinetics of these chemicals, so it makes understanding the pharmacodynamics much more difficult. For some endpoints, such as fetal and tissue measurements, we lacked protocols for the mass spectrophotometer.

While additional human data is needed to understand if we are testing within a human relevant dose range, we were able to determine that the maternal disposition for BPAF and BPS are very similar to BPA with the exception of the predominant route of elimination. This in turn helped to establish the proper dosing regimen for future reproductive and developmental studies. The primary route of BPAF and BPS fetal exposure is likely through placental transfer and metabolizing capabilities during early development are similar between males and females for this strain. The changes in expression of metabolizing genes may become disproportionate later in development, affecting the appropriateness of this model for all applications; however, these studies were not designed to address that hypothesis and should be investigated in future studies. Overall, these studies will help to pave the direction for future studies and human risk assessment.

	BPA (50 mg/kg)	BPAF (5 mg/kg)	BPS (5 mg/kg)
Serum			
AUC_{0-1} (mg*hour/L)	136.0	111.3	184.4
$AUC_{0-\infty}$ (mg*hour/L)	136.2	111.4	186.4
T_{max} (hours)	2	4	4
C_{max} (ng/ml)	1090.0 ± 270.0	108.3 ± 103.6	179.7 ± 78.3
$t_{1/2}$ (hours)	5.5	4.5	7.0
Urine			
T_{max} (hours)	2	4	4
C_{max} (ng/ml)	136300.0 ± 91304.2	9320.0	29800 ± 14268.9
$t_{1/2}$ (hours)	5.3	4.7	8.2
Amniotic Fluid			
T_{max} (hours)	18	18	18
C_{max} (ng/ml)	735.3 ± 70.4	20.2 ± 2.5	277.3 ± 41.4
$t_{1/2}$ (hours)	7.6	n.d.	6.9
Feces			
T_{max} (hours)	n.p.	4	4
C_{max} (ng/ml)	n.p.	334.2 ± 78.7	10.1 ± 2.0
$t_{1/2}$ (hours)	n.p.	6.0	6.3

Table 2-1: Toxicokinetic parameters of BPA, BPAF, and BPS in maternal serum, urine, feces and amniotic fluid. AUC (area under the curve), T_{max} (time and maximal concentration), C_{max} (maximal concentration), $t_{1/2}$ (half-life), n.d. (not determined), n.p. (no protocol was available at the time of analysis). Mean ± SD.

	BPA (ng/ml)		BPAF(ng/ml)		BPS (ng/ml)	
Age	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
PND 3	3.70 ± 1.18 (3)	3.33 ± 0.93 (3)	<LOQ (3)	0.08 ± 0.02 (3)	0.11 ± 0.07 (4)	0.15 ± 0.13 (3)
PND 4	1.12 ± 0.15 (4)	2.17 ± 1.67 (3)	<LOQ (3)	0.15 ± 0.16 (4)	0.10 ± 0.06 (4)	0.08 ± 0.01 (4)
PND 5	1.10 ± 0.56 (3)	1.10 ± 0.61 (3)	0.13 ± 0.11 (4)	0.26 ± 0.22 (4)	0.12 ± 0.05 (4)	0.12 ± 0.06 (4)

Table 2-2: Total bisphenol serum concentrations in female and male offspring following a single in utero exposure on GD16. Mean ± SD.

	Male		Female		Total Litter Size	Sex Ratio (% female)
	Body Weight (g)	**# per litter**	**Body Weight (g)**	**# per litter**		
Vehicle	1.46 ± 0.04	7.7 ± 0.8	1.35 ± 0.03	5.0 ± 0.7	12.75 ± 0.69	39.5 ± 4.9
BPA						
0.5 mg/kg	1.50 ± 0.04	6.8 ± 0.6	1.47 ± 0.09	5.4 ± 0.5	12.23 ± 0.51	44.4 ± 4.0
5 mg/kg	1.59 ± 0.06	5.7 ± 0.7	1.51 ± 0.05	5.7 ± 0.7	11.33 ± 0.86	49.8 ± 4.6
50 mg/kg	1.61 ± 0.03	6.4 ± 0.5	1.50 ± 0.03	5.4 ± 0.4	11.91 ± 0.69	37.3 ± 0.3
BPAF						
0.05 mg/kg	1.48 ± 0.05	6.4 ± 0.4	1.45 ± 0.06	6.4 ± 0.7	12.80 ± 0.76	49.1 ± 3.7
0.5 mg/kg	1.55 ± 0.06	6.7 ± 0.6	1.45 ± 0.05	6.2 ± 0.7	12.91 ± 0.55	47.4 ± 4.7
5 mg/kg	1.55± 0.07	4.8 ± 0.7	1.45 ± 0.07	6.2 ± 1.0	11.91 ± 1.09	55.1 ± 5.7
BPS						
0.05 mg/kg	1.53 ± 0.06	6.4 ± 0.5	1.48 ± 0.06	5.1 ± 0.4	11.50 ± 0.73	44.1 ± 2.5
0.5 mg/kg	1.47 ± 0.06	5.0 ± 0.8	1.42 ± 0.04	6.7 ± 0.8	11.70 ± 0.68	59.0 ± 5.1*
5 mg/kg	1.44 ± 0.03	6.2 ± 0.5	1.41 ± 0.04	5.8 ± 0.6	12.00 ± 0.85	48.1 ± 2.8

Table 2-3: PND 1 pup weights and litter outcomes following BP analogue *in utero* exposure. Vehicle (n= 4), BPA 0.5 (n = 3), BPA 5 (n = 4), BPA 50 (n = 3), BPAF 0.05 (n = 6), BPAF 0.5 (n = 5), BPAF 5 (n = 6), BPS 0.05 (n = 4), BPS 0.5 (n = 4) and BPS 5 (n = 5). Data are represented as mean ± SEM.

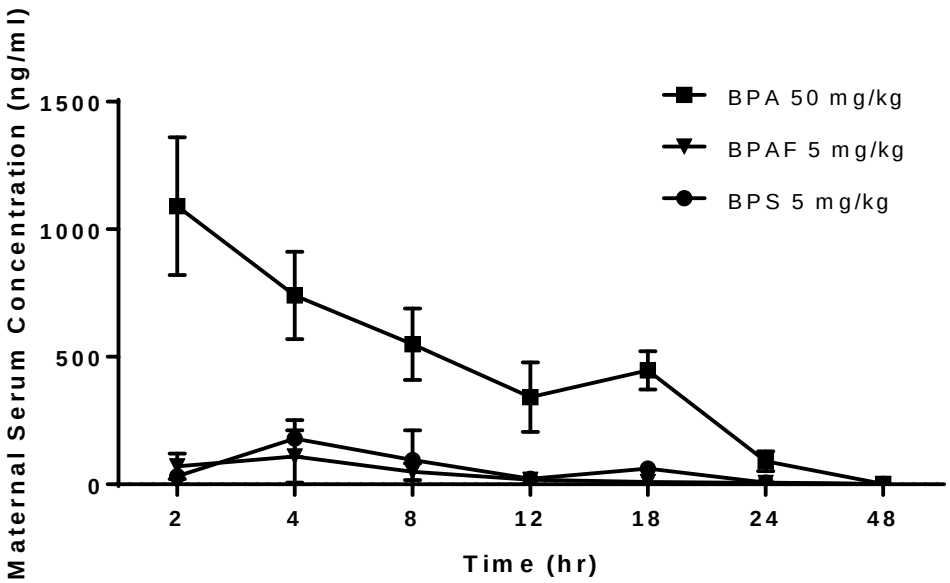


Figure 2-1: Time course of BPA, BPAF and BPS in maternal serum following a single oral administration to pregnant CD-1 dams. Mean ± SD for BPA (n = 3), BPAF (n = 3) and BPS (n = 3).

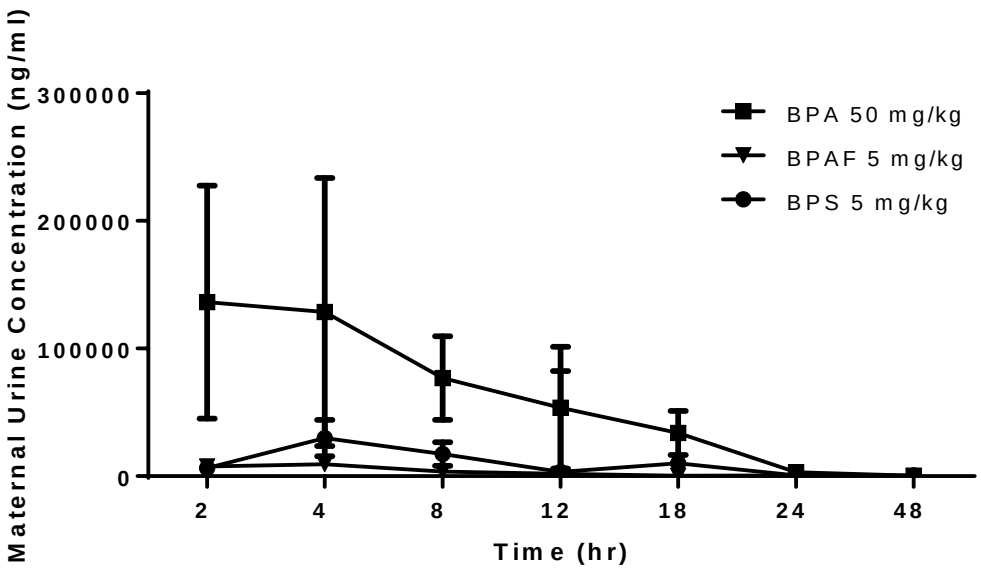


Figure 2-2: Gestational maternal urinary concentration following a one-time exposure to bisphenol analogues (2-48 hr) Mean ± SD for BPA (n = 2-3), BPAF (n = 1-3) and BPS (n = 3).

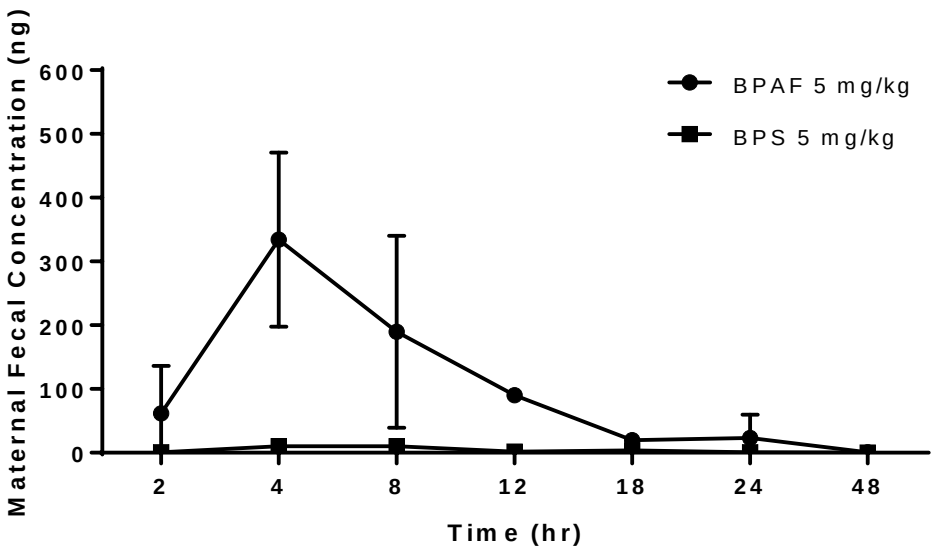


Figure 2-3: Maternal fecal concentration following a one-time exposure to bisphenol analogues (2-48 hr). Mean ± SD for BPAF (n = 3) and BPS (n = 3).

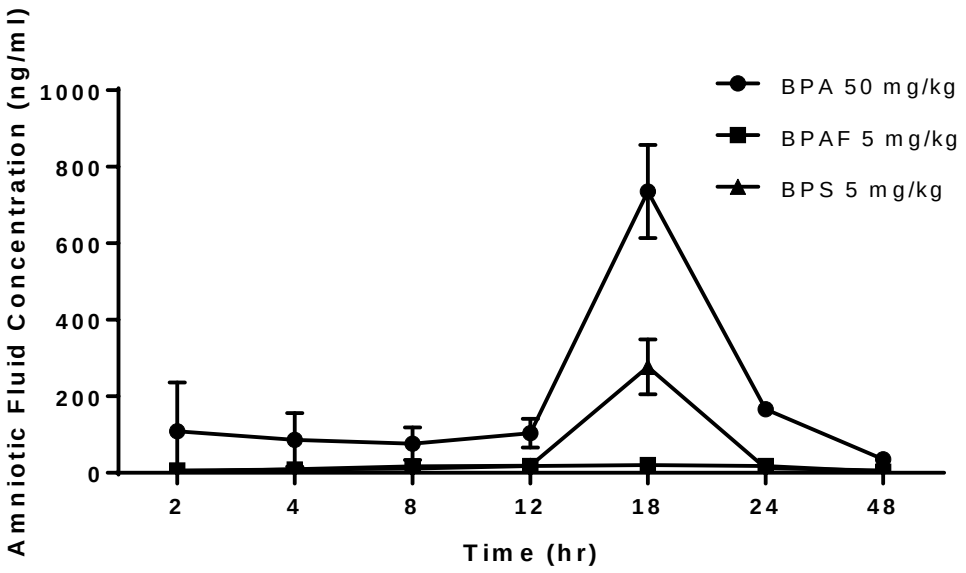


Figure 2-4: Amniotic fluid concentration following a one-time exposure to bisphenol analogues (2-48 hr). Mean ± SD for BPA (n = 3), BPAF (n = 2-3) and BPS (n = 3).

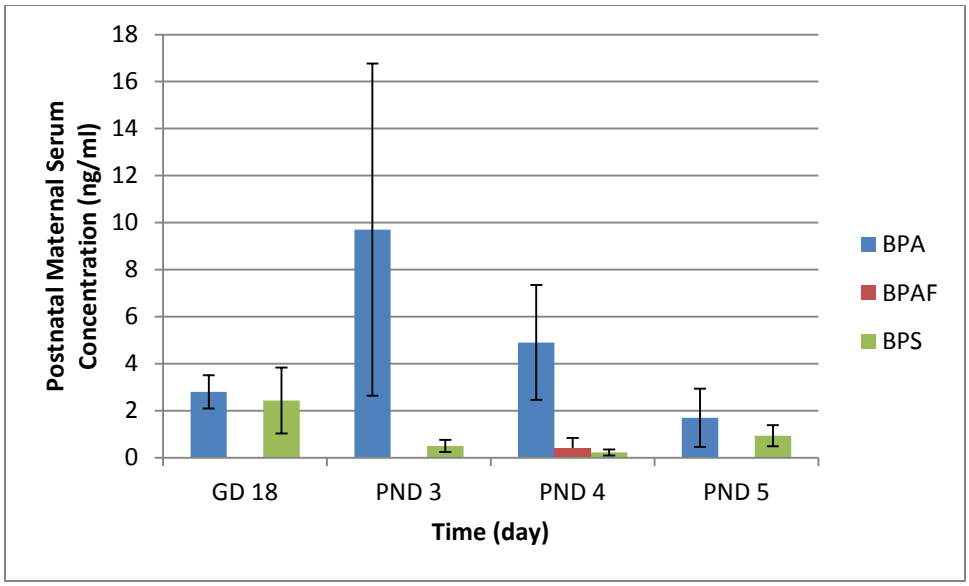
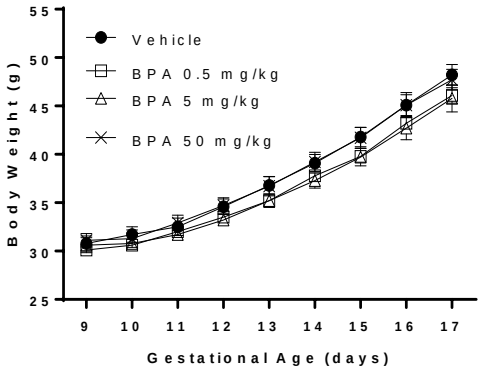
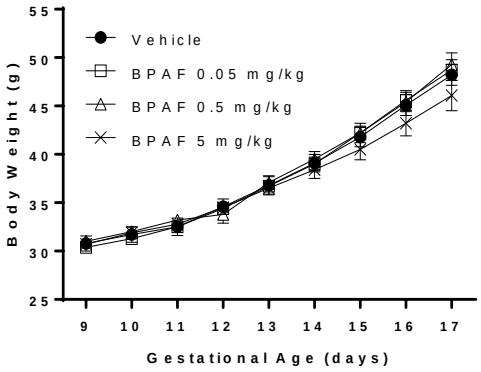


Figure 2-5: Postnatal maternal serum concentration following a onetime exposure to BP analogues. Gestational day 18 (GD 18) was added as a reference for comparison. Postnatal day (PND). Mean ± SD for BPA (n = 3), BPAF (n = 3) and BPS (n = 3).

A)



B)



C)

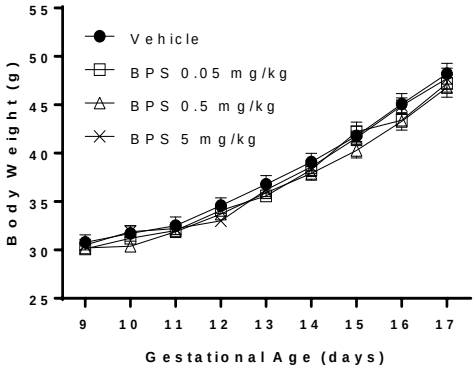


Figure 2-6: Maternal body weight gain during gestational BPA, BPAF, or BPS exposure. A) BPA, B) BPAF, and C) BPS. Vehicle (n = 13), BPA 0.5 (n = 13), BPA 5 (n = 12), BPA 50 (n = 13), BPAF 0.05 (n = 10), BPAF 0.5 (n = 12), BPAF 5 (n = 12), BPS 0.05 (n = 12), BPS 0.5 (n = 11), and BPS 5 (n = 13). Data are represented as mean ± SEM.

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CHAPTER 3

Precocious development, ductal hyperplasia, and mammary tumors in CD-1 mice following prenatal exposure to bisphenol analogues

OVERVIEW

The continued efforts to phase out Bisphenol A (BPA) from consumer products have been met with the challenges of finding safer alternatives. Several replacement analogs have been implemented including Bisphenol AF (BPAF) and Bisphenol S (BPS); however, both possess estrogenic characteristics higher than or similar to BPA and may equally pose a risk to the developing mammary gland, including an increased susceptibility to developing later life disease. This study aimed to determine whether early life exposure to BPA and alternatives could affect female pubertal mammary gland development and produce effects that would persist into adulthood. Timed pregnant CD-1 mice were exposed to vehicle, BPA (0.5, 5, 50 mg/kg), or BPAF (0.05, 0.5, 5 mg/kg) or BPS (0.05, 0.5, 5 mg/kg) via oral gavage between gestational days 10-17. Mammary glands were collected from female pups at PND 20, 28, 35 and 56, and at 3, 8, and 14 months for whole mount, histo-pathological evaluation, and qPCR; serum steroid levels were also measured at these same time points. Pubertal assessment included time at vaginal opening, time at first estrus, and mammary gland development. In the bisphenol exposed groups, accelerated mammary gland development was evident during early puberty and prolonged the presence of TEBs during early adulthood (3 months). By late adulthood (8 months and on), bisphenol-exposed female offspring exhibited varied morphology that included hyperplastic and inflammatory mammary lesions within and surrounding the ducts and stroma that were

especially prominent in the BPAF 5 mg/kg and BPS 0.5 mg/kg group. By 14 months, the incidence of epithelial proliferative lesions and inflammation of the mammary gland within these groups was significantly higher than in vehicle controls, with significant dose-related trends in the BPAF-exposed animals. These lesions included perivascular inflammation, lobuloalveolar, tubuloalveolar, and papillary hyperplasia, and squamous metaplasia. There were also a few diagnosed adenocarcinomas, squamous cell carcinoma, histiocytic sarcoma, and carcinomas in the bisphenol-exposed animals. Steroid levels were altered at limited time-points, were non-dose responsiveness and varied by hormone. Adult mammary mRNA levels of *Esr1*, *Pgr*, *Ar*, and *Gper1* genes from the classical estrogen pathway revealed minimal to no changes compared to vehicle control. Altogether, this data suggests that early exposure to BPAF or BPS may shift the window of development to make the mammary gland more susceptible to pre-neoplastic lesions at an incidence greater than that observed in BPA-exposed animals. It also suggests that early life events and/or non-classical estrogen pathways may play a critical role in mediating these phenotypes.

INTRODUCTION

Bisphenol A (BPA) is a chemical commonly used in the manufacturing of plasticizers, epoxy resins, thermal paper, dental sealants, lining of canned foods and, prior to 2012 was a component used in sippy cups and baby bottles. Several rodent studies have linked BPA exposure at human relevant doses to neurobehavioral deficits, reproductive alterations and hepatic tumors [1-6]. In addition, early and pre-pubertal exposures markedly increased epithelial tissue growth, decreased apoptosis and decreased the latency of preneoplastic and neoplastic lesions in the mammary gland [7-10]. There remains a controversial debate surrounding its

relevance to human health, as human exposures are low when measured in urine, and biological activity of BPA metabolites is poorly understood.

Between 2003 and 2004, BPA was detectable in urine samples of >90% of the general population [11, 12]. The 4th National Exposure Report published by NHANES and the CDC reported that the highest urinary geometric mean for total BPA in every age group occurred between 2003 and 2004 compared to samples collected between 2005 and 2010 [13]. During this time, higher urinary concentrations were observed in males compared to females, and in populations of Non-Hispanic blacks compared to Mexican Americans and Non-Hispanic whites. BPA has also been recovered in maternal serum, amniotic fluid, umbilical cords, and fetal cord blood indicating that BPA readily crosses the placenta [14-18]. Distribution into milk in humans and rodents has also been reported [18-21]. Exposure to BPA during the fetal, perinatal or the early formative years are of great concern because of BPA's weak estrogenic activity and known endocrine disrupting properties.

In 2010 the World Health Organization estimated that between the ages of 0-6 months breastfed infants were exposed to 0.3 µg/kg body weight BPA daily, while infants formula fed with polycarbonate bottles were estimated at 8x breast fed levels, and those given canned formula in polycarbonate free bottles were exposed to 0.5 µg/kg body weight [22]. This suggested that most of the exposure that infants acquired was through plastic bottles. Therefore, several initiatives have been put into place to minimize the use of BPA in certain products. In July 2012, the FDA banned the use of BPA in baby bottles and sippy cups, with an expected phase out date of July 2013 in infant formula packaging [23]. While these first steps were necessary, there was still evidence suggesting that infants and smaller children were exposed through additional routes including through gestation and lactation, inhalation of contaminated

dust or oral exposures through canned foods and beverages often consumed by older children [19, 24, 25]. The combination of FDA use restrictions and voluntary recalls by some manufacturers has led to the increased use of other bisphenol analogues that are similar in structure and activity to BPA.

Bisphenol AF (BPAF) was nominated for health effects assessment by the NTP in 2008 due its estrogenic potential *in vitro*. Activity assays have shown that BPAF (53.4 nM) binds to estrogen receptor alpha (ERα) ~20x greater than BPA (1030 nM) and is an agonist for ERα and full antagonist for ERβ [26, 27]. Its production is considered to be moderate (10,000-500,000 pounds) [28]; however, the six fluorine atoms located at the bridging carbon are likely to make it a persistent chemical and therefore make widespread human exposure inevitable. BPAF has been detected in contaminated air, soil, water and sediment downstream of factories involved in fluoro-elastomer production [29, 30]. Reports estimate that ~4300 occupational workers were exposed to BPAF between 1981and 1983, with one third of the exposed being women [31]. In addition, close to 1430 women in the molding and casting machine operations industry were thought to be exposed. More recently, BPAF was detected in the urine of a Chinese population downstream from a manufacturing plant [32]. The general population may be exposed through products used for dental sealant and composites and foodstuffs [25, 33].

Bisphenol S (BPS), another common BPA analogue, has been measured in thermal receipt papers, currency bills, and canned foodstuff, and is also used as a modifier for leather fiber, polymers, and as an epoxy curing agent [25, 34]. Similar to BPA, BPS was detected in 100% of indoor samples from U.S., China, Japan, and Korea indicating that oral, dermal, and inhalation are all potential routes of exposure [24]. Human exposure to BPS has been confirmed by urinary measurements in the U.S. and seven Asian countries with the highest concentrations

found in Japan, followed by the U.S. [35]. Urine levels from cashiers, an occupation that extensively handles receipt paper, were significantly increased in cashiers post-shift compared to pre-shift [36]. Intermittent low dose exposure to this chemical may pose an increased risk to this population, especially women of childbearing age.

The mammary gland is an essential tissue that is required for lactation and infant nourishment. Similar to other female reproductive tissues, it is influenced by many hormones and growth factors, with the primary drivers depending on the stage of development. Estrogens and progesterone are critical for ductal growth, elongation, branching, and differentiation and exposure to chemicals that can mimic these endogenous chemicals can alter normal mammary development. Similarly, when epithelial tissue from progesterone knockout mice (PRKO) was transplanted into stroma lacking PR, ductal development was stunted, indicating that expression of nuclear receptors in the stroma play a significant role in mammary development [37]. Mammary glands from ERα knockout mice (ERKO) develop rudimentary ductal structures but can grow normally in the presence of wild-type stroma that contains ERα [38, 39]. Studies of prenatal and perinatal exposure to human relevant low doses of BPA in rodents have caused changes in early postnatal mammary gland development that have persisted into adulthood and in some instances shifted the windows of susceptibility to developing preneoplastic and neoplastic mammary lesions [8-10]. While there is insufficient evidence in humans to delineate BPA's role for adversely changing the breast environment, there are a limited number of studies that have linked BPA exposure to breast cancer in humans. Canadian women working in occupational and industrial manufacturers of automotive plastics, food canning, agriculture, and bar-gambling all had elevated breast cancer risk, with the highest risk for premenopausal women working in food canning (OR = 5.70; 95% CI, 1.03-31.5) and automotive plastics (OR = 4.76;

95% CI, 1.58-14.4) [40]. These women were likely exposed to a mixture of bisphenol analogues, among other industrial chemicals. There are several established and emerging breast cancer risk factors in pre-menopausal woman that include increased breast density, lifestyle, menstrual history and exposure to chemicals (i.e. DES, PAHs, and BPA) [41-43].

The most recent NHANES report (2011-2012) has shown an overall decline in the urinary BPA geometric mean concentration in both sexes and all ethnicities and age groups between 2003 and 2010 [44]. Similarly, urinary measurements from the U.S. general population between 2000 and 2014 showed a decline in BPA, but the percentage of samples detecting BPS and the average concentrations detected steadily increased, suggesting a change in exposure trends for BPS and BPA [12]. Although BPAF was below the limit of detection (<LOD) in the majority of the urine samples tested (98.4-100%, 2000-2014), BPAF has been extracted from mammary and abdominal tissue indicating that it may partition to adipose rich tissues, including the mammary gland [45]. To date, no study has evaluated BPAF and BPS effects on the developing mammary gland, nor any latent health repercussions that may be associated with early life exposures. The aim of this study was to determine if fetal exposures to BPAF or BPS at relatively low levels are capable of altering postnatal and adult mammary gland development of mice in a similar fashion to BPA. We report not only altered development of mammary tissue, but endocrine disruption and latent mammary tumor formation, suggesting these analogues are not suitable BPA replacements and may present a risk to the developing child.

MATERIALS AND METHODS

Chemicals and Dosing Solutions

All chemicals, except for Bisphenol AF (3B Pharmachem International Co. Ltd, Wuhan, P.R. China), were obtained from Sigma Aldrich (St. Louis, MO). The purity of all tested chemicals were confirmed by the NTP Chemistry/Chemical Contract and were found to be ≥ 97.5 % (BPS), 98% (BPAF), and ≥99.0 % (BPA) pure. All chemicals arrived in powder form and were placed in a DryKeeper (Sanplatec Corp, Osaka City, Japan) at 25% humidity. Dosing solutions were prepared daily by dissolving in pure sesame oil (Jedwards, International, Inc., Braintree, MA) and stored in clear glass vials with screw on caps to prevent exogenous bisphenol contamination.

Animals

Outbred timed pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC) and received on gestational day 8 (GD 8). All dams were acclimated a minimum of 2 days prior to dosing. Throughout the entire study, animals were maintained at a controlled temperature of 20-24^{o}C with ~40-60% relative humidity and a 12:12 light/dark cycle. Upon arrival, animals were weighed and placed into polypropylene cages containing laboratory bedding (Sani-Chip Hardwood Bedding, PJ Murphy Forest Products, Inc., Montville, NJ) that had been tested and know to be free of estrogen activity [46]. Animals were randomly allocated to treatment groups; maintain equal initial body weight means in each group. Exogenous dietary estrogens were minimized by providing AIN-93G (Harlan Laboratories, Indianapolis, IN) feed and tap water *ad libitum*. All animals were treated humanely and in accordance with the protocols of the National Institute of Environmental Health Sciences Animal Care and Use

Committee. Dams were weighed daily prior to dosing and dosing was based on daily body weight (BW). All chemicals were administered to the animals by a trained technician. The technician was blinded to chemical/dose (color coded) and animals received chemical exposure by concealed allocation. Any dam that produced a litter of < 4 pups total, litters with only males or who appeared unable to care for her litter was excluded from all final analyses. Female pups produced by these dams were followed up for up to 14 months.

Experimental Design

These studies were initially powered to detect changes in pubertal indices, such as vaginal opening and mammary development in female pups. Earlier disposition studies in the pregnant CD-1 dam revealed that the bisphenol serum half-lives ranged from 4.5 – 7.0 hours and therefore to ensure that chemical steady state was reached; animals were gavaged twice a day (Chapter 2). Therefore, beginning on GD 10, approximately when the rudimental epithelial ductal tree begins to form, timed pregnant dams were oral gavaged twice daily with BPA 50 (n = 11), 5 (n = 12), or 0.5 mg/kg bw (n = 13); BPAF 5 (n = 11), 0.5 (n = 11), or 0.05 mg/kg bw (n = 10); BPS 5 (n = 12), 0.5(n = 11) or 0.05 mg/kg bw (n=12); or vehicle control (pure sesame oil, n=12), in dose volumes of 10 µl/g bw until GD 17. Dosing was concluded on GD17 because this strain normally gives birth on GD 18, unlike other mouse strains whose gestation length is < 19 days [47]. The vehicle control group served as the common control group for all three compounds. Non-treatment related experimental conditions were identical across all study groups. The BPA dose range was based on a low observed adverse effect level (LOAEL) of 50 mg BPA/kg, a no observed adverse effect level of 5 mg/kg for BPA, and the reference dose currently set by the EPA, which is 0.05 mg BPA/kg/d [48, 49].

Physiologically relevant BPA levels, lower than the reference dose set by the EPA have resulted in abnormal mammary gland development during critical periods of development in several rodent models; however, there is no data to suggest an effective dose range for either BPAF or BPS. Therefore, a dose range that includes the BPA reference dose concentration (50 µg/kg/bw) was chosen to estimate the response in the mammary gland, considering that several studies suggested estrogenic potential of these two analogues [27]. These levels were also chosen because we anticipate that exposure to these chemicals will be lower compared to BPA exposure.

Parturition occurred on the eve of GD 18 and the next day was considered postnatal day 1 (PND 1). Litters were culled to 10 pups at PND 3; all attempts were made to maintain a minimum 5:5 male to female ratio, however, the maximum number of females was retained to ensure that there were a sufficient number of females for analyses. Female offspring were assessed for pubertal maturation beginning on PND 16 and weaned at PND 21. At PND 20, 28, 35, and 56 (1 per litter) and 3, 8, and 14 months, female offspring (mostly 1, but sometimes 2 per litter) were euthanized by swift decapitation to obtain trunk blood for serum analysis and mammary glands for RNA, whole mount, and/or /histopathological analysis. Although there was not a designated collection between 11 and 13 months or after 14 months of age, several animals became moribund or exhibited estrous cycling irregularities that prohibited collection during the scheduled collections. With the exception of the 14 months collection (at diestrus), all animals at each time point were collected in the estrus stage of their estrous cycle.

Mammary Gland Preparation and Analysis

The 4^{th} and 5^{th} inguinal mammary glands were removed and processed for whole mounts and contralateral glands were fixed in 10% neutral buffered formalin (Fisher Scientific, Fair

Lawn, New Jersey) for histopathology. Fixed glands were embedded in paraffin and cut into 5µm sections for hematoxylin and eosin (H&E) staining or immunohistochemistry. Whole mounts were prepared by flattening glands on a charged slide followed by fixation in Carnoy's solution, staining with carmine alum and defatting in xylene [50]. Qualitative and quantitative assessments were performed at PND 20 and 35, hallmark time-points often assessed in academic and contracted studies (of weaning and puberty). Qualitative developmental scores were assigned separately by two individuals using a scale from 1-4 (1=poor development and 4=best development) [51]. Depending on age and level of development, scores were based on lateral and longitudinal epithelial growth, presence/absence of terminal end buds (TEBs), branching density, budding, and appearance of ductal ends. Glands from each collection time point were separated and analyzed by chemical without knowledge of the chemicals identity or dose. Since the samples were compared to vehicle controls, the assessor was un-blinded to the vehicle control group. All glands were randomly evaluated by trained lab staff. Longitudinal growth, mammary epithelial area (MEA), duct length, and TEB count were quantitatively measured using ImageJ (https://imagej.nih.gov/ij/). Glands were also quantitatively assessed for branching density using ImageJ and the modified Sholl analysis method [52]. A detailed description of this method is illustrated by Stanko et al. [52]. Briefly, images of glandular epithelium are skeletonized, binarized, dilated and used to measure the MEA and longitudinal distance, defined as the most anterior position of the collecting duct to the most distal branch on the gland, using ImageJ. The total number of radial intersections (N) in the MEA was determined by Sholl analysis method. The branching density of the gland was then calculated using the formula N/MEA. TEBs were defined as buds that were ≥ 2x the diameter of its duct and are represented as TEBs/mm^2. All quantitative measurements were performed on the 4^{th} inguinal gland.

Upon examining whole mounts at 3 months, numerous TEBs were noted in some slides, which is unusual for this age of CD-1 mice (S. Fenton, personal communication). Therefore, all whole mounted glands were visualized by light microscopy (Leica Z16 APO, Leica Microsystems, Buffalo Grove, IL), and terminal epithelial duct ends greater than 2X the diameter of the duct were considered a terminal end bud and recorded as an occurrence. Whole mounts from 8 and 14 month old mice were also examined for abnormalities (potential lesions) using light microscopy as described above. Unusual development was recorded. It should be noted that the Sholl method and certain quantitative measurements are unable to be performed on these glands due to extensive growth and tissue density.

Contralateral fixed glands from mice of 3, 8, and 14 months old were embedded in paraffin, cut into 5 µm sections, stained with H&E, visualized on an Olympus BX41 (Olympus Scientific Solutions Americas Corp., Waltham, MA) and digitally captured on an Olympus DP70 camera. A board-certified veterinary pathologist (S.B.H) evaluated all histopathology samples and was blind to treatment.

Neoplastic and non-neoplastic mammary lesions were diagnosed using standardized nomenclature proposed by the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) [53]. On occasions where a definitive diagnosis could not be made, a pathology peer review group was convened to resolve the diagnosis. Microscopic lesions were graded using a standard four-point scale of minimal, mild, moderate, and marked severity grade criteria.

During the latter endpoints (3,8 and 14 months) some mammary gland whole mounts contained gross abnormalities that were often not noted in the single contralateral section

histopathology findings, all mammary gland whole mounts were sectioned for histopathology and assessed by a pathologist to diagnose the abnormality. A detailed description of this method has recently been published [54] (Appendix I). Briefly, mammary whole mounts were immersed in xylene overnight to remove excess Permount (Fisher Scientific), and allow for ease of scraping tissue off of the slide. Glands were halved at the midline, placed in cassettes, and processed in a succession of xylene and xylene: molten paraffin steps before final embedding. Glands were sectioned at 4 µm, stained with H&E and evaluated using the same criteria as the contralateral glands.

Detection and Monitoring of Puberty and Cyclity

Beginning on PND 16, all female offspring were checked daily for vaginal opening (VO), by trained observers, using the methods described in Goldman et al. [55]. Upon opening, cervical lavage using 1X PBS, pH 7.4 (Gibco, Waltham, MA) was conducted to determine the timing of first estrus and their estrous stage at VO. Morning lavage was continued until each animal exhibited estrus. Beginning on PND 24 and thereafter until 5-6 estrous cycles were achieved, soiled bedding from aged matched male control mice was collected into a homogenous mix and dispersed into all female cages to circulate male urinary proteins known to normalize the estrous cycle. From PND 63 until PND 84 the same group of animals was re-assessed for estrous cyclity by examining cervical cytology using the same methods.

Hormone Analysis

Necropsy occurred between 9 and 11 AM and in the same estrous cycle stage (estrus, except for 14 months at diestrus) to minimize effects of hormonal fluctuation. Briefly, following trunk blood collection in a BD Vacutainer SST Plus tube (BD Bioscience, Franklin Lakes, New Jersey), the blood was inverted and stored at room temperature for 30 minutes. Samples were centrifuged at 1100g for 15 minutes, serum collected in a fresh tube, and stored at -80^{o}C until processing.

Serum preparation and analysis were performed per the manufacturer's protocols. Samples were run on a Steroid Hormone Panel Kit from Meso Scale Discovery (MSD) (Rockville, MD) to determine estradiol, progesterone, testosterone, and DHEA concentrations. Samples and standards were added to 96-well plates pre-coated with antibody and incubated for 2 hours at room temperature. A SULFOTAG label tracer was added to each well which generates a signal to determine the analyte concentration. The plates were washed 3x with 1X PBS-T and after final wash, 150 ul of a 1X Read-Buffer added to each well and plates were immediately imaged on a Sector Imager 2400 System (Meso Scale Discovery, Rockville, MD). All samples were run in duplicate, on the same day. Standards for all hormones were supplied in in the Meso Scale Discovery reagent kit, with the exception of testosterone (sample range 0.1ng/ml - 16 ng/ml) that was purchased from Steraloids (Newport, RI).

RNA Preparation

RNA was extracted from frozen mammary tissue by homogenizing the gland with Trizol using Lysing D Matrix tubes (MP Biomedicals, Germany). Samples were homogenized in a MP

Biomedical Fast Prep-24 SG at 6.0 m/sec for 40 s intervals. Samples were homogenized a total of 2-3 times and placed on ice in between each interval. Samples were transferred to a clean tube and centrifuged to remove debris and the lipid layer. Following the manufacturer's protocol, chloroform was added to obtain the organic layer and RNA was precipitated with isopropanol and washed with 75% ethanol. All samples underwent on-column DNAse I digestion using the RNA Mini Kit (Qiaqen, Germany). RNA quantity and integrity were measured on a Nano Drop 2000c and Bioanalyzer. Samples with RNA integrity numbers ≥ 7.9 were used for PCR. One microgram of RNA was reverse transcribed with the High Capacity cDNA Synthesis Kit (Applied Biosystems, Warrington, UK) and amplified with the BioRad Thermocycler. cDNA was mixed with Taqman Universal PCR Master mix, No AmpErase UNG, and Taqman probes shown in Table 3-1 (Roche, Branchburg, NJ). Amplification was performed on a QuantStudio 7 Flex PCR (Applied Biosystems, Foster City, CA) and analyzed using QuantStudio Real-Time PCR Software and Microsoft Excel 2010. Mean Ct values ≥ 35 or with a standard deviation of ≥ 0.5 between duplicates were not included in the final analyses. *Cdkna1* was used as the housekeeping gene for all samples. All analyses were performed using the $2^{-\Delta\Delta Ct}$ method and are illustrated as the fold change relative to vehicle control.

Statistical Analysis

Unless noted, all data are represented as mean ± SEM. The dam was considered the unit of measurement. In nearly all cases only one pup per dam was sampled at any given time point. When multiple pups per dam were evaluated in the same analysis of quantitative endpoints, such as timing of vaginal opening and first estrus, mixed effects analysis of variance (ANOVA) with Dunnett's test was used to account for potential litter effects. TEB occurrence was statistically

evaluated using one-sided Cochran-Armitage trend tests across vehicle control and each compound's dose groups and one-sided Fisher's exact tests comparing the vehicle control group to each dose group. ANOVA with Dunnett's multiple comparisons test was applied to quantitative endpoints for which one pup per litter was assessed. Hormone measurements were log transformed prior to statistical analyses to improve normality. Mean severity scores were calculated for 3, 8 and 14 month lesions whenever applicable and one-sided Fisher's exact tests were performed to compare lesion incidences in each dose group to the vehicle control group. All analyses were performed using SAS 9.3 (Cary, NC). All Graphs and tables were generated using Microsoft Excel and GraphPad Prism and statistical significances were denoted at $p \leq 0.05$.

RESULTS

Female Offspring Body Weight Changes. Body weight was assessed on PND 20, 28, 35, and 56 and at 3, 8, and 14 months for all female offspring prenatally exposed to vehicle, BPA, BPAF, or BPS (Figure 3-1). No differences among groups were observed at PND 20 or 28 but at PND 35 the BPAF 0.5 mg/kg group was significantly smaller compared to controls. By PND 56 average body weights for this group were similar to vehicle control, however, BPS 5 mg/kg females were approximately 6 grams smaller than controls (17%). By 3, 8, and 14 months no significant differences were observed between any exposure group and the vehicle control group.

Pubertal Assessment. All females were assessed for timing of pubertal development (including vaginal opening (VO), first estrus and mammary development). VO occurred between PND 25 and 26 in all groups with no significant differences observed among the groups (Figure 3-2). Similarly, time to first estrus in BPA, BPAF or BPS exposed groups did not differ significantly from the vehicle control group. Estrous cyclity was monitored for 3 consecutive

weeks between PND 63-83. The number of cycles for each exposed group and the vehicle control group were not significantly different (data not shown), so the mean number of days in each estrous stage was compared between the vehicle control and exposed groups (Table 3-2). There were no significant differences between groups for the number of days spent in estrus and in diestrus over that three-week span, these females spent nearly 40% more time in diestrus compared to estrus. On average, animals were observed in proestrus only 5-10% of those days, and The BPA 5 mg/kg group experienced a significant decrease in the number of days spent in proestrus compared to vehicle control group but since the mouse spends such a limited time in proestrus this may not be biologically relevant.

Bisphenol analogues hasten pubertal mammary gland development. Prenatal exposure to BPA, BPAF, or BPS influenced development of the mammary gland in female offspring evaluated on PND 20 (Figure 3-3). Specifically, mammary glands from females in BPAF (0.5 and 5 mg/kg) groups exhibited increased longitudinal growth, branching density, TEB counts, as well as TEB/mm^2. Control glands averaged ~2.3 TEBs per gland on PND 20, while animals in the BPA 5 mg/kg and BPAF 5 mg/kg groups averaged ~10 TEBs. Differences in mammary epithelial branching density differences were also observed between control vs. exposed (BPA 0.5 mg/kg, and BPS 0.05 and 0.5 mg/kg groups). Mammary epithelial area (an indication of lateral and longitudinal growth) in the exposed groups did not differ significantly from the vehicle control group. Qualitative developmental scoring assessments mirrored the quantitative findings. BPA 5.0 mg/kg and BPS 5.0 mg/kg exposed groups had significantly increased developmental scores compared to controls indicating an accelerated phenotype (Table 3-3). On PND 20, all BPAF glands exhibited significantly accelerated development, and number of TEBs and branching points increased in a dose dependent manner (Figure 3-4).

At PND 28, only the BPAF exposed groups demonstrated significantly accelerated development. By PND 35, the BPA 50 mg/kg, BPAF 0.05 and 5 mg/kg, and every BPS exposed group exhibited developmental scores that were significantly increased compared to the vehicle control group. In comparing vehicle control whole mounts to BPS-exposed glands on PND 35, TEBs were apparent in all control glands, however, as BPS exposure dose increased, distance between the 4^{th} and 5^{th} glands decreased until the 4^{th} and 5^{th} glands had grown together (5.0 mg/kg BPS). It was also noted that epithelial growth had either approached or surpassed the lymph node, something that was observed in few control glands (Figure 3-4). When glands were evaluated three weeks later (PND 56), the control and low doses of all treatment groups had caught up to the accelerated pace of development seen in earlier ages, and only BPS 0.5 mg/kg group still exhibited an advanced mammary phenotype.

Prenatal exposure to BPA, BPAF, and BPS leads to latent effects in the mammary gland. Mammary gland whole mounts and histological sections from female offspring were also evaluated between early and late adulthood to determine the occurrence and incidence of mammary lesions following prenatal exposure to BPA, BPAF, or BPS. At 3 months gross lesions were not observed in whole mounts for any chemical. Similarly, the contralateral glands exhibited no microscopic pre-neoplastic lesions. However, TEBs were observed in approximatively 41-75% mammary glands in each of the exposed groups (exception is BPAF 5 mg/kg group, 14%) compared to only 25% of vehicle control glands (Figure 3-5). Differences in TEB occurrence were not statistically significant using yes/no criterion. Those whole mounts containing TEBs were removed from slides and sectioned to determine if the TEBs were typical or exhibited disrupted growth patterns. Most TEB were confirmed to be within normal range, however mixed cell inflammation, was apparent in several of the exposed groups (BPA 50

mg/kg, BPA 5 mg/kg and BPAF 0.05 mg/kg). At the completion of the study ~15% of the mammary glands collected had a single or a combined diagnosis of inflammation with 45% (68/150) of the findings occurring at ≥ 14 months (Table 3-4 and Figure 3-6). By 14 months of age, most cases of inflammation were diagnosed by the presence of perivascular lymphocytes and were observed in all groups including the vehicle control (3 of 65 diagnoses), however, the occurrence was more prevalent in the chemically exposed groups and determined to be statistically significant in the animals from the BPS 0.5 mg/kg and BPAF 5 mg/kg groups.

By 8 months, epithelial proliferative lesions were evident in the exposed groups that were absent from vehicle control animals. One animal in the BPAF 5 mg/kg group presented with ductal squamous metaplasia at 8 months, and by 14 months numerous types of proliferative lesions in the ductal tissue were observed in exposed groups (Table 3-4-3-7 and Figure 3-6), with a significant trend in overall lesion development in the BPAF exposed groups, and significantly higher incidence in the BPAF 5 mg/kg group than in the vehicle control group. Although the BPS-exposed groups a significant trend, the BPS 0.5 mg/kg group developed significantly more proliferative lesions than the vehicle controls. The BPS exposed group had a significantly higher incidence of lobuloalveolar hyperplasia than vehicle controls, whereas the BPAF 5 mg/kg group saw an increased incidence of ductal squamous metaplasia, and a significant trend for that diagnosis. A total of twenty-four exposed animals were found to have lobuloalveolar hyperplasia of the mammary gland between 8 and 16 months (17 from timed necropsies and 7 from moribund animals).

No tumors were detected in any vehicle control group mammary glands at any time up to 14 months (Table 3-5) and spontaneous mammary tumor development in CD-1 mice is rare, especially in the ages necropsied in this study. Histopathology identified tumors as early as 11

months (Table 3-4) in mammary glands of prenatally exposed groups. Although statistical significance was not achieved for any chemical or dose group, potentially because this study was not powered for tumor formation, eight different tumor types were identified in exposed groups. Adenocarcinomas were identified as the reason for early necropsy of two moribund animals in the BPS 5 mg/kg group. The multiple tumor types included squamous cell carcinoma (n=2), papillary carcinoma (n=1), adenocarcinoma (n=2), carcinoma (n=1), and histiocytic sarcoma (n=1), and a benign fibroadenoma (n=1) (Figure 3-7). These lesions were identified between 11 and 16 months.

Serum hormone levels during puberty and late adulthood following prenatal exposure to BPA, BPAF, or BPS. Serum estradiol, progesterone, testosterone and dehydroepiandrosterone (DHEA) were measured on PND 20, 35, 56, and at 3, 8, and 14 months in prenatally exposed female offspring (Figure 3-8a and 3-8b). Prior to VO at PND 20, serum estradiol levels in all BPAF-exposed groups and the high and mid dose BPS-exposed groups were 90-160% higher than vehicle control levels. Progesterone levels were also raised in BPAF 5 mg/kg and BPS 0.05 mg/kg groups prior to VO. Similarly, BPS 0.05 mg/kg and BPA 50 mg/kg groups demonstrated elevated DHEA levels. By PND 28, estradiol, progesterone, and DHEA serum levels in all treated groups were similar to vehicle control levels, with the exception of testosterone which was significantly decreased in BPAF 5 mg/kg and BPS 0.05 mg/kg groups. Interestingly, at PND 35, testosterone levels were still decreased however, not in the same groups as at PND 28. Testosterone levels at PND 35 in the BPA 5.0 mg/kg, BPAF 0.05 mg/kg and BPS 0.5 mg/kg and 5 mg/kg groups were significantly decreased compared to vehicle controls. By PND 56, the only significant differences were in testosterone and DHEA for the BPA 50 mg/kg group. In contrast to increases in early serum levels between treated and control groups, all notable differences

between treated and vehicle control groups in early adulthood were reductions (Figure 3-9). At 3 months, progesterone levels were reduced in BPS 5 mg/kg groups. DHEA was also reduced by 27-42% in at least one dose group for each compound. Estradiol (BPS 0.05 mg/kg), testosterone and DHEA (BPS 0.5 mg/kg) were also reduced at 8 months, but no dose-related trends were evident. Serum hormone measurements from 14-month old animals revealed no significant differnces between vehicle control and treated groups. Overall, there was no trend over time or within a chemical/dose group that were detected for any of the serum measurements even though the females were carefully estrous cycle staged prior to necropsy.

Nuclear receptor expression in BP analogue treated female offspring. Since BPA, BPAF, and BPS are all considered to act as endocrine disruptors, we wanted to test whether an early life exposure could alter nuclear receptor expression in the mammary gland, and potentially pose as a mechanistic underpinning for phenotypic effects. *Esr1*, *Pgr*, *Ar*, and *Gper1* expression was measured in mammary gland tissue by RT-PCR at 8 and 14 months (Figure 3-10). At the 8 month evaluation, *Esr1* expression was significantly decreased in BPA 0.5 mg/kg and 50 mg/kg and BPAF 0.05 mg/kg groups. Exposure to BPA 50 mg/kg group resulted in downregulation of *Ar* whereas exposure to BPAF 0.05 mg/kg and BPS 5 mg/kg reduced *Pgr* expression. No significant differences in steroid receptor expression were observed at 14 months between exposed and vehicle control groups.

DISCUSSION

Altered pubertal mammary gland development and increased susceptibility to tumors following early life exposures to BPA have been illustrated in several rodent studies [7, 8, 56].

Our findings herein suggest that other estrogenically similar bisphenol analogues may target endocrine dependent tissues; specifically, the mammary gland. In these studies, we provide evidence that limited prenatal exposure of female mice to BPA, BPAF, or BPS caused accelerated pubertal mammary development without altering other pubertal indicators (VO, timing to first estrus and cyclity). This is not the first report of the mammary gland being a sensitive pubertal end-point [57, 58] (Appendix II). We observed proliferative effects in the mammary gland that persisted into adulthood and later life, such as significantly increased incidences of inflammation and proliferative epithelial lesions (lobuloalveolar hyperplasia, ductal squamous metaplasia). We also report the development of adenocarcinomas in the BPS 5 mg/kg group that appeared at < 12 months of age.

Compromised mammary gland development before birth may account for the many alterations and manifestations that arise in the gland later in life, especially following endocrine disrupting exposure. This study showed that by PND 20 mammary glands from treated groups had an increased number of TEBs, branching density and TEBs per area of gland; these characteristics occurred in a dose dependent manner and persisted into young adulthood (PND 56; 8 weeks.), and were concomitant with a pre-pubertal rise in serum estradiol levels. There is particular interest in the TEB because they are highly proliferative structures that are prominent early during puberty and contain cells sensitive to the effects of carcinogens and endogenous hormones critical for cell proliferation [59]. Their presence and differentiation are carefully orchestrated through a series of paracrine, endocrine, and autocrine events; however, the shift of these events can alter the timing and longevity of their presence. An increase in TEB counts and TEB/ductal area within one month of birth following low level BPA exposure has been previously observed [5, 8, 56], but we report here the extended presence of TEBs into adulthood

for all three bisphenols tested. In this study, TEBs were still present in 41-75% of mammary glands from nearly every chemically exposed group at 3 months. Although TEBs were still observed in 25% of the vehicle controls and were not considered significantly different from chemically exposed glands, their mere presence over multiple estrous cycles introduces intermittent exposures to endogenous hormones that increases the risk for later life tumor development.

Increasing evidence suggests that the role of the immune system is critical in the manifestation of breast cancer. We noted an increased incidence of inflammatory infiltrates comprised primarily of lymphocytes, plasma cells, and macrophages in the mammary gland of every chemically treated exposed group, with a the suggestion of a dose response trend ($p=0.06$) in the BPAF group, and significant increases compared to vehicle controls in the BPAF 5 mg/kg and BPS 0.5 mg/kg exposed groups. Macrophages play an essential role in the mammary microenvironment during normal development and breast cancer progression. Recently, Fischer et al. [60] reported that following a prenatal exposure to BPA (5 mg/kg), ovariectomized CD-1 female mouse offspring that were given estradiol (300 ng) were shown to have down regulation of chemokines (i.e. Cxcl2, Cxcl4, Cxcl14 and Ccl20), interleukins and interferons (IL1β, IL1rn, IL7R, lrg1 and lfr9), and leukocyte markers (CD45, CD19, Ly6G and FSP1) at 2 months of age. Downregulation of many of these genes reduces the immune systems timely recognition and response for destroying abnormal cells. Similarly, reduction of apoptotic genes allows for uncontrolled cell proliferation and could promote an environment that is tumor friendly. With respect to our data, the increased incidence of inflammation throughout adulthood mammary development that we observed may have been influenced by early changes in the immunomodulatory response. Further studies will be necessary to evaluate this mechanism.

Contrary to these findings, Moral et al. [61] showed up-regulation of immune response genes in the mammary gland after 50 days (5 mg BPA/kg) and 100 days (250 mg BPA/kg) in rats given BPA concentrations 5-50x higher than those used in our study. These changes may be associated with the administered doses. Species differences may also play a role, but we could not rule out compensatory gene changes in response to the infiltrates. The fact that over 90% of the inflammation diagnoses occurred in doses ≤ 5 mg bisphenol/kg in our study may suggest that inflammatory responses are sensitive end points for BPA and the other analogs. It should also be noted that macrophages and other leukocytic infiltrates are necessary during pubertal and adult human breast development for the formation of the first TEBs; macrophages regulate and maintain an immunostimulatory presence throughout the estrous cycle [62, 63]. A potential shift in important immune responses may suggest the reason for the extended presence of the TEBs at 3 months and requires further investigation.

Lobuloalveolar hyperplasia was also observed in every treated group with the exception of BPAF 0.05 mg/kg and BPS 0.05 mg/kg by 14 months. In rodents, lobuloalveolar hyperplasia often resembles the mammary gland from pregnant female rodents or pseudopregnancy and in some but not all cases may lead to an advanced hyperplastic state of the mammary gland. Vandenberg et al. [64] observed increased alveolar buds and lobuloalveolar units as early as 3 months in CD-1 females following a perinatal exposure to BPA (0.25, 2.5 or 25 μg/kg). By 9 months, intraductal hyperplastic lesions or “beaded ducts” were prevalent in multiple animals. BPA also produced lobuloalveolar hyperplasia in PND 90 and 140 mice exposed through a gestational (250 μg/kg) or gestational/lactational (0.25 and 25 μg/kg) exposure [10]. Mammary glands from rat offspring exposed to estradiol from gestation to PND 90 developed these structures, also [65]. Lobuloalveolar hyperplasia is considered a non-neoplastic lesion and there

is no association between these structures and tumor formation to date, but they frequently develop following exposure to hormones and endocrines disruptors. Therefore, we believe there is a link between bisphenol exposure and the development of lobuloalveolar hyperplasia formation in the mammary gland and that prolactin is possibly playing a role. We hypothesize that since exposure to estrogenic xenobiotics caused hyperprolactinemia and lobuloalveolar hyperplasia in female rats [66]. This potential mechanism warrants further investigation.

Several rat studies have examined the effects of early BPA exposure on the formation of preneoplastic and neoplastic lesions [67, 68]. Fetal exposure to low doses of BPA via a different route of administration (Alzet osmotic pumps) than was used in our study produced ductal hyperplasia and carcinoma *in situ* in Wistar-Furth at PND 50 and 95 that were confirmed to be actively proliferating [69]. Similarly, Sprague-Dawley rats developed mammary adenocarcinomas following gestational only and a combination of gestational and lactational exposure to BPA [10]. The few studies shown to induce mammary tumors following BPA exposure in a mouse model have involved a mammary tumor initiator (i.e. DMBA). Keri and Lozado et al. [70] showed that following prenatal exposure to 25 or 250 µg/kg BPA exposed female offspring given DMBA had a significantly higher predisposition for DMBA induced mammary tumors. In our study no animals from BPA dosed dams developed a tumor of any type, and we presume that we used doses that were too high to obtain the same results from those previous studies. However, we demonstrated that BPA tends to increase the rate of development of the mammary gland, with our lowest dose maintaining one of the highest developmental scores within the BPA-exposed group.

When this research began, the only human bisphenol data that existed was on BPA. One report recently published measured serum BPS in cashiers pre- and post-shift [36] but besides

this there are no reports of BPAF and BPS serum measurements from the general human population. Therefore, estimating a dose for human relevance relied on using BPA data, and thereofe our lowest BPAF and BPS dose are at the calculated EPA reference dose for BPA at 50 ug/kg/bw (0.05 mg/kg/bw) daily [71]. An adenocarcinoma was identified as early as 11 months in the BPS high dose, with a second finding a month later in the same group. However, it was the BPS 0.5 mg/kg group which presented with a multitude of tumors likely arising from one or a combination of the many cell types that comprise the mammary gland. The CD-1 strain is an outbred strain that has been shown to develop spontaneous mammary tumors in both control male and females at 18 months or 2 years, however, many of these lesions were not identified until the animals were two years old, with the exception of one analysis where one female developed a mammary adenocarcinoma at 41-45 weeks [72-75]. While it is possible that the two year animals developed tumors that went undetected, the fact that none of our vehicle controls at 3, 8, or 14 months developed a neoplasm may suggest that these tumors may truly be due to early life exposure to the bisphenols.

BPA is considered a weak estrogenic compound and therefore we were interested in determining if there would be changes in serum hormone levels well after prenatal exposure to BPA, BPAF, or BPS. Both BPAF and BPS have been shown to be agonists for ERα and produce proliferative effects in MCF-7 cells [27, 33, 76, 77]. In our study, serum estradiol, progesterone and DHEA levels were increased very early during puberty (PND 20) to complement the simultaneous mammary gland morphological observations. At PND 28 and 35, only testosterone levels were altered and were mostly reductions; by PND 56 increases in estradiol and DHEA were observed in the BPA 50 mg/kg group. By 3 months no dose-related trends were noted, however, all significant hormone changes were noted as reductions. DHEA was reduced in every

group except BPA 50 mg/kg, BPAF 0.05 mg/kg and BPS 0.05 mg/kg and 5 mg/kg groups at 3 months and BPS 0.5 mg/kg at 8 months. In humans and other primates, the adrenals serve as a main contributor to DHEA secretion which can then be converted to both estrogens and androgens in target tissues, including the ovaries [78]. DHEA and other androgens have been implicated as having inhibitory effects against mammary proliferation and since the levels of circulating DHEA tend to decrease between the ages of 20 and 50, there is reason to believe that the incidence of breast cancer and its progression may, in part be due, to this decline [79-81]. *In vitro* studies in the H295R steroidogenesis assay showed that BPA, Bisphenol B (BPB) and 4-cumylphenol increased 17β-estradiol and estrone levels. BPS did not cause any changes to any of these hormones but increased progesterone (744%) and 17α-OH progesterone levels (BPS, 1676% vs. BPS, 22%) compared to BPA and other analogues including Bisphenol F, Bisphenol E and BPB [82].

The mammary gland is composed of various tissue and cell types that are governed by endocrine, autocrine and paracrine signals. Therefore, there is probably not one definitive mechanism of action for BPA and mammary tumor formation and the same may go for BPAF and BPS. ERα is expressed in fetal stromal cells but not epithelium [83]. This suggests that since estrogenic pathways are less influential in the epithelium during fetal mammary gland development that the stroma plays a major in role in the signaling changes seen in the epithelium. It is quite possible that these early life mechanistic changes are driving the altered phenotypes observed at puberty and in later life. Mammary gland gene expression analysis of *Esr1*, *Pgr*, *Ar*, and *Gper1* as well as serum hormone levels showed very few changes at 8 months and no changes at 14 months that would point to a direct classical estrogen mediated mode of action. In a previous study at PND 50, BPA treated animals (250 ug/kg) had no change in protein

expression of ERα, however, PR-A, which is vital for ductal elongation, and the downstream co-regulators SRC-1, SRC-2, and SRC-3 were all significantly increased [67]. Rosenmai et al. [82] also showed that while BPS increased estrogenic activity *in vitro* that it was the least potent and only showed a decreasing trend in activity for the AR assay. These changes are not very surprising since BPA has been shown to have a stronger binding affinity to ERRα and BPAF to ERβ as an antagonist at lower doses [27].

Our data did not indicate that pubertal timing or body weight during pre-puberty and adulthood were altered following early exposure to the three analogues as administered. Similar results were obtained following pre-pubertal exposures to BPA (10 mg/kg) in CD-1 mice [84]. Body weight, vaginal opening and the length of estrous cyclity were all similar to controls. In a similar study, Nah et al. [85] showed that when females were administered BPA (0.1, 1, 10, and 100 mg/kg) during pre-puberty no changes in body weight gain in the two low dose groups occurred, while reductions were seen in the two highest dose groups. Perinatal exposure to drinking water containing 1 mg/L of BPA resulted in increased PND 1 body, perigonadal white adipose tissue and brown fat weights in female but not male Sprague Dawley rats [86]. Several lipogenic and adipogenic genes were also altered in the adipose tissue. Interestingly, all dose groups experienced accelerated VO compared to controls. BPA did not affect weight gain or VO in our animals; however, paracrine signaling is essential for normal mammary gland development. Alterations of these same genes in our chemically exposed mammary glands could suggest a potential mechanism of action. In a study in rats, perinatal exposure to low doses of BPA (0.1 and 1.2 mg/kg) showed increased neonatal body weights in both males and females (PND 4 and 7) but this trend only persisted into adulthood for the lowest dose (females only)[87]. Furthermore, the high dose animals exhibited significant reductions in the number of

estrous cycles and percent of animals with regular cycles at 4 and 6 months. Considering that we observed no changes in the BPA 50 mg/kg group nor the lowest BPAF and BPS dose groups (1/2 concentration of the lowest BPA concentration used in the previous study), we can only suggest that the timing of exposure was played a critical role in the differences observed. The only study that examined the estrous cycle for BPAF showed that concentrations ≥ 30 mg/kg caused cycling irregularities and also reduced body weights in both sexes [88]. Conflicting findings involving BPA urinary levels and precocious pubertal and mammary gland development have also been reported in epidemiological studies [89, 90]. Differences in rodent and human effects suggest that in addition to amount and timing of exposure that there are populations that may be more susceptible to changes induced by these three chemicals.

Our findings suggest that BPAF and BPS use in materials used by women of child-bearing age or infants/children should be reconsidered. The fetal mammary gland is a sensitive target organ for these chemicals. Our data have shown that BPAF and BPS prenatally exposed female animals developed proliferative epithelial lesions by mid-life and a significant inflammatory response that may predispose them to tumor formation later in life. In fact, two animals in the high dose BPS group developed adenocarcinomas prior to one year of life, and triggered a necropsy at 14 months of age in the remaining animals. The majority of neoplasia incidents in this study (7/8) occurred in the BPS exposed animals. Although TEB occurrence did not reach significance compared to vehicle controls, the extended presence of these structures, the significant pre-pubertal spikes in serum estradiol, and the altered immune responses (i.e. increased perivascular inflammation) may play a role in the alterations that were observed later in life. This is the first report of BPAF and BPS effects in the mammary gland and these findings warrant further studies to determine relevance of these findings for human breast cancer

susceptibility. It is important to note that animals from this study were only exposed during the late fetal period when mammary bud formation was taking place. Therefore, our future studies will focus on epigenetic and/or stem cell changes that may have occurred during this critical period, in hopes of informing us of the modes of action involved in these observed later life effects.

Table 3-1: Taqman Gene Primer List used for Mammary Gland Samples

Gene	Identifier	Vendor
Esr1	Mm00433149_m1	Applied Biosystems
Pgr	Mm00435628_m1	Applied Biosystems
Ar	Mm00442688_m1	Applied Biosystems
Gper1	Mm02620446_s1	Applied Biosystems
Cdkna1	Mm04205640_g1	Applied Biosystems

Table 3-2: Days spent in estrus, diestrus and proestrus during PND 63-83 following *in utero* exposure to BPA, BPAF, or BPS

	Estrus (d)	**Diestrus (d)**	**Proestrus (d)**
Vehicle	7.2 ± 4.0	11.7 ± 3.3	2.0 ± 1.0
BPA 0.5 mg/kg	7.6 ± 2.9	11.4 ± 2.7	1.2 ± 1.3
BPA 5 mg/kg	7.7 ± 2.7	11.0 ± 2.4	1.0 ± 0.6*
BPA 50 mg/kg	6.1 ± 2.4	12.1 ± 3.1	1.4 ± 1.2
BPAF 0.05 mg/kg	7.3 ± 2.2	11.2 ± 2.9	1.5 ± 1.2
BPAF 0.5 mg/kg	7.0 ± 2.3	11.7 ± 2.6	1.6 ± 1.0
BPAF 5 mg/kg	5.6 ± 2.4	12.8 ± 1.4	1.4 ± 0.7
BPS 0.05 mg/kg	7.5 ± 3.3	11.2 ± 3.8	1.7 ± 1.0
BPS 0.5 mg/kg	6.0 ± 3.7	12.4 ± 4.0	1.3 ± 1.3
BPS 5 mg/kg	6.7 ± 2.0	11.4 ± 2.2	1.7 ± 1.3

Data are presented as the mean number of days in that stage ± SEM. Dam is the unit of measurement. Litter numbers (n): Vehicle (n = 9), BPA 0.5 (n = 12), BPA 5 (n = 12), BPA 50 (n = 9), BPAF 0.05 (n = 9), BPAF 0.5 (n = 11), BPAF 5 (n = 11), BPS 0.05 (n = 12), BPS 0.5 (n = 11) and BPS (n = 10). Statistically significantly different from the control group by Dunnett's test at *$p < 0.05$;

Table 3-3: Qualitative pubertal mammary gland development scores of female offspring exposed in utero to BPA, BPAF, and BPS

	Vehicle	**BPA**		
		0.5 mg/kg	**5 mg/kg**	**50 mg/kg**
PND 20	2.03 ± 0.07	2.41 ± 0.12	2.79 ± 0.22 ***	2.45 ± 0.23
PND 28	2.33 ± 0.18	3.00 ± 0.25	1.92 ± 0.22	2.50 ± 0.26
PND 35	1.92 ± 0.21	2.50 ± 0.33	2.46 ± 0.22	2.89 ± 0.21*
PND 56	2.39 ± 0.15	3.00 ± 0.12	2.79 ± 0.26	3.11 ± 0.22
		BPAF		
		0.05 mg/kg	**0.5 mg/kg**	**5 mg/kg**
PND 20	-	2.77 ± 0.27**	2.79 ± 0.19***	2.80 ± 0.17***
PND 28	-	2.79 ± 0.33	2.57 ± 0.18	3.25 ± 0.23*
PND 35	-	3.12 ± 0.35*	1.96 ± 0.18	3.05 ± 0.46*
PND 56	-	2.95 ± 0.17	2.78 ± 0.28	2.93 ± 0.37
		BPS		
		0.05 mg/kg	**0.5 mg/kg**	**5 mg/kg**
PND 20	-	2.44 ± 0.14	2.38 ± 0.19	2.69 ±0.24**
PND 28	-	2.75 ± 0.22	2.38 ±0.19	3.04 ± 0.20
PND 35	-	3.21 ± 0.23**	3.00 ± 0.23**	2.88 ± 0.23*
PND 56	-	2.96 ± 0.34	3.22 ± 0.22*	2.61 ± 0.21

Evaluations on postnatal days (PND) 20-56 demonstrate advanced development in bisphenol treated animals. Glands are scored on a scale of 1 (poor development) - 4 (best development). (-) Denotes that there was a common vehicle control group for all chemicals. Data are presented as the mean ± SEM. Dam is the unit of measurement. Litter number= n (1 animal/litter/time point). Vehicle (n = 5-9), BPA (n = 5-10), BPAF (n = 4-10) and BPS (n = 5-10). Statistically significantly different from the control group by Dunnett's test at *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 3-4: Mammary gland diagnosis at time of death following prenatal exposure to BPA, BPAF, or BPS

Time of Death (Weeks)	1-10	11-20**	21-30	31-40*	41-52	Total between 53 and 68 weeks*†	Total between 1-68 weeks
Total number of animals		92	4	55	27	150	328
Inflammation		3		1	6	68	78
Ductal Squamous metaplasia				1		15	16
Lobuloalveolar hyperplasia				1	4	19	24
Miscellaneous (cyst, keratin, hemorrhage)						9	9
Histiocytic Sarcoma						1	1
Fibroadenoma						1	1
Papillary Carcinoma						1	1
Squamous Cell Carcinoma						2	2
Adenocarcinoma/Carcinoma					2	1	3
Total Neoplasia					2	6	8

*Includes animals from scheduled necropsies at 3 months (12 wk), 8 months (32 wk) and 14 months (56 wk).

**Refer to Deaths.xlsx to describe animals that were included but no official diagnosis was made due to the animal dying and autolyzing issues (Appendix III).

†Some animals may had have had multiple diagnoses

Table 3-5: Proliferative epithelial lesions and tumors following prenatal exposure to BPA, BPAF, or BPS

Exposure (mg/kg bw/day)	Incidence		
	Total Lesions	Cancer	Diagnosis (age)
Vehicle	0/27	0/27	None
BPA 0.5	4/34	0/34	Lobuloalveolar hyperplasia (12 and 14 months (3x)
BPA 5	5/31	0/31	Lobuloalveolar hyperplasia (8, 12 (2x) and 14 months (2x))
BPA 50	1/21	0/21	Lobuloalveolar hyperplasia (14 months)
BPAF 0.05	1/33	0/33	Squamous metaplasia (14 months)
BPAF 0.5	3/34	0/34	Lobuloalveolar hyperplasia (14 months) Squamous metaplasia (14 months (2x)
BPAF 5	12/37	1/37	Lobuloalveolar hyperplasia (14 (3x) and 15 months) Squamous metaplasia (8 and 14 months (6x)) Squamous cell carcinoma (14 months)
BPS 0.05	1/24	0/24	Squamous metaplasia
BPS 0.5	15/34	4/34	Carcinoma (14 months) Histiocytic sarcoma (14 months)* Lobuloalveolar hyperplasia (14 months (5x))* Papillary carcinoma/hyperplasia, papillary (14 months) Squamous cell carcinoma (14 months) Squamous metaplasia (14 months (4x))*
BPS 5	6/33	2/33	Adenocarcinoma (11 and 12 months) Fibroadenoma (14 months) Lobuloalveolar hyperplasia (11, 14 and 16 months)

(x) number of animals with diagnosis

*Some animals in the BPS 0.5 mg/kg group had more than one lesion

Table 3-6: Lesion Incidences

	Vehicle		BPA				BPAF				BPS		
3 months	**0**		**0.5**	**5**	**50**		**0.05**	**0.5**	**5**		**0.05**	**0.5**	**5**
Number examined	**8**		**11**	**11**	**9**		**9**	**8**	**7**		**9**	**9**	**9**
Inflammation, mixed cell	0		0	1 [1.0]	1 [1.0]		1 [1.0]	0	0		0	0	0
8 months													
Number examined	**5**		**8**	**5**	**5**		**7**	**5**	**6**		**3**	**5**	**5**
Lobuloalveolar hyperplasia	0		0	1 [1.0]	0		0	0	0		0	0	0
Inflammation, mixed cell	0		0	0	0		0	0	1 [1.0]		0	0	0
Squamous metaplasia, ductal	0		0	0	0		0	0	1 [1.0]		0	0	0
14 months													
Number examined	**13**		**14**	**11**	**6**		**14**	**18**	**22**		**11**	**18**	**13**
Carcinoma	0		0	0	0		0	0	0		0	2	0
Fibroadenoma	0		0	0	0		0	0	0		0	0	1
Histiocytic sarcoma	0		0	0	0		0	0	0		0	1	0
Lipoma	0		0	1	0		0	0	1		0	0	0
Cyst	0		0	0	0		0	1	0		1	0	0
Cyst lined by squamous epithelium	0		0	0	0		0	0	2		0	1	0
Duct dilation	0		0	0	1		0	0	0		0	0	0
Hemorrhage, focally extensive	0		0	0	1		0	0	0		0	0	0
Inflammation, lymphoplasmacytic perivascular	2 [1.0]		5 [1.0]	3 [1.0]	3 [1.7]		7 [1.0]	7 [1.0]	5 [1.0]		2 [1.0]	11* [1.3]	3 [1.0]
Inflammation, mixed cell	1$^{\$\$}$ [1.0]		0	0	0		1 [1.0]	2 [1.0]	8 [1.2]		2 [1.0]	6 [1.3]	2 [1.0]
Inflammation, neutrophilic	0		0	0	0		0	0	1 [1.0]		0	0	0
Inflammation, not specified	0		0	0	0		0	0	1		0	0	0
Keratin	0		0	0	0		1	0	0		0	0	1
Loose keratin	0		0	0	0		0	0	1		0	0	0
Lobuloalveolar hyperplasia	0$^{\$}$		3 [1.0]	2 [2.0]	1 [1.0]		0	1 [1.0]	3 [1.7]		0	5* [1.2]	1 [1.0]
Lymph node: inflammation, neutrophilic	0		0	0	0		0	0	0		0	1 [3.0]	0

*Differs from the control group by Fisher's exact test at $p < 0.05$; ** $p < 0.01$.

$^{\#}$ Significant trend for BPA by the Cochran-Armitage trend test; $^{\#\#}$ $p < 0.01$.

$^{\$}$ Significant trend for BPAF by the Cochran-Armitage trend test; $^{\$\$}$ $p < 0.01$.

$^{\%}$ Significant trend for BPS by the Cochran-Armitage trend test; $^{\%\%}$ $p < 0.01$.

Numbers in brackets, [ ], are the mean severity scores where 1 = minimal, 2 = mild, 3 = moderate.

Table 3-6: Lesion Incidences (cont.)

14 months	Vehicle		BPA				BPAF				BPS		
Lymph node: Inflammation, mixed with eosinophilic crystals	0		0	0	0		0	0	0		0	0	1
Lymph node: squamous cell carcinoma or met from zymbal's gland	0		0	0	0		0	0	1		0	1	0
Lymph node: vascular angiectasis	0		0	0	0		0	0	0		0	1	0
Lymph node: increased cellularity, plasma cells	0		0	0	0		0	0	1		0	0	0
Papillary hyperplasia, multifocal	0		0	0	0		0	0	0		0	1	0
Squamous metaplasia, ductal	0[$$]		0	0	0		1 [1.0]	2 [1.0]	7* [1.4]		1 [2.0]	4 [1.2]	1 [1.0]
Tubuloalveolar hyperplasia	0		0	0	0		0	0	1 [1.0]		0	0	0

*Differs from the control group by Fisher's exact test at $p < 0.05$; ** $p < 0.01$.

[#] Significant trend for BPA by the Cochran-Armitage trend test; [##] $p < 0.01$.

[$] Significant trend for BPAF by the Cochran-Armitage trend test; [$$] $p < 0.01$.

[%] Significant trend for BPS by the Cochran-Armitage trend test; [%%] $p < 0.01$.

Numbers in brackets, [ ], are the mean severity scores where 1 = minimal, 2 = mild, 3 = moderate.

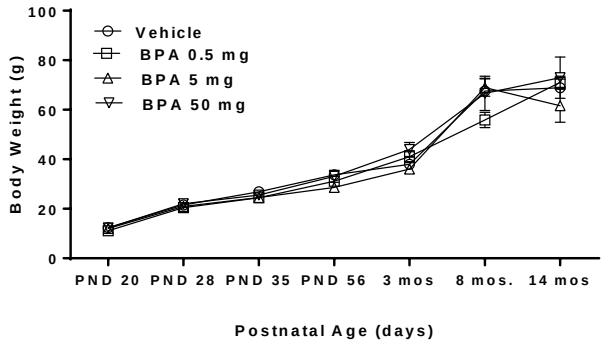
Table 3-7: Combined Incidences (p-values)

	Vehicle	BPA			Vehicle	BPAF			Vehicle	BPS		
14 months	**0**	**0.5**	**5**	**50**	**0**	**0.05**	**0.5**	**5**	**0**	**0.05**	**0.5**	**5**
Number examined	**13**	**14**	**11**	**6**	**13**	**14**	**18**	**22**	**13**	**11**	**18**	**13**
Inflammation, all types	3 (0.156)	5 (0.385)	3 (0.590)	3 (0.257)	3 (0.062)	8 (0.079)	9 (0.126)	14 (0.023)	3 (0.159)	4 (0.395)	15 (0.001)	4 (0.500)
Neoplasias	0	0	0	0	0	0	0	0	0 (0.287)	0 (--)	2 (0.329)	1 (0.500)
Epithelial proliferative effects	0 (0.375)	3 (0.124)	2 (0.199)	1 (0.316)	0 (<0.001)	1 (0.518)	3 (0.182)	10 (0.004)	0 (0.500)	1 (0.458)	7 (0.012)	2 (0.240)
Lymph node, all effects	0	0	0	0	0 (0.076)	0 (--)	0 (--)	1 (0.629)	0 (0.287)	0 (--)	2 (0.329)	1 (0.500)

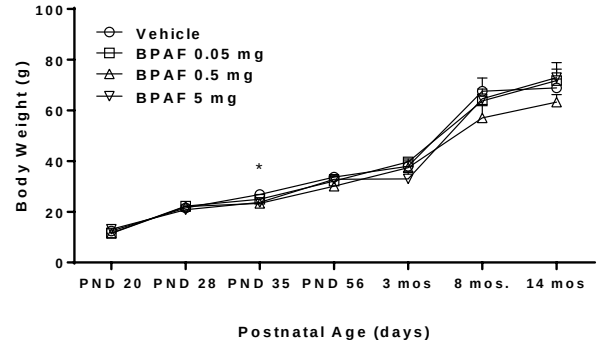
Trend p-values are in parentheses in the Vehicle column; pairwise p-values are in parentheses in the Dose columns. All p-values are one-sided.

(--) indicates that the pairwise p-value cannot be calculated

A)



B)



C)

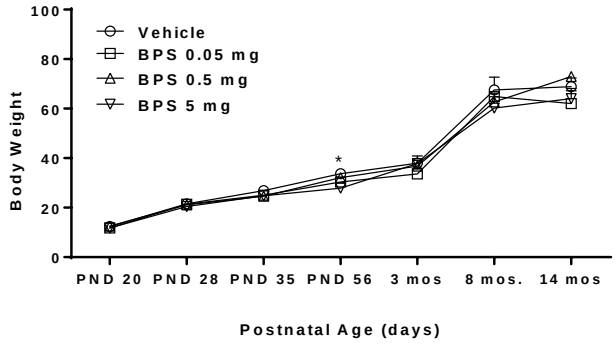


Figure 3-1: BPA, BPAF, and BPS exposures on pubertal and adult weight gain in CD-1 female offspring. $p < 0.05$ is statistically significant compared to vehicle control using ANOVA and a Dunnett's t-test. N=4-10 litters/dose group.

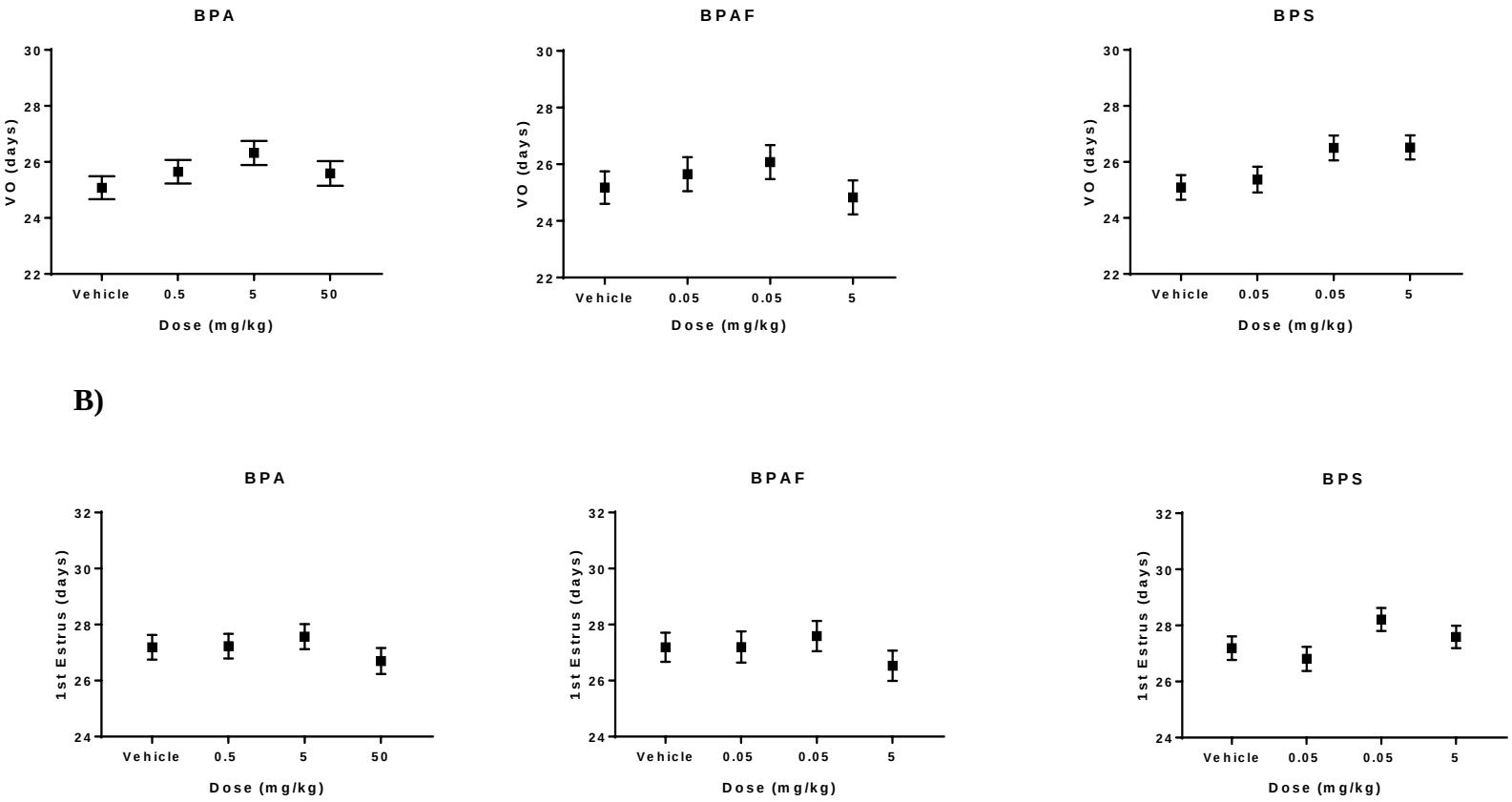


Figure 3-2: Pubertal assessment in CD-1 female offspring prenatally exposed to BPA, BPAF, or BPS**.** Data are reported as mean age with standard error bards (days old) at **A)** Vaginal opening (VO) and B) Occurrence of first estrus for the various doses of bisphenol. Time to VO and first estrus were compared using mixed effects ANOVA, accounting for litter effects. N= 4-10 litters/dose group. No statistical differences were observed when bisphenol exposed animals were compared to vehicle controls using mixed effects ANOVA with a Dunnett's test. N=4-10 litters/dose group.

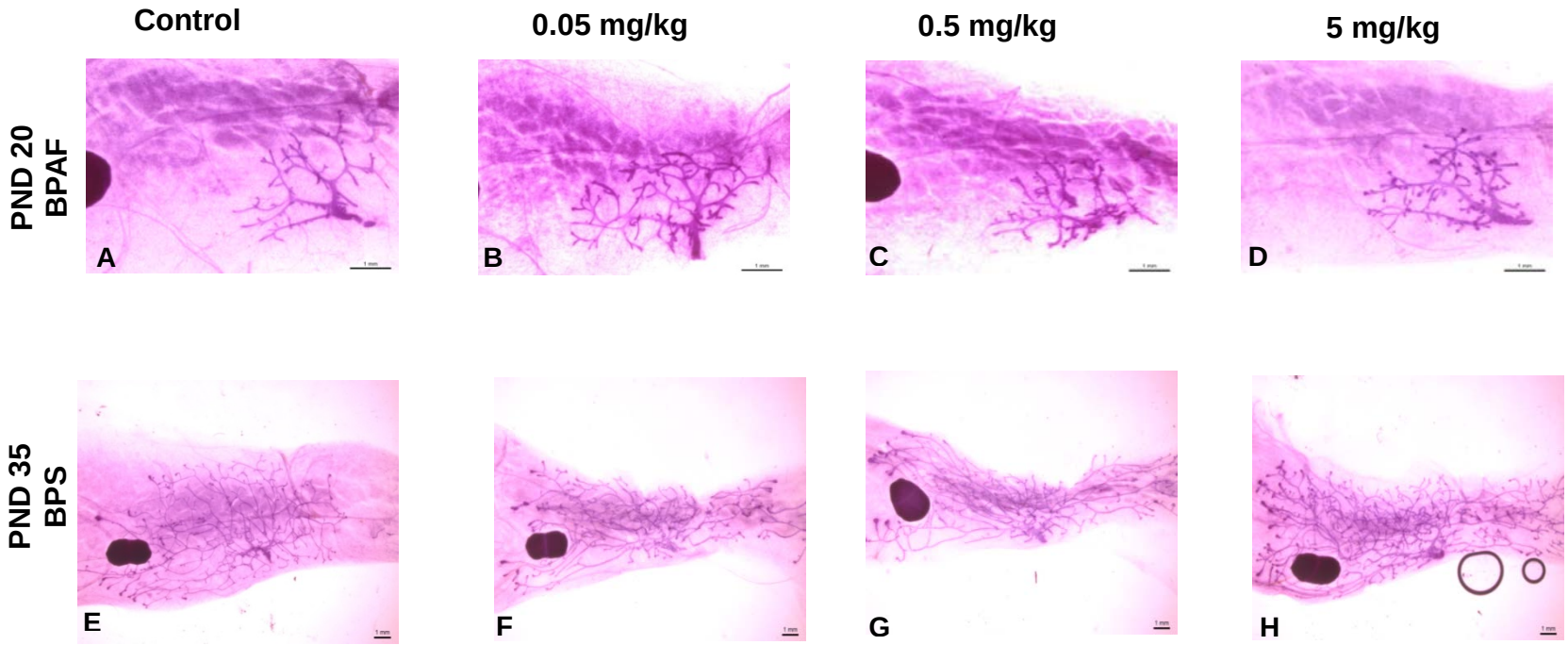


Figure 3-3: Mammary gland whole mounts from BPAF females on PND 20 (top panel) and BPS females on PND 35 (bottom panel). Representative image of A and E) Vehicle Control, B) BPAF 0.05 mg/kg, C) BPAF 0.5 mg/kg, D) BPAF 5 mg/kg, F) BPS 0.05 mg/kg, G) BPS 0.5 mg/kg and H) BPS 5 mg/kg.

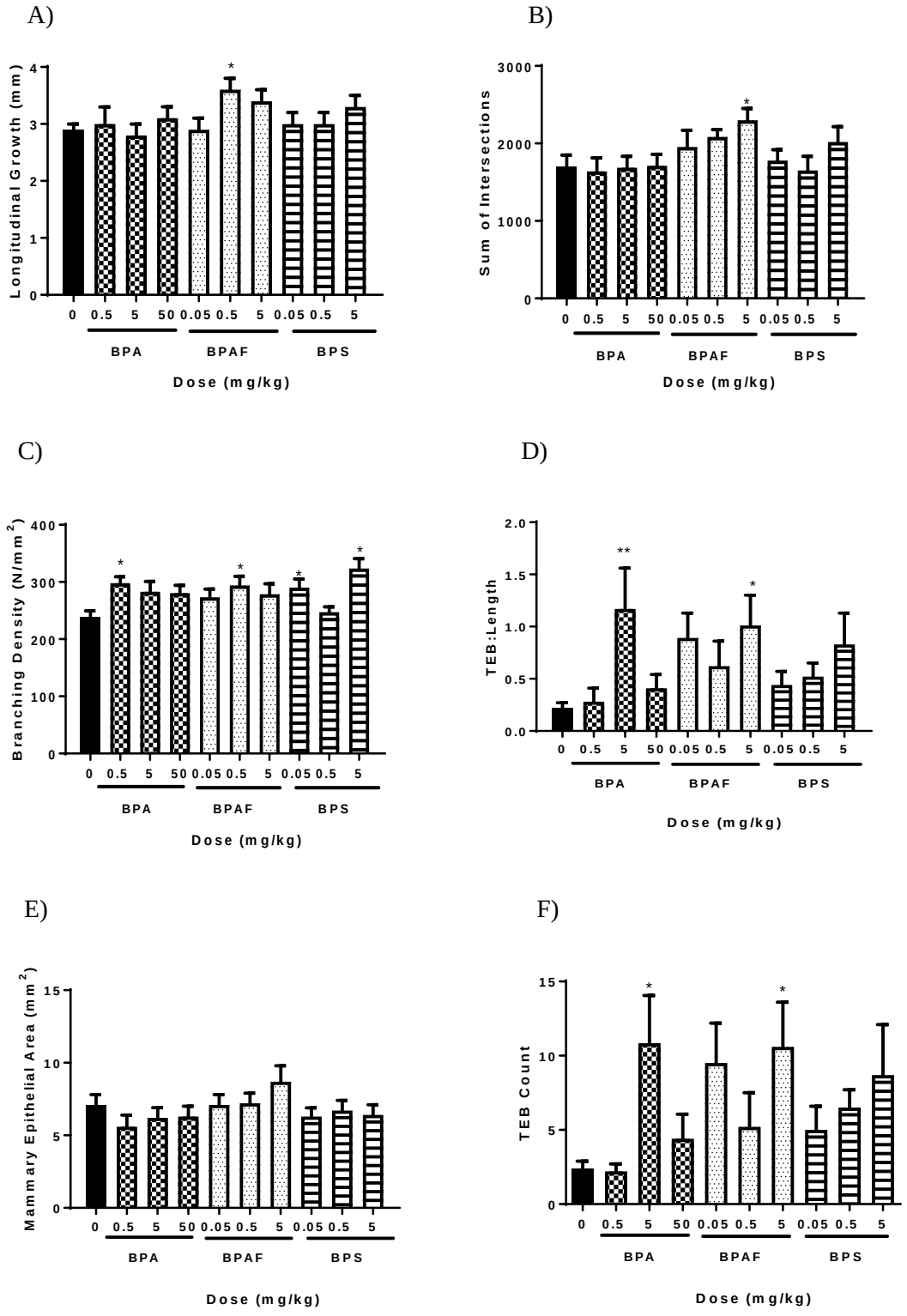


Figure 3-4: Sholl analysis and quantitative mammary gland parameters in BPA, BPAF, or BPS treated female offspring at PND 20. Mean ± SEM. $p < 0.05$ is statistically significant compared to vehicle control using ANOVA and a Dunnett's t-test. Vehicle (n = 8-10), BPA (n = 9), BPAF (n = 5-8) and, BPS (n = 8-10).

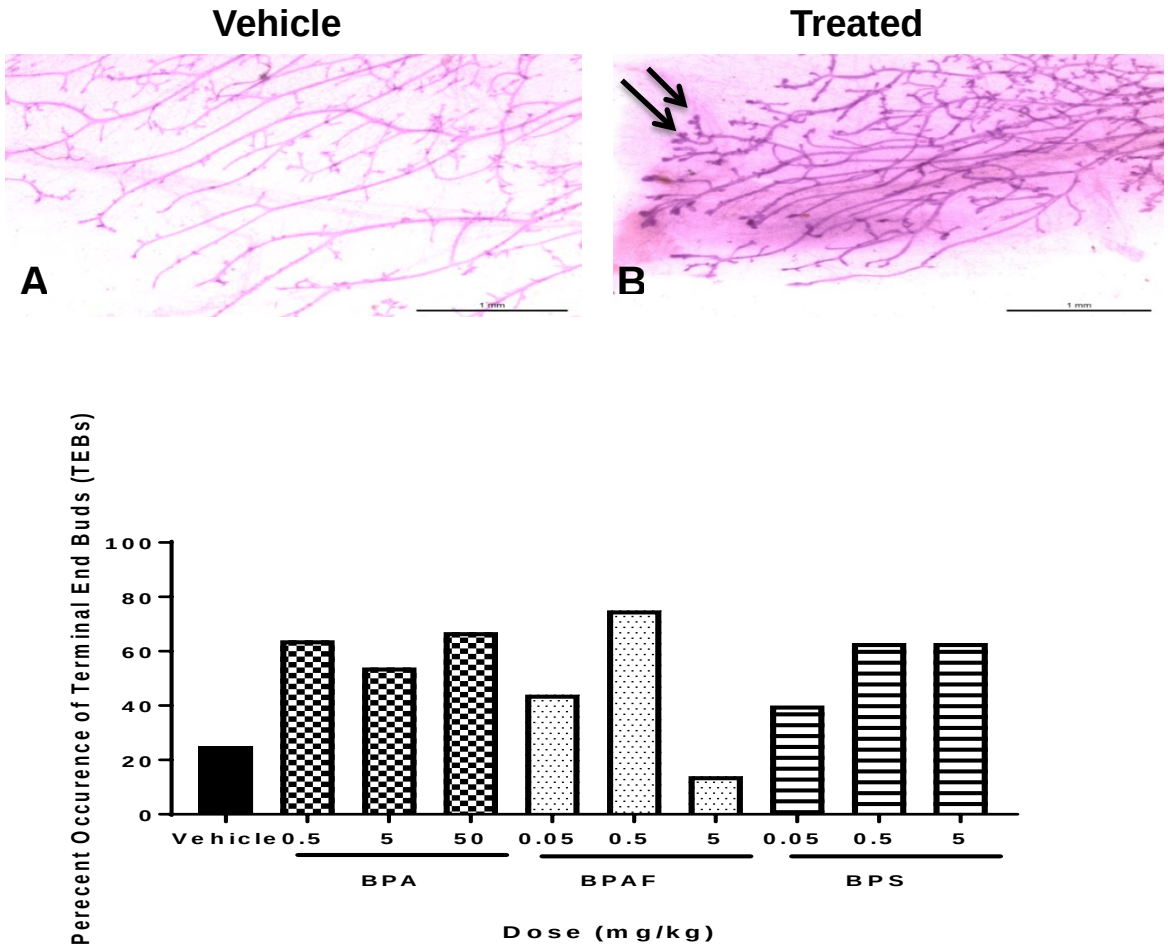


Figure 3-5: Increased occurrence of terminal end buds in mammary glands of 3-month old females prenatally exposed to BPA, BPAF, or BPS. Representative image of A) Control and B) Treated mammary whole mount. Occurrence is represented as a percentage of total animals. Vehicle (n=8), BPA 0.5 (n=11), BPA 5 (n=11), BPA 50 (n=9), BPAF 0.05 (n=9), BPAF 0.5 (n=8), BPAF 5 (n=7), BPS 0.05 (n=10), BPS 0.5 (n=8), and BPS 5 (n=8).

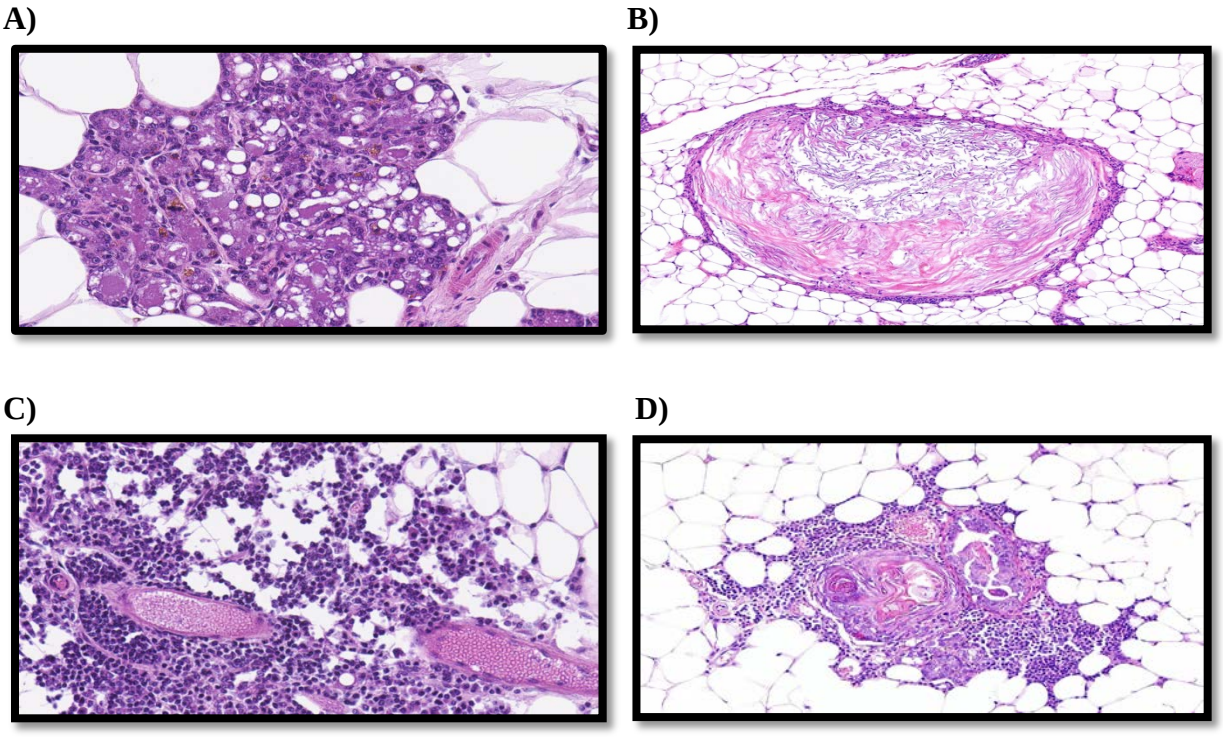


Figure 3-6: Histological evaluation of non-neoplastic mammary lesions following early life bisphenol analogue exposure. Representative images illustrate A) Lobuloalveolar hyperplasia, B) Cyst, C) Lymphoplasmacytic Perivascular Inflammation and D) Squamous Metaplasia, ductal

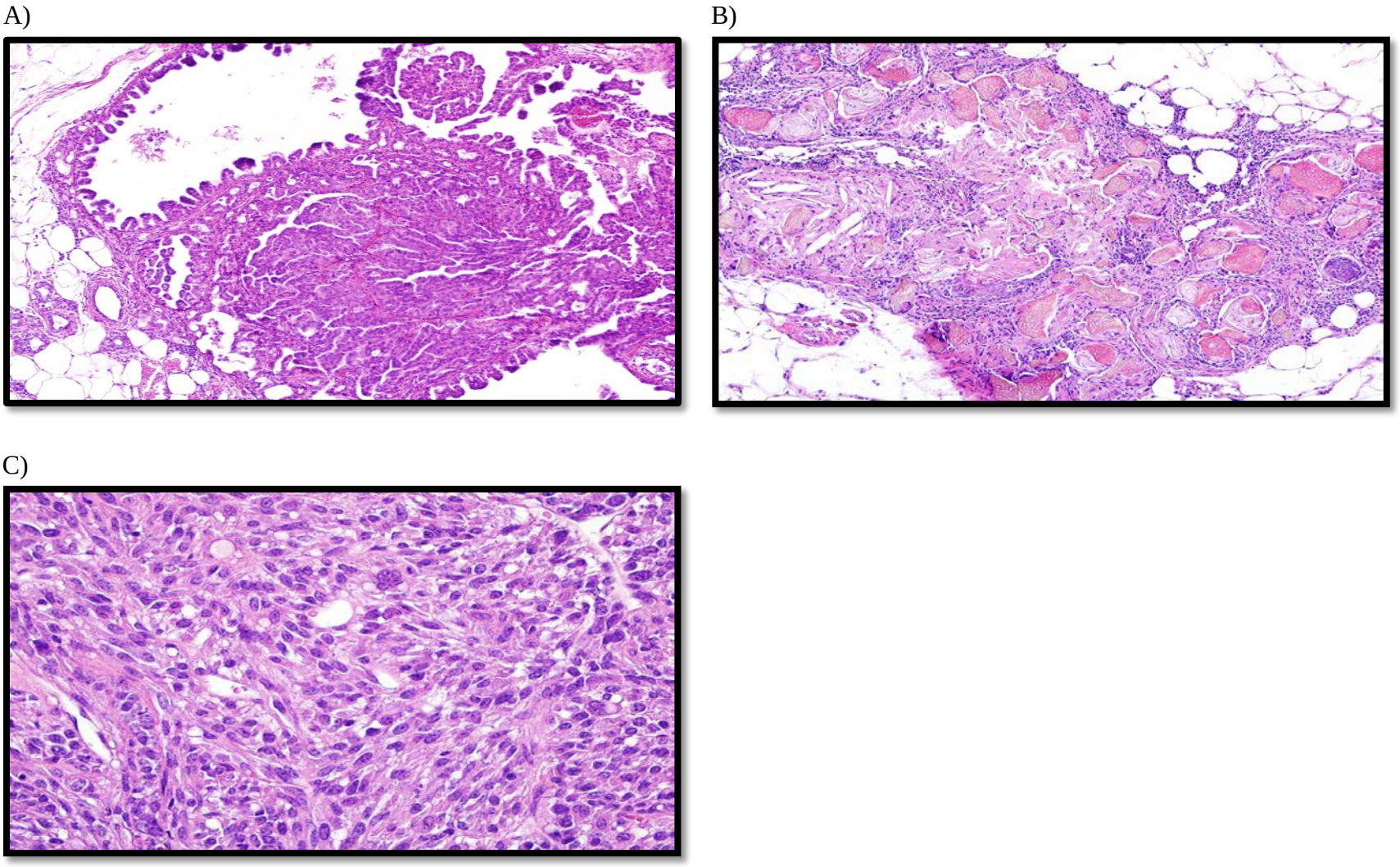


Figure 3-7: Histological evaluation of neoplastic mammary lesions following early life BP analogue exposure. Representative images illustrate A) Adenocarcinoma, B) Squamous cell carcinoma, and C) Histiocytic sarcoma.

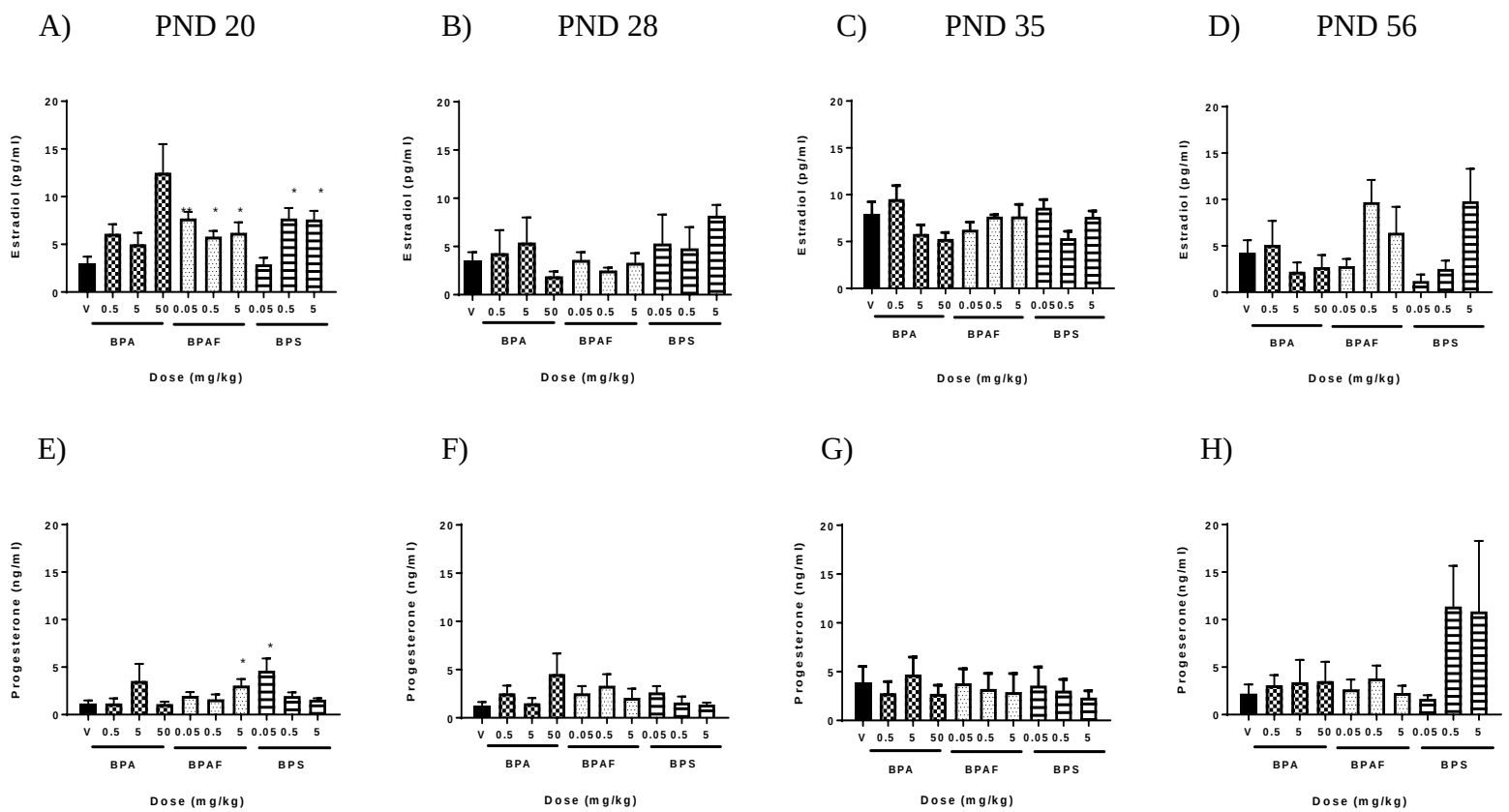


Figure 3-8a: Steroid serum levels from bisphenol analogue-exposed females during pubertal development (Estradiol and Progesterone). A, E) PND 20; B, F) PND 28; C, G) PND 35; and D, H) PND 56. Mean ± SEM. n=3-4 per group. *p<0.05 is statistically significant compared to vehicle control by Dunnett's test.

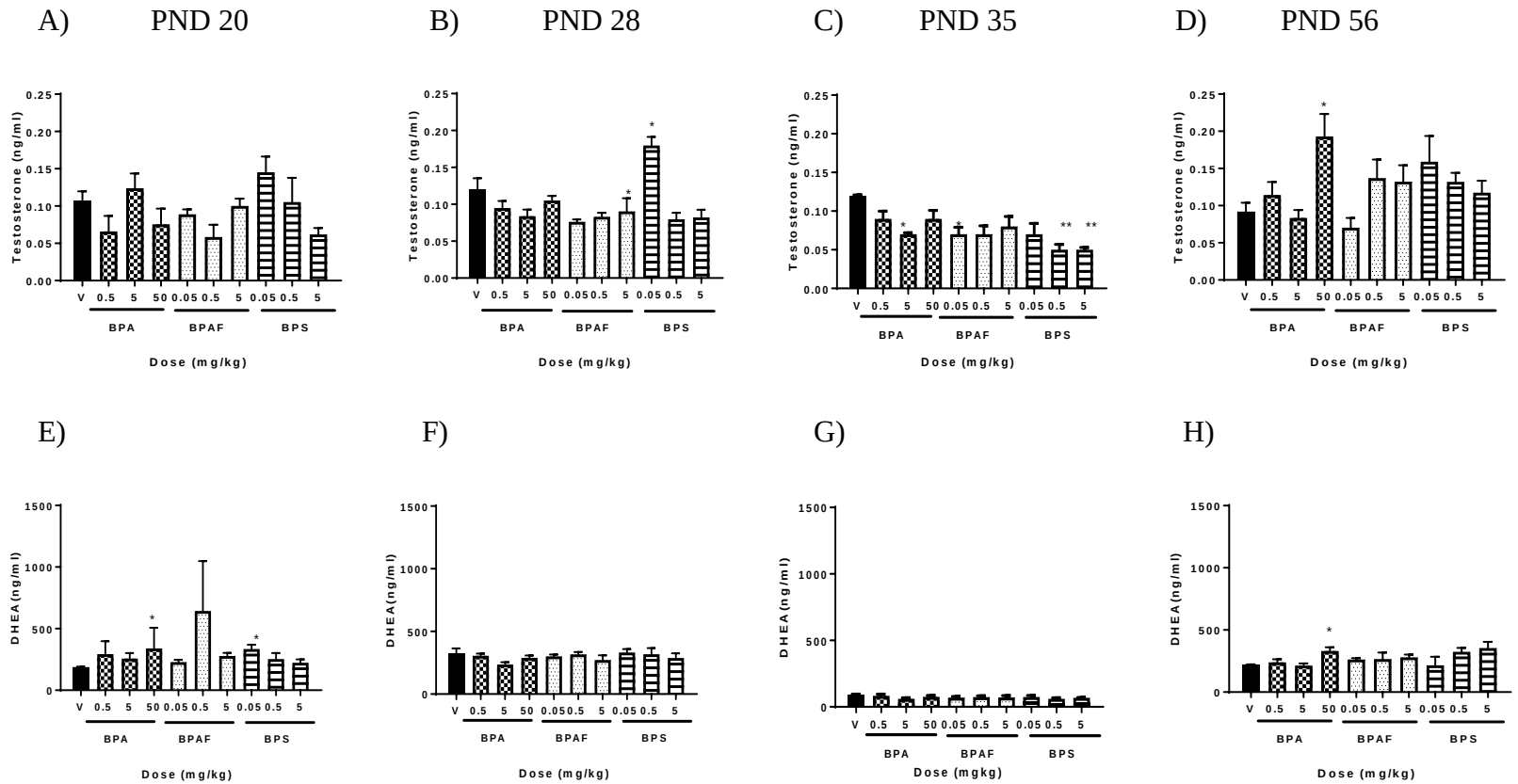


Figure 3-8b: Steroid serum levels from bisphenol analogue-exposed females during pubertal development (Testosterone and DHEA). A, E) PND 20; B, F) PND 28; C, G) PND 35; and D, H) PND 56. Mean ± SEM; n=3-4 per group. *p<0.05 is statistically significant compared to vehicle control by Dunnett's test.

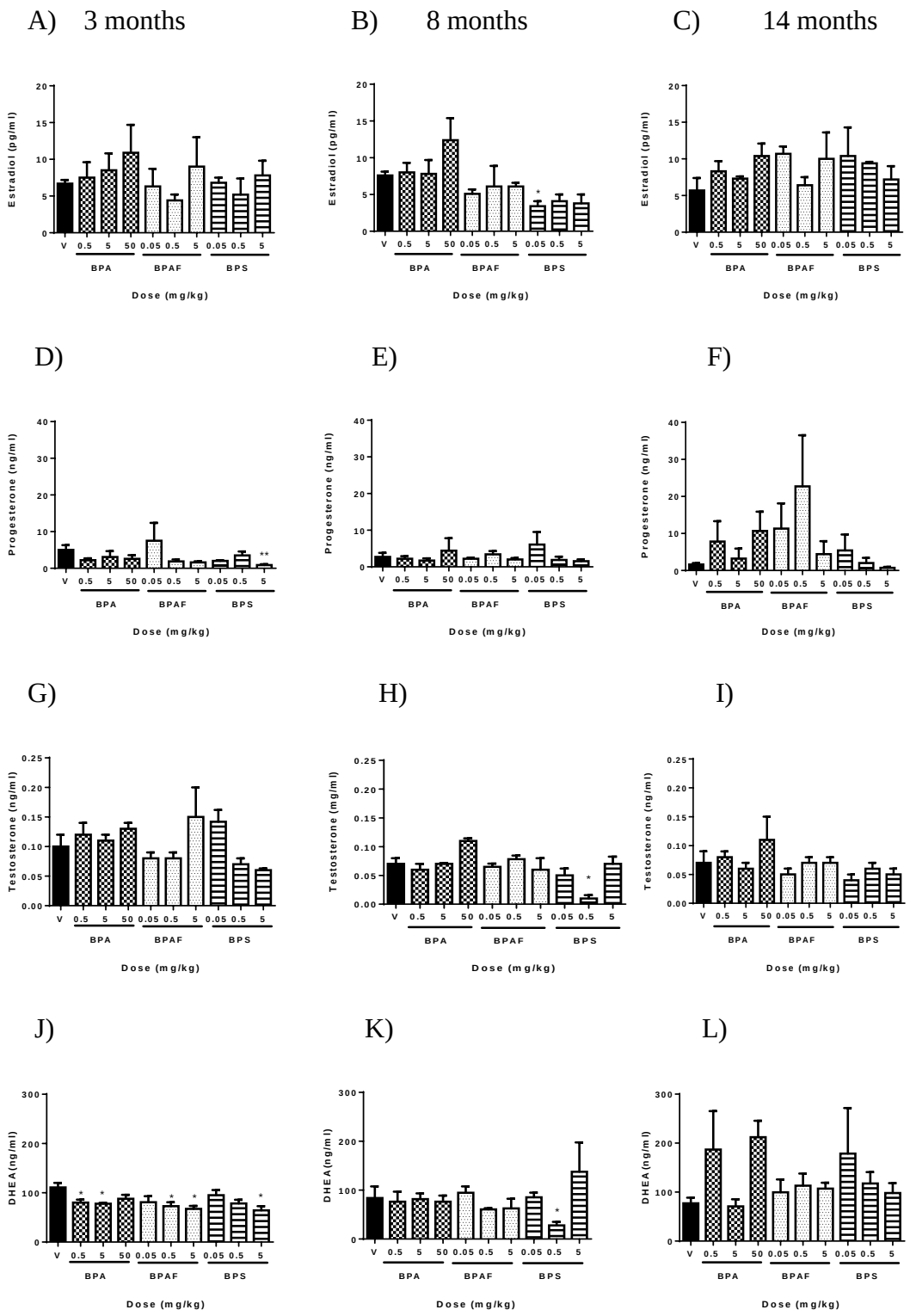
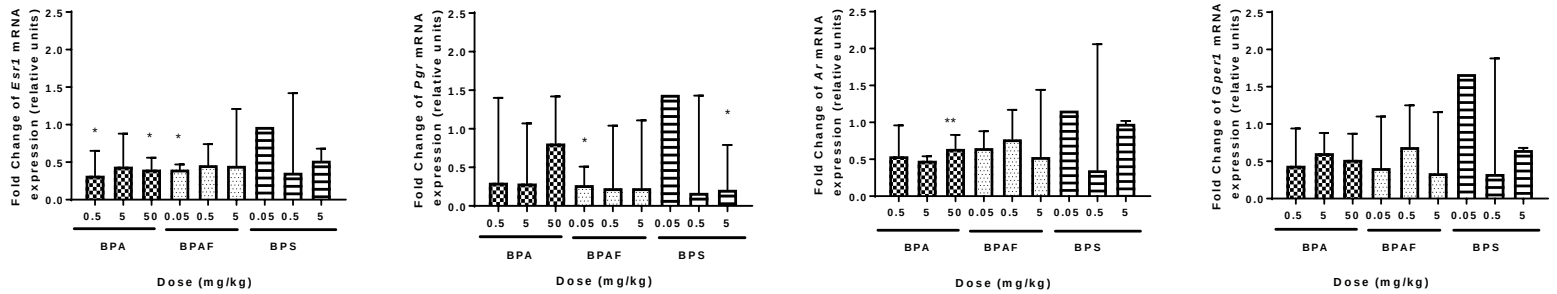


Figure 3-9: Effects of prenatal BPA, BPAF, or BPS exposure on female serum hormone levels at 3, 8, and 14 months. A, D, G, and E) 3 months; B, E, H, and K) 8 months; and C, F, I, and L). 14 months. Mean ± SEM. n=2-6. *p<0.05 and **p < 0.01 is statistically significant compared to vehicle control by Dunnett's test.

A)



B)

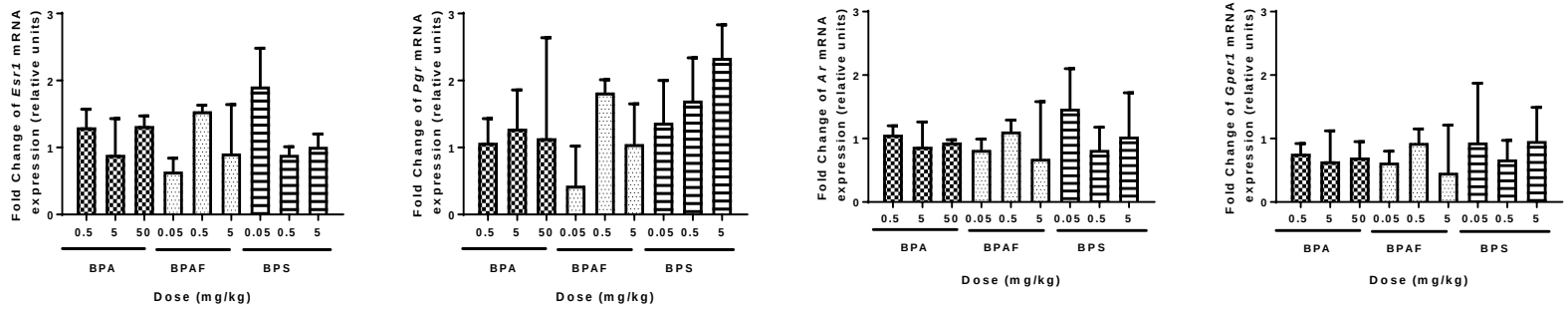


Figure 3-10: Expression of steroid receptor mRNA in the mammary gland at A) 8 and B) 14 months. Mean ± SEM. n= 1-4 * $p < 0.05$ and ** $p < 0.01$ is statistically significant compared to vehicle control.

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CHAPTER 4

Isolation and characterization of mammary stem cells following prenatal bisphenol analogue exposures in CD-1 mice

OVERVIEW

Early life exposures to human relevant doses of bisphenol A (BPA) in various rodent models have resulted in altered mammary development with an increased susceptibility to tumorigenesis. The proliferative wave of mammary stem cells (MaSC) during puberty and their increased susceptibility to damage during specific windows of development may serve as a plausible explanation for these changes. The mammary-specific mode of action of BPA or its sulfonated and fluorinated analogs, BPS and BPAF, respectively, remains elusive. We hypothesize that early life exposure to bisphenol analogs may play a role in "reprogramming" the stem cells, an effect that cannot be visualized until puberty when branching morphogenesis predominates. Previous findings identified increased epithelium during puberty following exposure to BPAF and BPS and therefore the main objective of this study was to identify and quantify MaSC changes in the peri-pubertal CD-1 mouse to determine if these effects correlated with observed morphological changes. Timed pregnant CD-1 dams were gavaged 2x daily with vehicle (sesame oil), BPAF (5 mg/kg) or BPS (0.5mg/kg) between gestational days 10-17. A subset of control female offspring was evaluated on postnatal days (PND) 19, 22, 28, and 35 for mammary morphological changes using whole mounts. On PND 19, 22, 28, 35, and 56 mammary glands from the remaining control animals, BPAF and BPS and were removed, dissociated, and sorted using flow cytometry based on the epithelial markers EpCAM and CD49f

to obtain the basal/myoepithelial and luminal populations. Sorted cells were transplanted into cleared fat pads of 3-week old recipient female mice using limiting dilution, assessed for gland regeneration 6-8 weeks later, and the mammary repopulating unit frequency (MRU) determined. Whole mammary glands from a subset of each group were assessed for *Wnt1*, *Wnt5a*, *Lrp5*, and *Notch1* expression. The percentage of MaSC was greatest at PND 22, but was significantly decreased at PND 35 following treatment with both chemicals. Neither BPAF nor BPS altered percent progenitor or stromal cells at any time during puberty compared to controls. Transplant regeneration yielded pubertal (PND 22-56) MRU frequencies of 1/1099-1/6988 (vehicle) compared to 1/4123-1/31997 (BPAF) and 0-1/20901 (BPS). *Lrp5, Wnt5* and *Notch1* gene expression were significantly decreased at PND 22. Taken together these data suggest that pubertal morphological changes associated with prenatal BPAF and BPS exposure are not driven by increased pubertal MaSC and progenitor populations. Lineage tracing of fetal MaSC may reveal different results.

INTRODUCTION

Breast cancer is a complex and heterogeneous disease that remains the second leading cause of death in women in the U.S. with < 25% of the cases resulting from heredity and genetics [1]. Established factors such as age, sex, ethnicity, and parity also alter a woman's risk for breast cancer, but other factors like lifestyle choices, high breast density, and incidental/accidental or occupational exposure to chemicals may further compound an individual's susceptibility. Breast cancer is a disease that targets the epithelial fraction of the gland, and these cells also respond to many environmental and chemical exposures. Therefore, great interest lies in understanding the associations between disease risk and communication of the breast epithelial and stroma that occur across a lifetime and may be modified by early life influences from these non-heritable

factors. The mechanisms that drive these events are at the forefront of mammary biology and breast cancer research and are critical to prevention/intervention, diagnosis, and treatment.

The mammary gland is an intricate tissue that involves extensive crosstalk between multiple cell and tissue types including adipose tissue, endothelial, stromal, immune, and epithelial cells. Normal mammary gland development occurs at a rate comparable to other organs and tissues but remains in a quiescent state until puberty. Mammary gland growth is reactivated prior to other aspects of puberty (i.e., first estrus or menses). In rodents, neuronal excitation and inhibition initiate pubertal development to initiate the release of gonadotrophin releasing hormone (GnRH) [2]. Influences from both trans-synaptic and glia to neuron pathways rely on the kisspeptin peptide, an excitatory amino acid, to become activated. Kisspeptin neurons have been found to expressed and regulated by the gonadal steroids estrogen receptor alpha and beta (ERα and β), progesterone receptor (PR) and androgen receptor (AR). When these gonadal steroids are released by the ovaries they signal the kisspeptin neurons [3]. As a result, the level of neuronal activity through gamma amino butyric acid and opioid peptides is reduced. In response to estradiol and progesterone release from the ovaries and GnRH activation, puberty is initiated and estrogen receptors in the epithelium and stroma of the mammary gland are increased causing increased terminal end buds (TEBs) and lumen formation, stromal remodeling, and ductal elongation. The TEBs are highly mitotic terminal ends [ex., terminal end buds (TEB) in mice and terminal ductal lobular units (TDLU) in girls]. Cap cells occupy the basal surface of the TEBs and have been interpreted to represent a pluripotent stem cell population that are capable of undergoing differentiation into ductal and myoepithelial mammary cell types [4]. Several studies have identified and characterized the pubertal mammary stem cell (MaSC) and progenitor cells in the mouse mammary gland [5-10]. Although spikes in fetal- and pregnancy-induced MaSC

exist, it is unclear whether these are the same populations that peak during puberty, since they perform unique functions during each phase of development [11-14]. Regardless of developmental stage, there is mounting evidence to support the concept that mammary carcinogenesis originates from a MaSC population or cancer stem cell population (CSCs) [5, 15-17]. These cells often have longer life spans with retained proliferating capabilities that results in a higher mutation rate probability [18]. Accumulation of mutations and aberrant signaling in the pathways associated with stem cell maintenance and differentiation are thought to be at the root of tumorigenesis [19-22]. The stage at which mutations occur may also dictate the tumor subclass (i.e. basal-like, luminal, ERRB2+) [23] and gaining a better understanding of these events will play a pivotal role in both diagnosis and treatment of a resulting cancer.

Bisphenol A (BPA) is one of the very few chemicals that has been extensively studied for effects on the developing mammary gland. Used in the production of plasticizers, epoxy resins, dental sealants, thermal receipt paper, and canned food linings, BPA is also an established endocrine disruptor. Several studies have linked BPA to neuroendocrine and neurobehavioral effects, reproductive tract abnormalities, and altered mammary gland development with progression to mammary carcinogenesis in rodent models [24-28] . Women involved in the manufacturing of plastics and food canning were found to have an increased breast cancer risk when factors including age of menarche, years of employment in the industry, parity and other lifestyle risks were considered [29]. Compared to case controls from the same area, women who manufactured plastics and food canning had significant odds ratios of 2.68 and 2.35, respectively, and premenopausal women were also found to have a higher estrogen receptor status of their tumors, suggesting a link between their breast cancer subtype and exposure to estrogenic chemicals. While it cannot be ruled out that these women may have been exposed to

other monomers including phthalates or polybrominated diphenyl ethers, these data suggest that there is an association between exposure to bisphenols and breast cancer risk.

Perinatal and pubertal exposures to BPA in rodent models have been linked to an increased number of TEBs, enhanced ductal extension and branching points, and increased sensitivity to estradiol in ovariectomized females [30-32]. Developmental BPA exposures also increased the incidence of pre-neoplastic and neoplastic lesions, while decreasing the time to tumor formation [28, 33-35]. Additional efforts have been undertaken to understand BPA's effect on the rodent mammary transcriptome and epigenome [30, 36], and only recently has the MaSC and progenitor cells been implicated as potential targets. Perinatal BPA exposure has been shown to increase total cell populations without altering individual population counts, increase progesterone expression in luminal cells, and induce genes involved in stem cell function [37]. Three-week peripubertal BPA exposures in Balb/C mice increased MaSC populations by 6 wk, delayed the increase of luminal progenitor cells seen at 4 mo, and decreased the time to neoplasm formation in DMBA induced transplant cells [38]. Taken together, there is a risk of altering the trajectory of cells involved in tumor formation during critical periods of development, such as puberty, when MaSC are more abundant.

In recent years, several structurally similar bisphenol analogues, including Bisphenol AF (BPAF) and Bisphenol S (BPS), have been detected in foodstuffs, dust and water and recovered in human samples [39-41]. Both chemicals have been shown to induce proliferative effects *in vitro* and *in vivo* and were also estrogenically comparable or enhanced compared to BPA [42-44]. Our group has shown that early life exposure to BPAF and BPS altered early pubertal mammary gland development that persisted into adulthood (Chapter 3). BPAF and BPS increased numbers of TEB, prolonged the presence of TEBs (3 mo), and increased the incidence

of preneoplastic and neoplastic lesions without altering pubertal onset or serum steroid hormone levels (i.e. estradiol, progesterone, DHEA, and testosterone). Although similar to BPA in its effects on the mammary gland, the definitive mechanism(s) and cellular pathways remain undefined and unexplored for these bisphenol analogues. From our experience ER, PR, and AR expression in the mammary gland during time points when hyperplasia, inflammation and neoplastic lesions were present remained unchanged. Therefore, it is possible that the mechanisms driving the changes later in life are independent of classical estrogen signaling and that many of these changes may be occurring much earlier in development. Still, with the increasing similarities between BPA, BPS, and BPAF it is possible that they have many similar or overlapping mechanisms, including the role of the MaSC.

Although the MaSC have been implicated as potential targets for tumor initiation only a handful of studies have attempted to draw a connection between this population and BPA [37, 45, 46]. In this study, CD-1 female offspring that were prenatally exposed to BPAF, BPS or vehicle control were evaluated during puberty to define the normal MaSC population, determine whether changes in this and other mammary cell populations correspond with previously reported morphological alterations and investigate potential pathways that may be involved. In this study the MaSC population was greatest at PND 22 in mice exposed to vehicle and both bisphenols, with no significant differences between groups. This population of cells also had a decreasing trend throughout pubertal development regardless of chemical treatment. This decrease resulted in a reduction of successful transplants and MRU frequency. Assessment of stem cell maintenance and renewal genes, *Wnt1, Wnt5a, Lrp5,* and *Notch1* revealed no changes or significant reductions at only PND 22. Overall, our data suggests that although there is clear

evidence that perinatal BPAF and BPS exposure increase epithelial cellularity that this may not facilitated by a larger pubertal MaSC population.

MATERIALS AND METHODS

Animals

Timed pregnant CD-1 mice were obtained from Charles River Laboratories (Raleigh, NC). Upon arrival, animals were weighed and randomly assigned to the vehicle or a treatment group. All dams were acclimated to the animal facilities a minimum of 2 days prior to dosing. Pregnant dams were individually housed in polypropylene cages and received AIN-93G feed (Harlan Laboratories) and tap water *ad libitum*. Bisphenol contamination in the animal environments was minimized as previously described (Chapter 2). Animal facilities were maintained on 12:12 hr light-dark cycles, at a controlled temperature (20-24ºC) with 40-60% relative humidity. All animals were treated humanely and in accordance with the protocols published by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

Chemicals and Dosing

BPAF was obtained from 3B Pharmachem International Co. Ltd (Wuhan, P.R. China) and BPS was obtained from Sigma Aldrich (St. Louis, MO). Purity for both chemicals was ≥ 97.5%, as previously confirmed (Chapter 2). Chemicals were dissolved in sesame oil (Jedwards, International, Inc., Braintree, MA) daily and administered to the animals by blind allocation in a volume of 10 µl solution/g body weight or pure sesame oil for vehicle control animals.

Experimental Design

Timed pregnant CD-1 dams were oral gavaged twice daily between gestational days 10 and 17 (GD 10-17) with vehicle (sesame oil), BPAF (5 mg/kg) or BPS (0.5mg/kg). Three separate dosing blocks were designed to retrieve an adequate amount of mammary epithelial tissue for the transplant studies. Except for Block 2, each block of exposed dams included vehicle n=4-9, BPAF 5 mg/kg n=9-12, and BPS 5mg/kg n = 9-12. These single doses of bisphenol analogues were chosen as they promoted significant increases in pre-neoplastic lesions and were also dose groups in which neoplastic lesions were evident in previous studies (Chapter 3). Gavage was twice daily to avoid episodic doses that would be cleared from the body by the next day (Chapter 2). The latter half of fetal growth was targeted as the mammary bud develops, forms its first stalk, and produces a lumen during the chosen exposure period [47]. CD-1 dams gave birth on the eve of GD 18 and postnatal day 1 (PND 1) was considered the next day. All litters were equalized to 10 pups on PND 3 with as many female pups as possible per litter. On PND 19, 22, 28, 35, and 56, the left and right 4^{th} and 5^{th} inguinal mammary glands of female offspring were excised for cellular dissociation and sorting. These glands are the preferred choice for carmine stained whole mount assessment and are not intermixed with muscle, unlike the thoracic glands. All animals greater than 22 days old were collected in the stage of diestrus when the stem cell populations have been shown to be at their maximum [48]. Mammary glands that were not collected for dissociation were snap frozen in liquid nitrogen and stored at -80^{o}C for future use.

Mammary gland dissociation and stem cell enrichment

The dissociation protocol was adapted and modified from Prater et al. [49] . Briefly, excised mammary tissues were washed in 70% ethanol to eliminate contamination. For each block, 4-10 animals/dose group from different litters were euthanized to obtain the 4^{th} and 5^{th} inguinal glands. Higher animal numbers were used during earlier collections (PND 22) due to less epithelial tissue per gland. Tissues were digested in a solution consisting of collagenase-hyaluronidase (1X-final concentration) (Sigma Aldrich, St. Louis, MO), DMEM/F-12 (Life Technologies, Grand Island, NY) and gentamicin (50 µg/ml) and incubated at 37 ºC for 14-16 hr The cell pellet was collected by brief centrifugation at 450 x g for 5 min (Thermo Scientific Sorvall Legend XFR centrifuge, Langenselbold, Germany). Red blood cells were lysed using Ammonium Chloride solution (Stem Cell Technologies, Cambridge, MA) and cleaved by successive trypsin (Life Technologies) and Dispase/DNase I (Stem Cell Technologies) washes (37 °C). Cells were then washed in 10 ml of 4° C HF [Hanks' Balanced Salt Solution (500 ml)/HEPES (10 mM)/fetal bovine serum (2% FBS)], filtered through a 40 µm cell strainer and centrifuged. 2 ml of HF were added to the pellet after the removal of supernatant and the cells were counted on a Z2 coulter particle count and size analyzer (Beckman Coulter, Indianapolis, IN).

Stem and progenitor cell enrichment was performed using the EasySep Mouse Mammary Stem Cell Enrichment Kit (Stem Cell Technologies) per the manufacturer's instructions. This kit helps to separate mammary epithelial cells from other cells through a process known as negative selection. In this case, non-epithelial cells become bound to the tube and removed from the epithelial enriched supernatant. Briefly, 100 µl of the EasySep mouse enrichment epithelial cocktail was added to the cell suspension acquired from dissociation and incubated on ice for 15

min, followed by two additional 15 min incubations with 1) EasySep Biotin selection (200 µl), then 2) EasySep magnetic nanoparticles (100 µl). HF (100 µl) was added to the mixture to bring the volume to 2.5 ml and placed in the EasySep Magnet (Stem Cell Technologies) for 5 min. With the tube still in the magnet, the enriched cell suspension was transferred to a new tube and placed on ice. To ensure that all or at least most the epithelial population was isolated, the tube in the magnet was removed and HF (2.0ml) was added, gently triturated to mix the contents, and returned to the magnet for another 5 min to remove all the undesired populations. The cell supernatant/suspension in the magnet was poured into the tube with the supernatant/suspension from the first separation until a total volume of 4.5 ml was achieved; the enriched cell suspension was centrifuged at 350 x g for 5 min. The HF media was discarded and 2.5 ml of cold new HF was added to the cells and placed in the magnet to select out any remaining non-epithelial populations. The transferred cells were centrifuged and recounted.

Cell Staining and Flow Cytometry

The cell pellet was suspended in 3 ml of cold HF with 10% normal rat serum (Invitrogen), incubated on ice for 10 min and 1.7 ml of cold HF was added to stop the reaction and bring the total volume up to 5 ml. It should be noted that due to limited antibody, we were only able to stain a select number of cells. Preliminary studies showed that 10-20 million cells provided an adequate number of each sorted population. To ensure that one population was not overrepresented, the cell suspension was vigorously triturated and then distributed into 5 ml polystyrene tubes at a density of 1.0-2.0 x 10^7 cells for the flow sorted sample and stained with EpCAM and CD49f (Table 4-1). A total of 6 tubes are prepared for individual cell titrations at a concentration of 0.5-1.0 x 10^6 cells and included one tube for 1) cells that received no staining,

2) cells stained with propidium iodide stain (PI) to measure cell death, 3) Isotype Control (EpCAM), 4) Isotype Control (CD49f), 5) CD49f, and 6) EpCAM. All tubes were incubated for 30 min covered on ice and washed with HF (4°C) followed by a 5 min centrifugation at 450 x g. The supernatant was poured off from each tube and each cell pellet was re-suspended in 500 µl of HF. Prior to FACS sorting, PI (5 µl/1 x 10^6 cells) was added to its designated tube.

Stained cells were sorted on a BD FACSAria II (BD Biosciences, San Jose, CA). Briefly, sorted cells were gated on PI^- to obtain a viable population, and then by forward scatter (FSC) and side scatter (SSC). Cells were then gated by SSC high vs. SSC width followed by FSC high vs. FSC width to obtain single cells as cell clumping can inhibit proficient sorting. To separate the basal, luminal, and stromal populations from one another the samples were sorted per their fluorescence in the fluorescein isothiocyantate (FITC) and Allophycocyanin (APC) channels. Luminal cells were identified as $EpCAM^{high}CD49f^{med}$, stromal cells were $EpCAM^-CD49$, and basal/myoepithelial cells were $EpCAM^{med}CD49f^{high}$. The highest 20% fluorescing cells within the basal population contained the mammary repopulating unit cells (MRU) (Figure 4-2). FACS Diva Software 6 was used for data acquisition and analysis. All cell populations were collected in HF pre-coated chilled 1.7 µl microcentrifuge tubes. Cells were recounted and centrifuged at 450 x g for 5 min to form a pellet. The pellet was then re-suspended into a trypan blue (10%)/PBS (65%)/Growth Factor Reduced Matrigel (25%) mixture and diluted into cell concentrations that ranged from 1-500 cells/µl for transplants.

Mammary Transplantation and MRU Frequency Analysis

Three-week old female CD-1 mice were obtained from Charles River Laboratories. Animals were pre-operatively anesthetized with isoflurane gas and received 0.1 mg/kg

buprenorphine HCl (i.m.) for pain. During fat pad clearing and cell transplants, all recipient animals received a constant flow of isoflurane gas at 2.5% with oxygen to ensure they remained sedated. Briefly, a small incision was made around the nipple where the fat pad was retrieved using blunt forceps. The mammary gland was cauterized between the 4^{th} and 5^{th} inguinal glands, caudal to the lymph node (reference point) to ensure that all endogenous epithelial tissue from the recipient's 4^{th} and 5^{th} gland was removed. Transplanted cells were injected in the fad pad of the recipient's 4^{th} gland. The trypan blue/PBS/Growth factor and cell population mixture was injected into the cleared fat pad in a volume of 10 µl with stem cell concentrations from 1-500 cells/µl (10-5,000 total cells/transplant). Three to five mice per cell concentration were transplanted for each chemical or vehicle (total of all mice = 20-25 per block). The skin was sutured and recipient mice were placed on a heat pad until they awoke. Once mice were appeared normal they were returned to regular cages lined with Diamond soft recovery bedding (Envigo, Madison, WI). Transplant animals were given the same diet as the animals used for mammary harvesting. Animals were monitored daily for the first week following surgery and once weekly thereafter. Mammary glands from transplant animals were recovered 6-7 weeks later, as described above. Glands were placed on a charged microscope slide, fixed in Carnoy's solution, stained with carmine alum, cleared in xylene, mounted with Permount (Sigma Aldrich) solution, and coverslipped.

Transplant examples are shown in Figure 4-1. Successful transplants consisted of epithelial tissue that grew in a concentric direction and appeared to not be a result of endogenous epithelium. Transplants were considered failures if the fat pad was improperly cleared leaving epithelial tissue or unilateral growth. Each successful transplant for vehicle or BPAF/BPS treatment were recorded and entered into L-Calc software (https://www.stemcell.com/l-calc-

software.html) to determine the mammary repopulating unit frequency (MRU). Frequency is calculated from the total of successful transplants and total number of attempted transplants using a Poisson distribution.

RNA preparation and RT-PCR

Snap frozen mammary tissues were immediately placed in Trizol (Sigma Aldrich), homogenized at 6.0 m/sec for 40 sec. and incubated on ice for 5 min (3x). The Trizol supernatant was transferred to a clean microcentrifuge tube and spun for 10 min to remove the fat layer. The clean supernatant was then extracted for RNA per the manufacturer's recommended protocol and purified with the Qiaqen RNA Mini Kit (Qiaqen, Germany) and DNase I treatment. RNA was quantified with a Nano Drop 2000 (Agilent, Germany). One microgram of RNA was reverse transcribed using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA) and amplified in a Bio-Rad thermocycler (Bio-Rad, Hercules, CA), per the manufacturers recommended setting. cDNA was mixed with Taqman (Applied Biosystems) Universal PCR Master Mix, No AmpErase UNG (2x), Taqman 20x gene expression assay mix (*Wnt5a, Wnt1, Notch1,* and *Lrp5,* Table 4-2) and RNase free water. Transcripts were measured on a Quantstudio 7 Flex (Applied Biosystems) and analyzed in Excel. All data output was recorded as a threshold value (C_t) and fold change was calculated using $2^{-\Delta\Delta Ct}$. Briefly, all samples were normalized to the reference gene (*Cdkna1*) and ΔC_t was calculated using the formula Δtarget gene C_t – Δreference gene C_t. Samples with Ct values ≥ 35 or with a duplicate standard deviation of ≥ 0.5 were omitted from analysis.

Statistical Analysis

The dam is considered the unit of measurement; therefore, if two animals were retrieved from the same dam then the average of their measurements was used in the analyses. All data is represented as the mean ± SEM. FACS sorted fraction data is represented as the number of sorts completed. A student's t-test was performed to determine statistical significance ($p \leq 0.05$). Fold change was calculated using Microsoft Excel 2010 and graphs were constructed in GraphPad Prism v7.

RESULTS

Identifying the MaSC population in CD-1 females during normal pubertal development. Normal pubertal mammary gland development in CD-1 mice occurs just after the third week of life when the HPGA becomes active. Estrogens from the ovary and potentially the mammary fat pad itself promote epithelial proliferation within the cell, while progesterone increases lateral ductal growth. During puberty, duct ends form the highly mitotic TEBs, which house the MaSC population. Using mammary gland whole mounts, normal development was observed from pre-puberty to mid-puberty (Figure 4-3A). At PND 19, both 4^{th} and 5^{th} inguinal glands were entering the time of rapid growth, however, TEBs were not typical and very few secondary branches were present. By PND 22, TEBs were easily identifiable and much more branching had occurred even though there was still a significant gap between the two glands. Vaginal opening, an outward sign of puberty in mice, occurred within a couple of days of this breast development stage (Chapter 3). At 5 weeks of age (PND 35), ductal growth had increased exponentially; 4^{th} gland growth either approached or surpassed the lymph node and the distance between the 4^{th} and 5^{th} glands decreased to the point that they had grown together and ends had differentiated. Because

the glands grew together, contact inhibition occurred and TEBs become terminal ducts. Ducts that had grown to the edge of the fat pad had persistent TEBs. Assessment of these glands for TEB count showed a linear increase in the number of TEBs from PND 19-28 but slightly decreased by PND 35, which may reflect the changes that occurred due to 4th and 5^{th} glands growing together (Figure 4-3B).

Prior to determining the effects of BPAF and BPS on the MaSC population, it was necessary to characterize the specific cellular populations during normal pubertal development in control CD-1 mice. To confirm that basal cells were indeed the population that contained the stem cells, mammary transplants were conducted using basal, luminal, and stromal cells (*luminal and stroma data not shown*). Stromal cells did not re-constitute any glands (0/3) whereas luminal cells did (2/4), and basal cells reconstituted a gland in 14 out of 37 transplants. The high percentage of luminal reconstitution is because only four transplants were conducted. More transplants would have adjusted for this discrepancy. Three to twenty five females per treatment group were euthanized depending on the postnatal age evaluated (PND 19, n = 15; PND 22, n= 10-15; PND 35, n= 9-20; and PND 56, n = 3-10). It should be noted that following FACS sorting, a subset of each cell population was removed from the sample and isolated in Trizol for future PCR analysis. PND 19 animals did not contain many epithelial basal cells because they have not entered puberty and therefore required more animals to acquire more cells.

MaSCs were successfully sorted via FACS by gating the viable mammary cell sample with EpCAM APC and CD49f FITC. The basal population was identified by EpCAMmedCD49fhigh (Figure 4-2) and the 20% highest fluorescing cells within this population are considered to contain 70-80% of the mammary repopulating unit cells (MRU) [49, 50]. This population is capable of both self-renewal and differentiation. A quantification of this population

in control animals revealed that at PND 19 approximately 13% of the mammary population consisted of basal cells (Figure 4-4). Within three days, the population had increased by 3-fold to 40%. Following this peak there was a precipitous decrease in the population to almost pre-pubertal percentages and this trend continued into adulthood (PND 56).

Mammary transplants from the sorted basal cells collected from PND 19-56 were conducted in three separate blocks as previously mentioned to determine the MRU frequency in CD-1 pubertal females. Within each block, four collection and transplant time-points were attempted where approximately 3-5 animals per cell concentration were transplanted into recipient mice (~20-25 animals/time-point/block). Although basal cells were detected by FACS at PND 19, those cells were unable to reconstitute a gland at any cell concentration. Therefore, the MRU frequency for PND19 was unable to be calculated. Further analysis at PND 19 was discontinued. The highest frequency achieved was at the next collection time point (PND 22), which was also the collection point where the highest percentage of basal cells was collected. During this time 1/2,159 basal cells was calculated to effectively reconstitute a mammary gland in control animals. With increased age, this ratio decreased considerably by approximately 3-4 fold. A comparison of MRU frequencies found in CD-1 mice in this study and mouse strains previously reported is shown in Table 4-3. Compared to the outbred CD-1 mouse, the FVB and C57Bl/6 mouse could reconstitute the mammary gland at much lower frequencies (ex. 1/100-1/200) at postnatal ages that spanned from 8-12 weeks old [51], an age similar to 56 d within our study that provided inefficient reconstitution results.

BPAF and BPS effects on the MaSC Population and MRU frequency. We previously reported that when pregnant dams were exposed to BPAF and BPS the mammary glands of their female offspring had accelerated development compared to vehicle controls (Chapter 3). At PND

20, mammary glands from BPAF exposure showed increased branching density, number of TEBs, TEBs/area of mammary gland, and longitudinal growth that persisted into early adulthood (PND 56). Similarly, BPS glands were significantly more developed than controls as shown by the developmental qualitative scores. Other pubertal indices, such as timing to vaginal opening and first estrus, were unchanged relative to controls. Those significant developmental increases in epithelial structures led us to isolating the MaSC fraction of the mammary glands from females prenatally exposed to BPAF and BPS by flow cytometry to assess whether these cells were contributing to increased branching and development. MaSC counts (%) were greatest at PND 22 for control, BPAF and BPS (33±18.5%, 29.5±11.3% and 26.9±18.3%); however, no significant differences in cell populations were observed between treatment and control animals (Figure 4-5). Overall, between PND 28 and 56, the fraction of basal cells per total cell number decreased in all groups including control. At PND 35, % BPAF and BPS basal cell counts statistically decreased compared to controls. Surprisingly, the lowest basal cell counts were obtained at PND 28 and 35 for BPAF and BPS. When the luminal and stromal cell fractions (consists of endothelial and immune cells and fibroblasts) were evaluated, they were also unchanged following early life exposure to BPAF and BPS. Luminal and stromal cell counts at all time-points were typically greater than the basal compartments, except for BPAF at PND 22. Luminal counts ranged from 9-40% for BPAF, and 26-40% for BPS, and were greatest at PND 56 for both chemicals. Stromal compartment fraction ranged from 33-67% for BPAF, and 36-61% for BPS, peaking at PND 28.

Using limiting cell dilutions, basal mammary epithelial cells from control, and BPAF (n=20-25 females/time-point/block) and BPS (n=20-25 females/time-point/block) exposed females were transplanted into the cleared fat pads of 3-week old female recipient CD-1 mice to

establish the MRU frequency and determine if differences between vehicle and treatment groups existed (Table 4-4). Similar to quantitative basal cell counts, MRU frequency in the control group was greatest at PND 22, decreased by PND 35 and maintained at the same level until PND 56. We expected the MRU population in BPAF and BPS groups to increase since we had observed increased TEBs and growth in the whole mounts (Chapter 3), however, frequencies were decreased to 1:4,123 at PND 22 for BPAF (vs. 1/2,159 in the controls) and could not be calculated for BPS due a lack of successful gland re-constitutions. The inability of basal cells from bisphenol-exposed mammary tissue to reconstitute glands caused MRU frequencies to continue to decrease with increased pubertal age and were at least 3-4x less compared to controls.

Pubertal BPA exposure has previously been shown to alter the MaSC population in female Balb/C mice [38]. Immediately following the last exposure, the animals experienced an increase in the % of basal cells and % of MaSC as measured in frequency of total epithelium. Because of these findings, a similar mechanism was hypothesized to occur following BPAF and BPS exposure, as these chemicals have also been shown to stimulate mammary gland growth and branching in our previous studies (Chapter 3).

During development, MaSC rely on critical genes to regulate stem cell maintenance, renewal, and luminal differentiation but these same genes are also thought to play a critical role in mammary carcinogenesis. Therefore, exposure to chemicals such as BPAF and BPS may have the capability of altering this population in several ways including 1) increasing the MaSC population, 2) altering critical genes that can drive the cells increased differentiation, or 3) exert genotoxic effects that can develop into mutations and drive cancer formation. *Wnt5a*, is found in the TEB and involved in directing branching morphogenesis but was significantly decreased at

PND 22 following BPAF and BPS treatment (Figure 4-6), even though these exposures increased the number of TEB at this age (Chapter 3). We also investigated *Wnt1*, a gene involved in stem cell renewal, and it was similar to vehicle expression. In the canonical Wnt/β catenin pathway, co-receptors *LRP5/6* are involved in normal mammary gland development and required for stem cell activity [52]. Interestingly, expression of *LRP5* was significantly decreased in glands from BPS females, but not BPAF-treated animals, at PND 22 at a time when basal cell counts were greatest for both chemicals. At other times, expression levels of *LRP5* were similar to controls. Lastly, *Notch1* and the Notch signaling pathway are also involved in stem cell maintenance and differentiation [53, 54]. When decreased, these pathways encourage the growth of the MaSC populations and breast cancer progression. Significant decreases in *Notch* family genes were observed in both bisphenol groups at the earliest time point (PND 22; Figure 4-6).

DISCUSSION

Early life exposures to BPA, BPAF and BPS are reported to cause advanced mammary morphological changes that manifest prior to puberty and lead to the increased risk of developing preneoplastic and neoplastic lesions (Chapter 3 and [28, 33, 34]). BPAF and BPS have been shown to be agonists for the estrogen receptor alpha [42, 55], however, the effects from bisphenol exposure that are observed in the mammary gland may not be solely due to classical estrogen signaling. In this study, we investigated alternative means by which uncontrolled proliferation may have been manifested, namely through increased basal cell (MaSC-containing) populations in BPAF- or BPS-exposed mammary tissue. Using prenatal bisphenol analogue exposures previously shown to induce inflammation, epithelial proliferative lesions, and neoplasia during adulthood (Chapter 3), it was determined that BPAF and BPS decreased or had

no effect on the MaSC or progenitor populations in the pubertal and young adult mammary gland. MaSC populations rose to their peak levels around the time of vaginal opening, and were maintained at lower levels into adulthood, just like that seen in untreated controls. Overall, these data suggest that the number of pubertal MaSC may not be critical in manifesting BPAF- and BPS-induced lesions in later life, even though the MaSC is known to be critical in epithelial expansion during puberty [56].

The CD-1 mouse (an outbred strain) was chosen for our studies because BPAF and BPS were shown to induce multiple abnormalities in the mammary gland of this strain in a previous study (Chapter 3), although most transplant studies are conducted in inbred mouse strains. A recent study that examined the effects of perinatal exposure to BPA in C57Bl/6 females revealed that total epithelial cell populations were increased following exposure, however, individual cell populations (basal, luminal and stromal) remained similar between treatment and control [37]. We similarly observed no changes in the cell populations (specific cell fraction/total of isolated cell populations) in treated and control mammary tissue, nor were total isolated cell numbers changed (*data not shown*) following dissociation or FACS sorting. Dissociation of the mammary gland is a harsh process. If the method is precisely followed, it would be expected that the ratio of living to dead cells would remain constant, but this may not always be the case. In a different study, the MaSC from BPA-exposed Balb/c mouse mammary glands were increased (6 weeks) compared to controls following pubertal exposure, but those changes were not sustained in glands obtained in later collections (2 and 4 mo) [38]. Disparate findings between studies [35, 36] may be attributed to differences in BPA's effects in various mouse strains, as well as dose or timing of exposure.

Another reason that we may not have seen effects of BPAF and BPS on MaSC populations is that we were looking at the wrong time. Basal and MaSC increases were only observed following the last day of a BPA pubertal exposure (6 wk) in previous studies of Balb/C mice [38], which suggests that BPA and possibly BPAF and BPS effects on these populations are short-lived. Considering that these chemicals were administered between GD 10 and 17 and mammary collections and transplants were not performed until 3-5 weeks later could mean that we had already missed the window for these changes. It also implies that these chemicals may have a much greater effect on the fetal mammary stem cells rather than the pubertal stem cells. A recent study using mammary glands harvested from CD-1 embryos identified and characterized a fetal MaSC population that peaks during late embryogenesis and may closely resemble specific human breast cancer subtypes [14].

When assessing a stem cell capability to self-renew and differentiate, the gold standard is to conduct mammary transplants, where the cell population of interest is injected into the cleared fat pad of a syngeneic pre-pubertal mouse. A successful transplant confirms that the cells within that population have stem cell characteristics. When this population was transplanted into recipient mice using differing concentrations of stem cells within an appropriate range, we determined the MRU frequency or exact number of stems cells required to fully reconstitute a gland. We became proficient at these methods prior to attempting transplants of treated animals. Multiple ages were used to isolate stem cells in each treatment group, as we wanted to explore whether prenatal exposures to BPAF and BPS would increase the MaSC fraction as postnatal age increased; this would have explained why we reported increased epithelial structures (TEBs and branching density, Chapter 3) in a previous study. It was hypothesized that an increase in this population by these chemicals would also increase the number of positive gland reconstitutions

and MRU frequency. Contrary to this theory, BPAF decreased MRU frequencies compared to controls and this trend continued into early adulthood (PND 56). We also could not have predicted that BPS transplants conducted at PND 20 and 35 were going to be unsuccessful, thus prohibiting us from determining the MRU and any changes from controls. One explanation for this outcome could have been due to the decrease in basal cell populations at PND 28. Considering that approximately 70-80% of the MRU population is located in the highest fluorescing 20% of the basal fraction [57], the fact that this population is decreasing over time would suggest that there are also fewer MRU-type cells. We should also highlight that the CD-1 model may have also been instrumental in the lack of changes observed. Cells from outbred strains may be more easily rejected during transplantation due to differences in the immune response because of genetic variability. Future studies should perform transplant studies in an inbred strain to assess whether lack of changes are due to chemical exposure or strain differences, as that could not be determined in this study.

Several genes and pathways that regulate MaSC renewal and differentiation are also critical for mammary tumor formation. Two common pathways regulating stem cells include the canonical Wnt/β catenin and Notch pathways. In one study, the MMTV-*Wnt-1* in transgenic mice increased the epithelial cellularity and percentage of cells in the basal fraction compared to the control. Following basal fraction transplants of both strains the MMTV-*Wnt-1* mouse increased the MaSC frequency from 1/86 (control) to 1/57 (MMTV-*Wnt-1*) [8]. *Wnt5a* has also been shown to be expressed in luminal ERα cells and the TEBs [21]. The increased number of TEBs following BP analogue treatment that was observed in prior studies (Chapter 3) would suggest a possible increase in this gene. In contrast to our expectations, *Wnt5a* was significantly decreased at PND 22 and unchanged at all other time-points. Similarly the Wnt co-receptor *Lrp5*

was decreased at PND 22 in BPS-treated samples, but unaffected for BPAF and BPS throughout the rest of pubertal development. MaSC are highly enriched in *Lrp5* and the decrease could suggest a decrease in the ability of the MaSC to self-renew. Although we did not observe any changes in the Wnt ligands and receptors we cannot eliminate the potential role of other Wnts on these populations; this includes *Wnt4* which is greatly influenced by progesterone and solely expressed in luminal cells [37, 58]. Previous findings have shown that serum progesterone was significantly increased in 3-week old BPAF (5 mg/kg) exposed females (Chapter 3). Therefore, paracrine mediated effects should not be discounted as a possible mechanism for BPAF and BPS induced mammary changes and will require further studies to confirm. Notch ligands are also found in the MaSC, while the Notch receptors are in the luminal/progenitor cells [53]. Analysis of the *Notch1* receptor in whole mammary glands showed statistically decreased levels only at PND 22 for both chemicals. Decreased signaling through *Notch1* has been shown to alter transcription by decreasing interaction with *Cbf-1,* resulting in the increase of the MaSC populations [53]. An important note to make about our observations is that they were conducted in whole mammary glands versus FACS sorted epithelial and stromal populations. Expression from whole glands may have masked subtle changes due to the several cell types that comprise the mammary gland. It is recommended that an assessment of gene expression changes in FACS-sorted populations should be conducted at these time points.

In addition to understanding the effects of endocrine disruption on the MaSC and luminal cells these studies sought to successfully identify the MaSC population in the CD-1 outbred strain. To date, no published reports exist for this strain and are likely due to the genetic variability associated with outbred strains. However, numerous reports have used the CD-1 mouse to report significant effects of BPA on the mammary gland [26, 59] making them an

important starting model system. More importantly, inbred strains that have identified the MaSC population have solely focused on late puberty to early adulthood populations. Since early pubertal mammary gland alterations following BPAF and BPS perinatal exposure were observed in CD-1 females, this study sought to use the same strain to assess the potential mechanisms of action. Basal cells accounted for approximately 30% of the total cell population at 3 weeks old compared to ≤ 20% of the population prior to puberty and during weeks 4-7. This early increase may be due to the surge of ovarian hormones that begin to circulate to stimulate mammary gland growth. Following enrichment with CD49f and EpCAM, MRU frequencies in the control CD-1 pubertal mouse were shown to range from 1/942-1/7354. 12-week old FVB mice enriched for the same markers had an MRU frequency of 1/100, while an 8-week gland enriched for $CD24^{+}CD29^{hi}$ had a MRU frequency of 1/200 [51]. Study comparisons reveal that in addition to differences in strain that timing and enrichment markers will highly influence MaSC populations. Therefore, great consideration should be taken when choosing a strain to assess basal and luminal properties.

This study is the first to explore the relationship between the MaSC and alterations associated with early life BPAF and BPS exposure. These findings suggest that the pubertal MaSC population may not be the direct target of early bisphenol exposure, at least not at the concentrations that were explored and considering our study design. In fact, the bisphenol treatment decreased the MaSC populations and the ability to repopulate the mammary gland in transplants. Indirect signaling from other cells (i.e. cytokines, adipocytes and fibroblasts) are also critical for normal and altered mammary development and further exploration of their roles will be invaluable.

Table 4-1: Flow Cytometry Antibodies Needed for Cell Fraction Sorting

Antibody	Dilution
EpCAM CD326 Alexa Fluor 647 Anti-mouse	1:4000
CD49f Alexa Fluor 488 Anti-human/mouse	6.25 µl/ 1.0 x 10^6 cells
Alexa Fluor 488 Rat IgG2a, κ Isotype Control Antibody	6.25 µl/ 1.0 x 10^6 cells
Alexa Fluor 647 Rat IgG2a, κ Isotype Control	1:4000

Table 4-2: MaSC-Related Gene Primer List

Gene	Identifier	Vendor
Wnt5a	Mm00437347_m1	Taqman
Wnt1	Mm01300555_g1	Taqman
Notch1	Mm00627185_m1	Taqman
Lrp5	Mm01227476_m1	Taqman
Cdkna1	Mm04205640_g1	Taqman

Table 4-3: Mouse Strain Comparison of MRU Frequency in Controls

	FVB (per gland)	FVB (per female)	C57Bl/6 (per female)	CD-1 (4 glands/female)
Age (weeks)	12	8	8	3-5
Total Epithelial	520,000	~2,000,000	900,00	N/D
MRU	20,000	N/D	N/D	N/D
CFC	80,000	N/D	N/D	N/D
Lin- Population	EpCAMCD49f	$CD24^{+}CD29^{hi}$	$CD24^{+}CD29^{hi}$	EpCAMCD49f
MRU/Total Cell	1/100	1/200	N/D	1/942-1/7354
Reference	Stingl/Watson [51]	Visvader/Lindeman [51]	Visvader/Lindeman [51]	Tucker/Fenton

MRU- Mammary repopulating unit

CFC – Colony forming cells

Table 4-4: MRU frequency following transplants of isolated MaSC from bisphenol analogue exposed mammary tissue

Exposure	PND 22 (n)	PND 35 (n)	PND 56 (n)
Control	1/2159 (29)	1/7354 (22)	1/6988 (15)
BPAF 5 mg/kg	1/4123 (28)	1/6237 (25)	1/31997 (20)
BPS 0.5 mg/kg	0 (26)	0 (31)	1/20901 (32)

n= total number of animals that received transplants. MRU frequency = (# positive transplant outgrowths/total # of attempted transplants)$_x$ + (# positive transplant outgrowths/total # of attempted transplants)$_{x+1}$, where x =cell concentration transplanted

A) B)

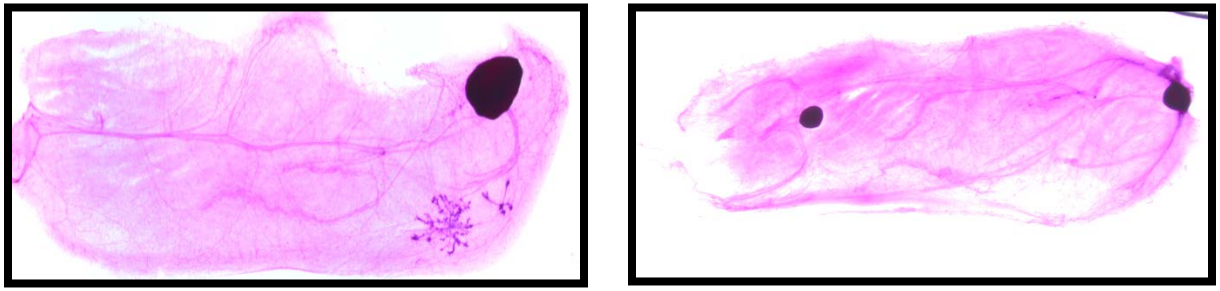

Figure 4-1: MRU Transplantation Analysis. Representative images of whole mounts showing: A) Successful MaSC transplant where growth originates from a central region and bi-directionality of ductal branching and B) Unsuccessful transplant where no epithelial tissue is present post-transplantation.

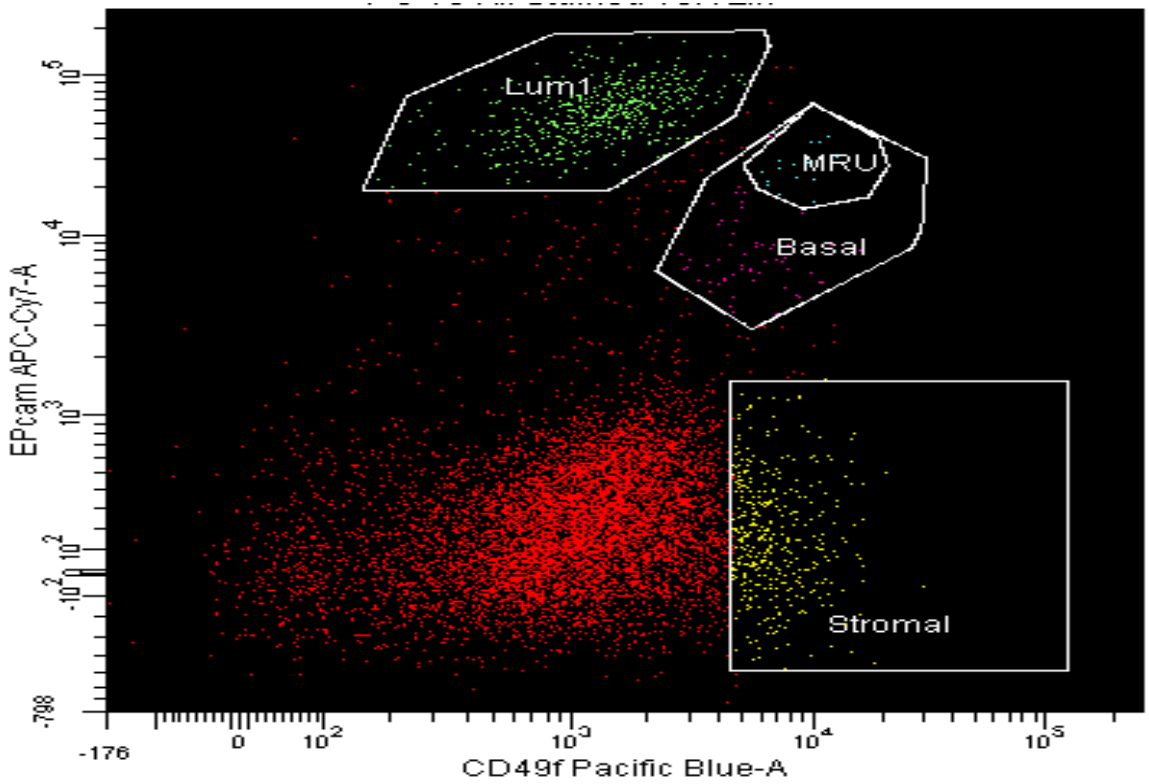

Figure 4-2: Representative Fluorescent Activating Cell Sorting (FACS) of isolated mammary cells stained with EpCAM and CD49f. $EpCAM^{high}CD49f^{med}$ (green) = luminal cells (Lum1), $EpCAM^{med}CD49f^{high}$ (pink; Basal) = basal/myoepithelial cells, $EpCAM^{-}CD49$ (yellow) = stromal. The highest 20% fluorescing cells (blue) within the basal population contains the mammary repopulating unit cells (MRU).

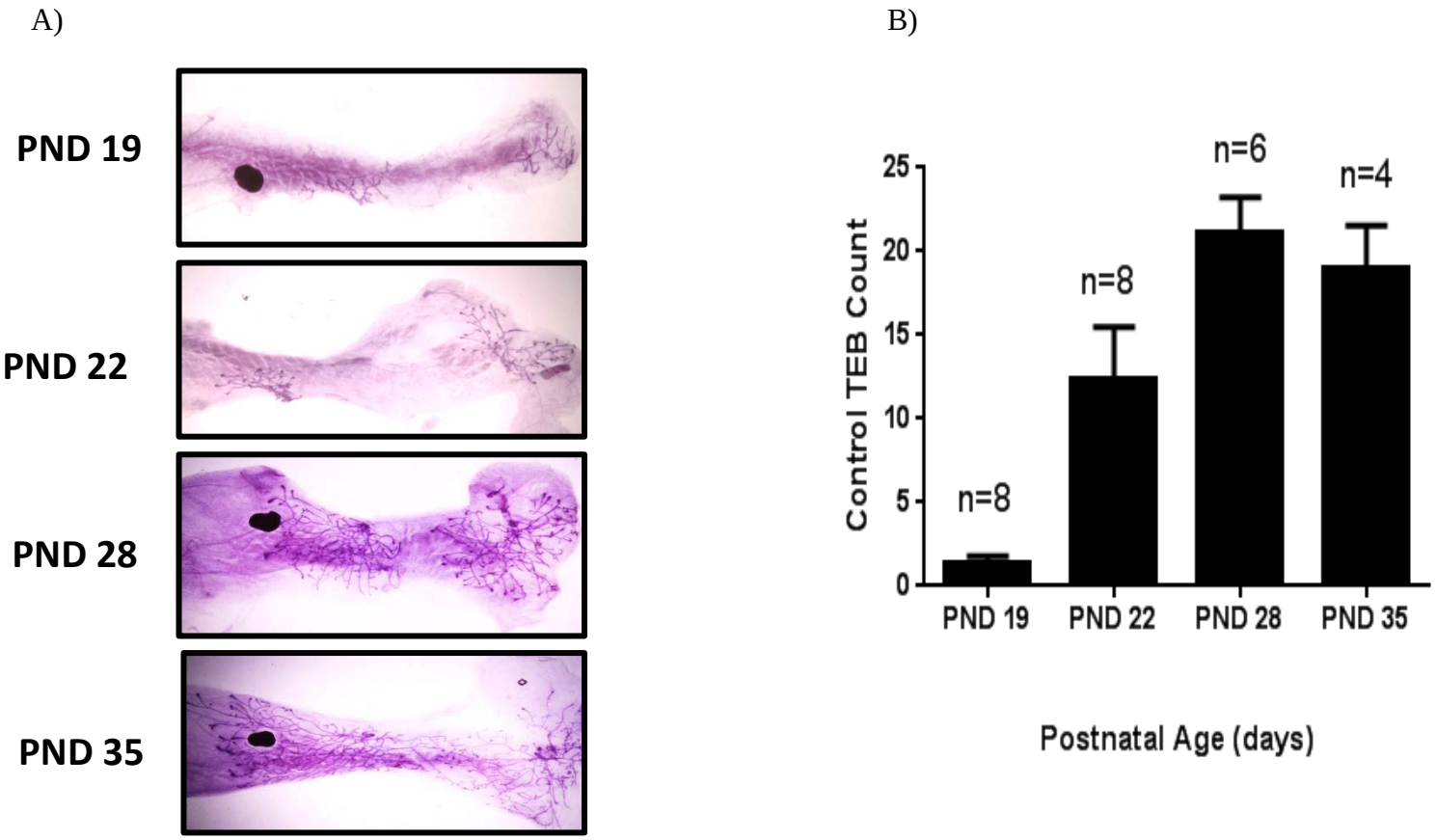

Figure 4-3: Peripubertal development in the untreated CD-1 female mouse mammary gland. A) Representative control mammary whole mounts showing progression of growth through puberty and B) Terminal end buds per gland. n=number of animals per time-point. Mean ± SEM.

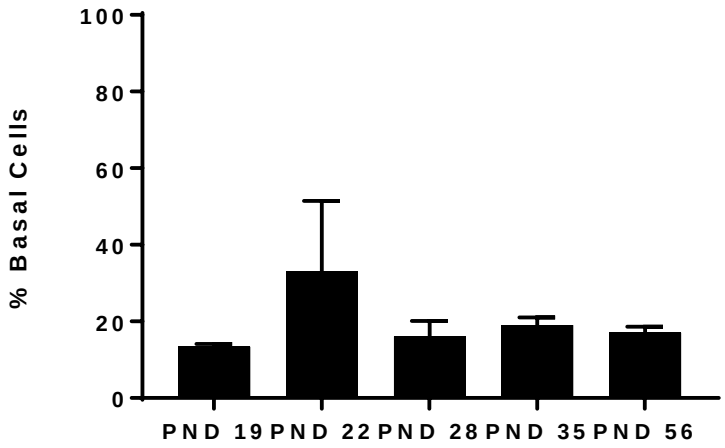

Figure 4-4: Mammary basal epithelial cell counts in control CD-1 female mice following FACs quantification (Peri-pubertal; PND 19-56). PND 19 (n=2), PND 22 (n= 3), PND 28 (n=4), PND 35 (n=7), and PND 56 (n=5). N= number of FACS sorts at each time-point.

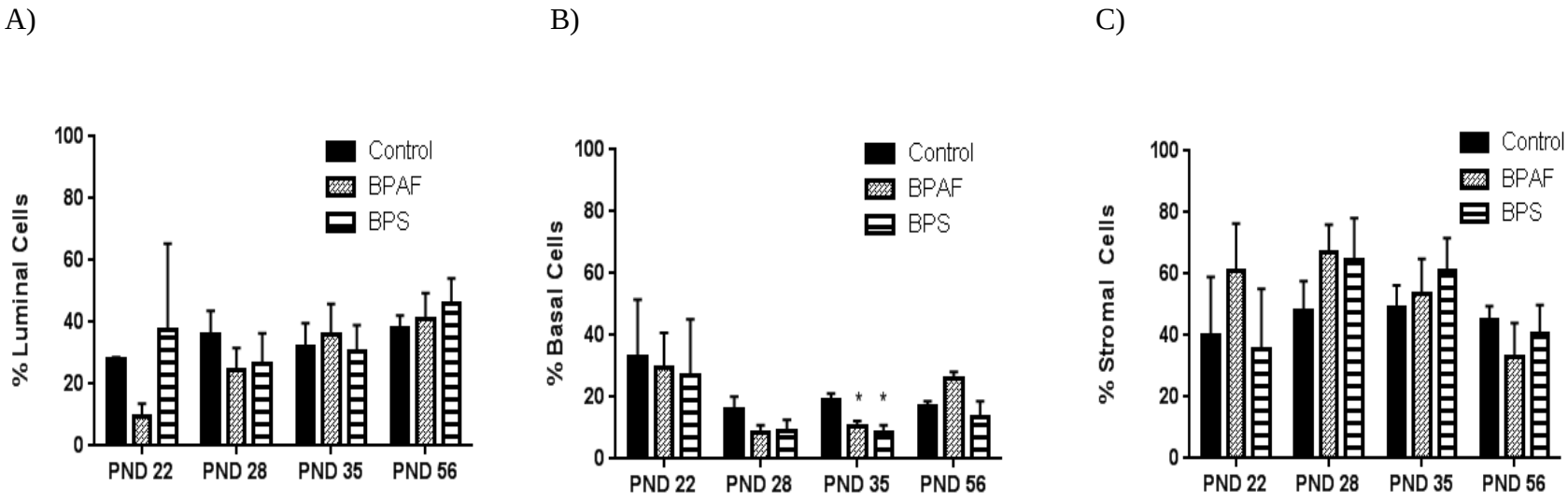

Figure 4-5: FACS sorted mammary cell fractions from control, BPAF or BPS early life exposure mice; evaluated over the course of puberty from PND 22-56. Quantitated fractions shown as a percentage of total isolated cells: A) Luminal, B) Basal, and C) Stromal. PND 22: Control (n = 2) BPAF (n = 3) and BPS (n= 3), PND 28: Control (n =4) BPAF (n = 3) and BPS (n = 3), PND 35: Control (n =7) BPAF (n = 3) and BPS (n = 3) and PND 56: Control (n = 5) BPAF (n= 3) and BPS (n= 3) where n= number of individual FACS sorts completed. All data are presented as the mean ± SEM. % cells were determined by # of cells per population/total # cells. Statistical significance was determined by student's t-test; ($p \leq 0.05$).

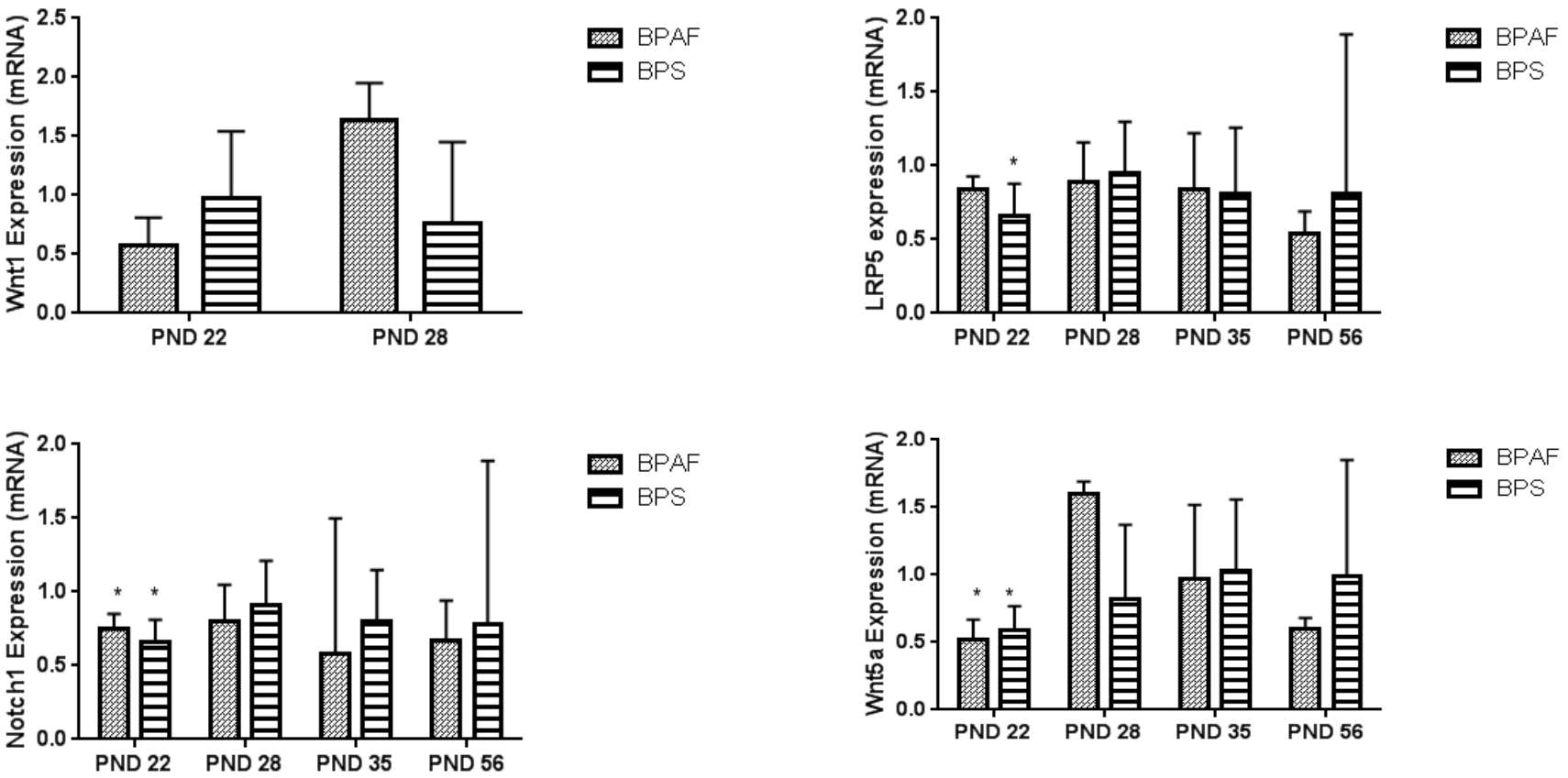

Figure 4-6: Whole mammary gland gene expression of stem and progenitor markers following prenatal exposure to BPAF (n=3) or BPS (n=3). These tissues were taken from animals used for cell fractionation shown in Figure 4-5. All data are presented as the mean ± SEM. Fold induction changes calculated relative to controls (shown as 1.0 on each graph) and all data was relative to the housekeeping gene *Cdkn1a* which does not change in expression over this time-frame in the mammary gland. N= 3-4. Statistical significance determined by student's t-test ($p \leq 0.05$).

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CHAPTER 5

Effects of prenatal exposure to BPAF and BPS on mammary gland gene expression in the CD-1 mouse

OVERVIEW

Bisphenol AF (BPAF) and Bisphenol S (BPS) are endocrine disruptors used to produce fluoroelastomers, plastics and epoxy resins. Similar to BPA, prenatal exposure to these chemicals altered pubertal mammary gland development and increased inflammation and proliferative epithelial hyperplasia, producing tumors in mid-life (Chapter 3). These studies explored gene signatures produced by BPAF and BPS at concentrations shown to induce morphological changes as early as PND22, aiming to determine early life markers of later life disease. Pregnant CD-1 mice were exposed to vehicle (sesame oil), 5 mg/kg body weight BPAF or 0.5 mg/kg body weight BPS via gavage from gestation days 10 to 17. Female offspring were euthanized at postnatal day 20, 34 and 55 and the mammary gland was collected. Whole genome microarray analysis was performed on glands collected at PND 34 and genes from selected pathways were validated by RT-PCR, using RNA from glands from all time-points, so that temporal gene expression could be determined. BPAF exposure altered expression of 44 genes, many of which were associated with immunity (B cell development and antigen presentation), but expression levels of evaluated genes were all unchanged compared to age-matched controls. BPS altered expression of over 400 transcripts that were involved in mitochondrial dysfunction, circadian rhythm, and the TRK/Rac signaling pathways. RT-PCR analysis of RNA from tissues

collected between PND 20 and PND 55 confirmed changes in all (*Aco2*, *Uqcrc1*, *Arntl*, *Nr1d1*, *Per3*, *c-jun*, and *Pi3k*) but one gene (*Map2k7*) identified by the microarray. Gene signature changes were also evaluated for the family of estrogen receptors (*Erra*, *Errg*, *Esr1*, *Esr2*) and several genes involved in inflammation and apoptosis (*Bax*, *Bcl2*, and *Il-6*), but were all similar to controls, with the exception of *Errg* at PND 34. This nuclear receptor was downregulated by BPS exposure. *Evi5* (BPS) and *Tscc2d1 (*BPAF*)*, two genes involved in epithelial cancer formation were also altered at PND 34. These data suggest that several mechanisms are responsible for early pubertal mammary gland changes; they include mitochondrial and circadian genes, and genes involved in cancer formation. Although no single pathway was related to pathology outcomes, these stated pathways may all contribute and manifest in gland malformations that eventually lead to pre-neoplasia and cancer.

INTRODUCTION

Bisphenol A (BPA) is a high volume production chemical that is incorporated into plasticizers, epoxy resins, thermal receipt paper and measured in many consumer products including foodstuff and beverages due to leaching from containers [1-3]. As a result, it is ubiquitous within the environment and has been detected in over 90% of urine [4] and serum [5, 6] samples collected from the general U.S. population. BPA was also detected in maternal serum, amniotic fluid, placenta, fetal sera, and umbilical cord [7-10] and breast milk [11-13], and it can traverse the placenta resulting in fetal exposure. Exposure to BPA during perinatal and peripubertal development and the resulting health effects have been a prime focus of this research field. The current lowest observed adverse effect level (LOAEL) is set at 50

mg/kg/bw/d and the no observed adverse effect level (NOAEL) is at 5 mg/kg/bw/d based on two multigenerational studies that used high BPA exposures [14, 15]. Regulatory guidelines were set according to systemic and offspring toxicity, however, reproductive toxicity, was not calculated as a factor when determining the margin of safety. The margin of safety for both humans and adults was deemed as “adequate” and safe at the current levels occurring in foods. Therefore, based on these data the EPA proposed a recommended daily intake for BPA of 0.05 mg/kg/d. However, several low dose *in vivo* rodent studies have linked BPA to abnormal reproductive development, fertility, neurobehavior, liver function, tumor formation, and pubertal mammary gland development that decreased the timing to and incidence of proliferative and neoplastic lesions during adulthood [16-25]. The 2010 decision by the FDA to ban BPA in baby bottles and formula packaging products [26], and a separate voluntary discontinued use by manufacturers agreement, has resulted in the replacement of BPA in products with chemicals that are structurally and estrogenically similar to BPA, including Bisphenol AF (BPAF) and Bisphenol S (BPS).

BPAF is moderately produced and used in the production of fluoroelastomers and consumer electronics [27]. The six fluorine atoms increase its thermal stability in many products but may consequently increase its persistence within the environment. Exposure to the general population is primarily through oral ingestion, although it may be transdermally absorbed and inhalation exposure to contaminated dust may occur [28-30]. Occupational exposure to BPAF in the molding and casting industry was reported, with nearly half of the ~4300 workers exposed identified as women [31]. The disposition and pharmacokinetics of BPAF are similar to BPA where it is readily absorbed, metabolized by conjugation, and quickly excreted [32]. However, it has been recovered in human urine samples at much lower percentages than BPA [33]. More

importantly, BPAF is more estrogenic than BPA in numerous assays, and it has a greater affinity for the estrogen receptor beta (ERβ) [34]. Therefore, the use of this chemical may create concerns like BPA, especially since very little is known about its affects at low exposures.

A sulfonyl group is situated in between the two phenolic rings, in place of the bridging carbon and methyl groups of BPA, to produce BPS. Many products including thermal receipts, currency bills, foodstuff and beverage containers, and epoxy resins contain BPS, leading to human exposure [3, 28, 35, 36]. In a study conducted by the CDC, urine from convenience samples of adults collected between 2000 and 2014 were tested for a series of bisphenols to determine exposure trends. BPS was detected in the urine in all collection years and increased in detection rate from 25% (2000) to 74% (2014) [33]. BPA remained the highest recovered bisphenol in urine in all years, but an increasing trend of BPS detection in the general population could suggests that exposure to BPS is also on the rise. In a recently published study, BPS was detected in human maternal and cord serum making that the first human confirmation of transplancental transfer [37]. Perinatal exposure in mice altered maternal behavior and produced alterations in the brains of F0 and F1 generations [38]. In addition to being estrogenic, BPS was found to be adipogenic in murine 3T3-L1 preadipocytes and female human primary adipocytes [39, 40]. Collectively, these new findings propose a role for BPS to induce affects in endocrine regulated tissues, especially when exposure is early in life.

Mammary gland effects following early life, human relevant, exposure to BPA are well documented in rats and mice. The mammary gland is only a rudimentary ductal structure during late gestation and early perinatal development. During puberty, the ovarian hormones estrogen and progesterone influence mammary epithelial structure, as well as endogenous hormones and growth factors, for promotion of ductal elongation and side branching, led by TEBs. TEBs are

highly mitotic structures and are the targets of transformation because they contain the mammary stem cell population [41]. Early life exposure to BPA led to morphological changes during pubertal development that included increased TEB formation, ductal extension, and branching points as well as a reduction in apoptosis [20, 24, 42]. These early life changes resulted in pre-neoplastic and neoplastic lesions in mammary glands [43, 44].

BPAF and BPS accelerated pubertal mammary development and induced preneoplastic and neoplastic lesions following prenatal exposures in CD-1 mice (Chapter 3). BPAF has been recovered in elution fractions of human abdominal and breast adipose tissue [45]. The estrogenicity and anti-adrogenicity [34, 46-49] of these chemicals are postulated to regulate certain endocrine tissues (i.e. mammary glands, ovaries and testes, etc.) by binding to the estrogen receptor (ER), androgen receptor (AR) or through non-genomic binding [50]. Liver injury following BPA exposure has been attributed to increased oxidative stress and reactive oxygen species (ROS) generation [51, 52]. However, in the mammary gland, the mechanism(s) leading to accelerated development and neoplastic formation are still poorly understood. This may be due to factors that include the complexity of the mammary gland and continuous changes in morphology that occur during different stages of development. For BPAF and BPS, these mechanisms have yet to be explored and one could assume that similar tissue challenges may arise.

To identify genetic and signaling alterations during pubertal mammary development which may act as early biomarkers to predict risk of later life abnormalities, tissues prenatally exposed to BPAF and BPS doses previously shown to significantly produce proliferative lesions and inflammation (Chapter 3) were evaluated by microarray and RT-PCR. Postnatal day 34

(PND 34) mammary glands were assessed by whole genome microarray and candidate genes were validated by RT-PCR of mammary gland RNA isolated at PND 20, 34, and 55.

MATERIALS AND METHODS

Animals

Timed pregnant CD-1 mice were obtained from the National Institute of Environmental Health Sciences (NIEHS) in-house breeding facility. This animal stock was originally obtained from the Charles River R16 facilities (Raleigh, NC). Dams were weighed, randomly assigned to a treatment group, and individually housed in polypropylene cages previously screened for estrogenicity. San-Chip Hardwood bedding (PJ Murphy Forest Products, Inc. Montville, NJ) was supplied in all cages. Dams received AIN-93G chow (Harlan Laboratories, Indianapolis, IN) and tap water *ad libitum* in bottles that were screened for estrogenicity prior to being given to each animal. All procedures and handling were in accordance with the NIEHS Institutional Animal Care and Use Committee. Animal rooms were maintained on a 12:12 hr light-dark cycle at a controlled temperature of 20-40°C with 40-60% humidity. Precautions were taken for exogenous bisphenol contamination as previously described in Chapter 2. Dams were weighed daily prior to dosing. Any dam that produced a litter of ≤ 4 pups was excluded from all analysis.

Chemicals

BPS ($\geq$ 97.5 % pure) was purchased from Sigma Aldrich (St. Louis, MO) and BPAF ($\geq$99.0% pure) was obtained from 3B Pharmachem International Co. Ltd (Wuhan, P.R. China).

Chemical purity was confirmed by the NTP chemical contract laboratory prior to dosing. Dosing solutions were prepared daily by dissolving the chemicals into pure sesame oil (Jedwards, International, Inc., Braintree, MA) followed by gentle vortexing (VWR, Scientific Products, Radnor, PA). All daily solutions were prepared in glass vials to reduce bisphenol contamination. All animals were administered sesame oil (vehicle), BPAF or BPS by blind allocation in a volume of 10 µl solution/g body weight.

Experimental Design

Following a 2-3 d acclimation period, timed pregnant dams (n=9-10) were administered a twice daily oral gavage of vehicle control, BPAF (5 mg/kg) or BPS (0.5 mg/kg) due to the chemicals' short half-lives and to ensure that the chemicals were not completely cleared prior to the next dosing day (Chapter 2). Exposure occurred between gestational days 10 and 17 (GD 10-17; the period when rudimentary mammary placodes form and a critical timing of chemical exposure on the developing mammary gland). In a 14-month developmental study, accelerated mammary development and mammary lesion incidences (i.e. tumors, hyperplasia, and inflammation) were highly observed in female offspring from these two dose groups (Chapter 3). Animals gave birth on the eve of GD 18 and the next day was deemed postnatal day 1 (PND 1). Litters were culled to 10 pups on PND 3 and on PND 20, 34 and 55 the inguinal mammary glands from both sides of the animal were removed from the female offspring, placed in RNALater (Invitrogen, Waltham, MA) at 4˚ C overnight and frozen at -80˚C for future use.

Total RNA isolation

Mammary tissues from PND 34 were homogenized in Trizol Reagent (Ambion, Carlsbad, CA) in 2 ml Lysing D Matrix RNase/DNase free tubes (MP Biomedicals, Eschwege, Germany) that contained sterile 1.4 mm ceramic spheres. All samples were homogenized on a MP Fast Prep-24 5G (MP Biomedicals, Santa Ana, California) at 6.0 m/sec in repeated 40 s intervals followed by 5 min on ice until no tissue was visible. The supernatant was transferred to a clean microcentrifuge tube and the remainder of the extraction was performed per the manufacturer's protocol (i.e., 200 µl chloroform, 500 µl isopropanol, and 1 ml 75% ethanol). RNA clean-up and DNase I digestion was completed using the RNeasy Mini Kit (Qiagen, Hilen, Germany). Total RNA quantification was performed on a NanoDrop 2000c (Thermo Scientific, Wilmington, DE) and RNA integrity was measured using the RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) per the manufacturers 'protocols. Samples with a RNA integrity number (RIN) $\geq$ 7.9 were considered acceptable for microarray analysis.

Microarray Preparation and Data Analysis

Five mammary glands each from vehicle, BPAF and BPS were chosen for analysis and processed on an Affymetrix Mouse Genome 430 2.0 platform (Affymetrix, Santa Clara, CA) by the NIEHS Molecular Genomic Core Laboratory (RTP, NC). Differentially expressed probe sets between experimental groups were determined using analysis of variance (ANOVA). For each time point, probe intensity data from GeneChip® Mouse Genome 430 2.0 arrays were read into the R software environment (http://www.R-project.org) directly from .CEL files using the R/affy

package [53]. Data quality was assessed using image reconstruction, histograms of raw signal intensities, and scatterplots of normalized data. Normalization was carried out using the robust multiarray average (RMA) approach [54] to form one expression measure per probe set per array. Briefly, the RMA approach adjusts the background of perfect match (PM) probes, applies a quantile normalization of the corrected PM values and calculates final expression measures using the Tukey median polish algorithm. Gene expression measures were expressed in the fixed effects ANOVA model using $Y_i = \mu + GROUP + \varepsilon_i$ [55] where Y_i represents $\log_2$ transformed expression data, μ is the mean for each array, GROUP is a 3 level factor identifying each experimental mouse group (untreated vehicle control, BPAF and BPS), and ε_i is random error. A modified F-statistic incorporating shrinkage variance components [56] was used to perform pairwise contrasts of interest (vehicle vs. BPAF and vehicle vs. BPS). *P* values were calculated by permuting model residuals 1,000 times using the R/maanova package [57]. False discovery rate (FDR) values were estimated in R using the p.adjust function based on the Benjamini-Hochberg step down procedure [55]. Unless otherwise noted, significant changes were determined at the FDR threshold of 0.1-0.2. Affymetrix probe sets were mapped to genes using Ingenuity Pathway Analysis (IPA) (Redwood, CA).

RT-PCR

Total RNA was extracted from frozen mammary tissues (3-5 tissues/treatment/time-point) in the exact fashion as the tissues prepared for microarray analysis. Briefly, glands were isolated with Trizol reagent, then purified and precipitated, following DNase I treatment, using an RNeasy Mini Kit. RNA quantity and integrity were performed using the methods described, with a quality cut-off above RIN $\geq$ 7.9. One microgram of RNA was reversed transcribed using

the High capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA) and amplified in the Thermocycler (Bio-Rad) per the manufacturers' instructions. Briefly, cDNA was mixed and prepared with Power SYBR PCR master mix (2x) (Applied Biosystems) and mouse primers identified by IPA or reference genes and measured on a QuantStudio 7 Flex (Applied Biosystems). Transcript analysis was performed on Quantstudio Real-Time PCR Software and Microsoft Excel 2010. Only samples with a CT value of ≤ 35 or standard deviation between duplicates of ≤ 0.5 were included in the final analysis. Fold change was calculated using $2^{-\Delta\Delta Ct}$. Briefly, the reference gene (RPL19) for each sample was averaged for each treatment group and normalized for each sample within the same group for each target gene to obtain the average delta CT (ΔCT). ΔΔCT was calculated using the formula ΔCT –Δvehicle. All results are represented as the log fold change.

Reference and target genes were purchased from Integrated DNA Technologies and include: *Nr1d1*, *Per3*, *Arntl*, *PikK*, *Map2k7*, *c-jun*, *Uqcrc1*, *Aco2*, *Erra*, *Errg*, *Esr1*, *Esr2*, *Bax*, *Bcl2*, *Il-6*, *Evi5*, *Rnf168*, *B3galt5* and *Tsc22dl*. Mouse homologs for *HLA-A* (*H2-Q4*) and *HLA-DRB5* (*H2-Eb1*) were identified by NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/) and the locus with the greatest homology to the human form was used to design the primer. The forward and reverse primers were input into the IDT PrimerQuest tool (https://www.idtdna.com/Primerquest/Home/Index). Primers with an amplicon length of ~100 base pairs and GC content of ~50% were chosen. A description of all primers used in this study is illustrated in Table 5-1.

Statistical Analysis

Unless noted, all analyses are represented as the mean ± SEM and the dam is considered the unit of measurement. All analyses are a comparison between vehicle control and chemical treatment and were calculated using at student's t-test (two tail) where $p \leq 0.05$ is considered statistically significant. All RT-PCR samples were run in duplicates for each gene. If the standard deviation of the duplicates was ≥ 0.5, the entire sample was removed from the analysis. All graphs were constructed in GraphPad Prism 7.

RESULTS

To correlate the morphological changes observed following prenatal exposures to BPAF and BPS with potential genes and mechanistic pathways, a genome-wide microarray was performed on mammary gland RNA from vehicle control, BPAF (5 mg/kg) and BPS (0.5 mg/kg) treated, PND34 CD-1 mice. The principal component analysis (PCA) for vehicle, BPAF and BPS are illustrated in Figure 5-1 and shows distinct separation between treatment groups for most of the tested samples.

Early BPS exposure alters genes involved in the circadian clock and mitochondria. Approximately 39,000 mapped transcripts were identified in the microarray. Once duplicate genes were removed and a single probe for each gene was accounted for, ~20,400 genes were used in the analysis. Filtering with an FDR ≤ 0.10 (10 %) returned 114 genes that were significantly altered between vehicle and BPS treatment. Ingenuity Pathway Analysis (IPA) ranks the Top Networks based on the number of altered genes from the data set and showed that BPS treated glands were highly associated with 1) developmental disorders, neurological

disease, and DNA replication, recombination, and repair (24 genes), 2) cell morphology, assembly, and organization (22 genes), and 3) lipid metabolism, small inflammatory response, and cellular function and maintenance (15 genes). A list of the top 10 up and down regulated genes is shown in Table 5-2. The Top Cellular and Molecular Functions identified were cell cycle and movement, DNA replication, recombination and repair and lipid metabolism (Table 5-3). When a less stringent FDR (≤ 0.20) was applied to the data set, the number of genes increased to 422. Similar to the 0.1 FDR analyses, the Top Networks included cellular assembly, DNA replication recombination and repair, RNA post-transcriptional modification and lipid metabolism. The Top Molecular and Cellular Functions included energy production, lipid metabolism and RNA post-transcriptional modification (Table 5-3). The increased gene set also showed that the Top Tox lists were mitochondrial dysfunction, fatty acid metabolism and increased transmembrane potential of mitochondria and mitochondrial membrane.

Several canonical pathways that contained overlapping genes were identified. Three pathways that were likely associated with mammary gland changes were further investigated and included mitochondrial dysfunction, circadian rhythm and neurotrophin/TRK/Rac signaling. Analysis of the circadian pathway showed up-regulation of *Period (Per3), Rev-ERBα (Nrid1), Dec,* and *Creb* (Figure 5-2). Alterations in mitochondrial genes mostly occurred in the inner membrane where Complex I-IV of the electron transport chain takes place (Figure 5-3). These genes included NADPH dehydrogenases (*NDUF*), succinate dehydrogenases (*SDHA-D*), cytochrome bc1 (*Uqcrc1* and *CYTB*), and cytochrome c oxidase (*COX3*). Several other genes located in the outer membrane and in the mitochondrial matrix were activated and included *Aconitase 2*, *GPD21* and *ATP5A1*. Map2k7 (-1.367) and PTPN11 (-1.70) expression were downregulated and associated with the Rac and Neurotrophin/TRK pathways.

Upstream regulators were also predicted based on gene expression changes from the data set and are assigned an activation (≥2) or inhibition (≤ 2) z-score. Based on p-values, the top five predicted upstream genes associated with inflammation/immunity, mitochondrial dysfunction and cancer were selected. The genes with an FDR ≤ 0.10 were tumor protein 53 (*TP53*), nuclear protein 1 (*Nupr1*), aryl hydrocarbon receptor nuclear translocator like 1 (*Arntl*), and Period 2 (*Per2*) (Table 5-4). Z-scores were only assigned to *Nupr1* (1.00) and *TP53* (z- score =0.48), but a definitive prediction could not be determined due to the scores. Top upstream predicted genes evaluated with an FDR ≤ 0.2 included the transcriptional regulators estrogen related receptor alpha (*Errg*), beta-estradiol, lysine demethylase 5A (*Kdm5a*), peroxisome proliferator alpha (*Pparα*), peroxisome proliferator co-activator 1 alpha (*Ppargc1a*, *Per1* and *Per2). Pparα* and *Ppargc1a* were predicted to be activated due to positive z-scores (2.80 and 2.46, respectively), whereas Kdm5a was predicted to be inhibited (z-score -3.05). Noteworthy was the fact that other members from the PPAR family (*Pparγ* and *Pparδ*) were all upregulated and predicted to be activated (z-score = 2.77-3.02), and insulin receptor (*Insr*) and insulin receptor substrate 1 (*Irs1*) were also predicted as activated (*data not shown*). Fibroblast growth factor 21 (*Fgf21*), a known biomarker for mitochondrial disease was predicted to be activated. *Rb1* is downstream of *Kdm5a* and functions to inhibit cell cycle progression, but also functions to drive cells toward a differentiated state. Cells that don't express *Rb1* experience decreased oxygen consumption and altered mitochondria function [58]. In this data set, *Rb1* had a positive z-score and was considered to be activated.

RT-PCR was completed on a select number of genes from each identified pathway as well as other genes from the Top Ten altered list. PND 34 glands were first considered in the validation effort, followed by RNA from tissues acquired on PND 20 and 55 (Table 5-5). The

PCR analysis confirmed that Aconitase (*Aco2)* and ubiquinol-cyctochrome c 1 (*Uqcrc1)* were both significantly up regulated at PND 34, but were unchanged at PND 20 and 55. Validation of circadian clock genes revealed that nuclear receptor subfamily 1 group D member 1 (*Nr1d1*) was increased at PND 34 and 55, whereas Period (*Per3*) was increased at PND 34, but no other time points. Aryl hydrocarbon receptor nuclear translocator like (*Arntl),* a downstream target of *Nr1d1* and *Per3,* was significantly reduced at PND 20. Selected genes that are common to the Neurotrophin/TRK and Rac pathways were also evaluated by PCR. *Pi3k* was increased at PND 34, whereas *Map2k7* was unchanged at all time-points. *c-jun* was not changed on the microarray, but at PND 20 was decreased. Ecotropic viral integration site 5 (*Evi5*) is a GTPase activator involved in cell proliferation and linked to adenocarcinoma and epithelial cancers; it was increased at PND 34, but decreased in the microarray. The family of estrogen receptors were assessed since BPS is estrogenic and previous studies showed that nuclear receptor expression was unchanged in late adulthood (Chapter 3). Estrogen receptor alpha and beta (*Esr1* and *Esr2*) and estrogen related receptor gamma and alpha (*Errg* and *Erra*) were analyzed by PCR at all time points; expression in treated and control glands were similar. Fischer et al. [59] showed that following prenatal BPA exposure that genes involved in immunoregulation were altered in the mammary gland. Although changes in the microarray were not apparent, we were interested in determining if we would observe individual genes changes with PCR since we saw increased histopathological findings of inflammation from our previous 14 mo. study (Chapter 3). The cytokine Interleukin-6 (*Il-6*) and two master regulators of cell death, B-cell lymphoma-2 (*Bcl2*) and Bcl2 like protein 4 (*Bax*) were analyzed. Interestingly, none of these genes were significantly different between treated and vehicle control glands evaluated at exposure to PND 20 and 55.

Early BPAF exposures results in transcriptional alterations in the immune system. The same analysis was performed on mammary glands from BPAF exposed mammary glands. When samples were filtered using a FDR ≤ 0.10 (10%) only 6 genes were altered (Table 5-6). Selective Top Networks identified by IPA included metabolic disease, lymphoid tissue structure and development, and cancer. Since there were a limited number of genes identified many of the Top Cellular and Molecular Functions were linked to cell cycle maintenance, structure and cell to cell signaling (Table 5-7). To increase our pool of candidate genes, the FDR was broadened to 0.20 (20%) and increased the number of altered genes to 44. This change identified inflammatory disease and response (20 genes) and cell cycle/interaction (17 genes) in the Top Cellular and Molecular Functions. In addition to the top functions that were determined at a FDR ≤ 0.1, energy production and cellular growth and proliferation were identified once the FDR was increased. Top Tox lists genes included PXR/RXR/RAR activation and AHR signaling.

Many of the altered genes were clustered into canonical pathways that involved immune regulation including antigen presentation, OX40 signaling and B cell development. The four genes related to these pathways were major histocompatibility complexes I and II, specifically *HLA-A*, *HLA-DRB5*, *HLA-DMB,* and *HLA-A*. All play a role in T4 and T8 antigen presentation and activation. HLA-A was the only gene found upregulated (4.61); HLA-DMB (-3.25), HLA-DRB5 (-6.85), and HLA-E (-2.26) were all downregulated.

A list of selective predicted upstream regulators is illustrated in Table 5-8. Very few genes were assigned a z-score prediction with the exception of myeloid differentiation primary response 88 (*Myd88*), a member of the death domain superfamily that is associated with several neoplasia's and lymphoid diseases. *Myd88* was downregulated and predicted to be inhibited (z-score = -2.16). Genes involved in the inflammatory response with significant p-value overlaps,

but not given a prediction, included *Il10ra*, *Il6*, *Il1b*, and *Ifng* (z-score = -1.98, -1.32, -0.70 and -0.99). The endocrine related gene *Erbb2* and chemical beta-estradiol (z-score = 0.15 and -0.53) were also considered to be potential upstream regulators.

Similar to BPS, select genes of interest from pathways identified by IPA were evaluated by RT-PCR. Mammary glands from PND 34, as well as PND 20 and 55, were analyzed to establish if the genetic changes were persistent. Selected genes and their expression levels can be found in Table 5-9. Genes involved in B cell development and antigen presentation were evaluated. The microarray identified the human HLA homologs; however, human primers do not amplify mouse tissues. Therefore, mouse homologs were identified by the NCBI database and validated using primers with a similar genetic sequence. *H2-Q4* was run for *HLA-A* and *H2-Eb1* for *HLA-DRB5*. At PND 34, *H2-Eb1* expression was unchanged, unlike its downregulation that was observed in the microarray. Although significance was not achieved at PND 20 and 55, *H2-Q4* trended towards significance (p = 0.06-0.07). Statistical changes were not observed at any time-point for H2-Q4. Beta-1,3-galactosyltransferase 5 *(B3galt5*) and TGF-β stimulated clone 22 domain family 1 (*Tsc22d*1) were within the top up and downregulated genes within the microarray and both play a role in diseases associated with adenocarcinoma, squamous cell carcinoma and carcinomas (Table 5-6). *Tsc22d1* was upregulated at PND 20 and unchanged at PND 34, although it was downregulated in the microarray. By PND 55, expression trended toward down-regulation (p = 0.07). *B3galt5* expression was unchanged at PND 34, although it was down-regulated in the array (-1.28). Studies that have confirmed BPAF as estrogenic also prompted us to evaluate the family of estrogen receptors [34, 48]. Estrogen related receptor alpha (*Erra*), estrogen receptor alpha (*Esr1*), and estrogen receptor beta (*Esr2*), were not altered between PND 20 and 55. Estrogen related receptor gamma (*Errg*) was significantly upregulated

at PND 34. The cytokine Interleukin-6 (*Il-6*) and two master regulators of cell death, B-cell lymphoma-2 (*Bcl2*) and Bcl2 like protein 4 (*Bax*) were analyzed. Interestingly, none of these genes were significantly different from vehicle controls during PND 20 and 55. *Il-6*, *Bax* and *Bcl2* were unchanged at all time-points.

DISCUSSION

Previous studies within this lab have identified increased TEBs, longitudinal growth and branching density that progressed into inflammation, hyperplasia and adenocarcinoma in mammary glands following prenatal BPAF and BPS exposure (Chapter 3). However, the mechanism in which this occurs has not been explored and still poorly understood in BPA exposed mammary glands. In this study, mammary glands from low dose prenatal exposure to BPAF and BPS were evaluated to understand mechanistic changes that were related to early morphological changes. Changes in immunoregulatory genes including *HLA-A* (*H2-Q4*) and *HLA-DRB5* (*H2-Eb1*) and a membrane bound estrogen receptor, *Errg*, were identified as possible mediators of effect in BPAF exposed mammary glands. In glands from BPS treated mice, genes involved in the circadian rhythm, mitochondrial dysfunction and TRK and Rac signaling were of interest. These data suggest that the dynamics of multiple gene signaling events are likely leading to the morphological alterations that affect later life pre-neoplastic and neoplastic mammary lesion development.

Interestingly, the number of genes that were identified as statistically altered by BPAF were ~8x (44 genes) lower (FDR $\leq$ 0.20) compared to changes observed in the BPS treated mammary glands (~400 genes). Animal to animal variability is the likely cause of this, as many

of the BPS samples clustered more closely to one another on the PCA, compared to BPAF glands where some samples were more like vehicle glands. If this is the case, then potentially increasing the sample size or excluding animals that were like controls may increase the number of altered genes. However, mammary glands following BPA exposure have also been shown to produce limited transcriptional changes [42, 60].

Since only a small number of genes were changed following BPAF exposure, limited pathways that involve these same genes were identified. These pathways included antigen presenting B-cell development, and OX40 signaling, and involved genes associated with the major histocompatibility complexes I and II (MHC). HLA-A is a part of the class I complex. When bound to CD8 this complex presents cytosolic proteins to CD8+ cytotoxic T lymphocytes. We chose the *H2-Q4* mouse homolog for validation because it shared 69% homology with human HLA-A. Downregulation of this class of genes in primary tumors, including breast tissue, are thought to promote tumor metastasis [61], because the tumor cells are able to evade immune targeting. *HLA-A*, however, was up-regulated in the microarray, but was unchanged with PCR. HLA-DRB5 and HLA-DM5 are a part of the complex II class and were both downregulated in the microarray. MHC complex II genes are found on many cell types including epithelial cells and are responsible for presenting extracellular proteins to CD4+ T cells by phagocytosis. PCR analysis of the HLA-DR5 homolog *H2-Eb1* showed no changes at PND 34, but exhibited a downregulated trend toward significance at PND 20 and 55. If this trend were to become significant during later development, then toxic cells may be able to successfully evade immune surveillance, increasing potential risk for tumor progression.

Several pathways involved in normal and abnormal mammary development and cancer were identified following BPS exposure. Genes from the circadian rhythm pathway were altered

(Figure 5-3). *Bhlhe40, Nr1d1,* and *Per3* were all upregulated in the microarray. We chose to evaluate *Nr1d1, as well as Per3,* and *Arntl,* which are downstream of *Nr1d1*. *Per3* and *Nr1d1* validated the microarray findings, and *Arntl* was significantly downregulated at PND 20 and 55. Women who work night shifts have increased breast cancer risk, thus this group of circadian rhythm genes may have impact on cancer development [62, 63]. Camacho et al. also showed that at EE2 (5.0 mg/kg), but not BPA, caused decreased expression of *Clock,* a transcription regulator that dimerizes with *Arntl,* in the mammary gland.

Over 15 genes related to mitochondrial dysfunction were altered in BPS-treated mammary glands including *Aco2* and *Uqcrc1* (Figure 5-4). *Aco2* is an enzyme that catalyzes citrate to isocitrate and has been shown in several studies to play a role in prostate cancer. Under normal conditions it is inhibited, but in cancer it is restored and increases citrate oxidation and decreases fatty acid oxidation [64, 65]. In the microarray, *Aco2* was upregulated in both the microarray and by PCR (1.90). Similarly, Uqcrc1 a component of the ubiquinol-cyctochrome c complex III was also upregulated in the microarray and via PCR (1.53). This gene is involved in catalyzing electrons from ubiquinone to coenzyme Q 10, and mutations in the mitochondrial DNA of the cytochrome bc1 family have been linked to breast cancer [66]. Therefore, early dysfunction of these genes could suggest a mechanism for the phenotypes observed in later life. The TRK and Rac pathways share many similar genes and are involved in cancer formation because they signal downstream to drive transcription of *c-jun*. *c-jun* is overexpressed in MCF-7 cells and involved in breast cancer metastasis [67]. *Map2k7* and *Pi3k* were downregulated in the microarray. These genes and c-jun were chosen for PCR validation. Contrary to the microarray, *Pi3k* was upregulated at PND 34 and *c-jun* was significantly reduced at PND 20 and 55.

BPA, BPAF, and BPS have all been identified as estrogenic due to their ability to bind to the estrogen receptor but only at concentrations much greater than 17β-estradiol therefore making them weak estrogens. Since these chemicals are estrogenic, and the mammary morphologies identified previously (Chapter 3) following exposure to these chemicals are similar to those produced by estradiol or EE2, it was thought that the mechanism for mammary effects were estrogen related. BPAF and BPS are agonists for ERα, but BPAF is considered a full antagonist for ERβ, and a predominant isoform has not been identified for BPS-related estrogenicity [34]. Therefore, we performed PCR on BPAF and BPS mammary glands with *Erra, Errg, Esr1,* and *Esr2,* even though the microarray did not show changes in these genes. BPAF has a greater binding affinity to ERα compared to ERβ [34], but is considered a full antagonist for ERβ and currently the preferential estrogen receptor for BPS binding has not been established. Our previous findings showed that following prenatal exposure to BPAF (5 mg/kg) and BPS (0.5 mg/kg) *Esr1* expression was unchanged at 8 and 14 mo. (Chapter 3). Similar to adult mammary *Esr1* expression, that in BPAF and BPS-exposed pubertal mammary glands was also unchanged. None of the other estrogen related genes were altered at any time-point except for *Errg* at PND 34 (increased 1.23 fold). These data suggest that effects of BPS and BPAF on the mammary gland may be indirectly mediated by the ERs, but no clear direct indication is apparent. Further testing will be required to determine this relationship.

We were also interested in the effects on BPAF and BPS on immune changes. As mentioned earlier, BPA exposure caused reduced expression of cytokines, chemokines, interleukins and leukocyte markers in the mammary gland [59]. This was also confirmed in rats by Camacho et al. [60] who showed decreased *Ccl11, Ccl5,* and *Il4ra* expression. In Chapter 3, BPAF and BPS caused inflammation in mammary glands that increased significantly with age,

and in some cases dose. Macrophages and leukocytes play a critical role in normal and breast cancer microenvironment [68, 69]. In our study, PCR was performed on BPAF and BPS exposed mammary RNA using a select number of genes from the Fischer et al. study [59], but *Il6*, *Bax* and *Bcl2* expression were unchanged. However, several genes were altered in the microarray, and the three that were chosen may not have been a good representation for these chemicals or pathway. It should be noted that several cytokines (*Ilb, Il1a, Il10ra,* and *Infg*) were all predicted upstream targets by IPA due to changes in other genes involved in immune regulation and those genes were not tested by PCR. Therefore, immune dysfunction in the mammary gland following BPAF and BPS exposure should still be considered as a potential mechanism, especially since we observed increased inflammation that was detected in cycling animals and persisted over time in previous studies.

Finally, we wanted to explore genes that played a role in cancer initiation, promotion, or progression. BPS exposure resulted in downregulation of *Evi5* and *Rnf168* in the microarray. *Evi5* was the only gene changed in PCR analyses and was upregulated, contrary to the microarray findings. *Evi5* is a GTPase activator that whose role in the cell involves proliferation and cell formation. When *Evi5* was ectopically expressed in hepatocellular carcinoma cells, tumor migration and invasion were reduced [70]. *Tsc22d1* and *B3galt5* were both downregulated in BPAF-exposed glands in gene arrays, but were unchanged when PCR was performed at PND34. *Tsc22d1* was however, upregulated at PND 20. *Tsc22d1* works as a tumor suppressor by inhibiting Ras/Raf activation. These conflicting findings between the microarray and PCR expression will require a further look at protein expression levels to determine *Evi5* and *Tsc22d1* roles in BPAF and BPS treated mammary gland, and possibly an evaluation of these genes at time points closer to when pre-neoplastic lesions and adenocarcinoma were developing in our

prior studies. Both genes have also been implicated in epithelial cancers and adenocarcinoma. It should be noted that several other genes that play a role in cancer were identified by both treatments, and will be evaluated in the future.

When comparing bisphenol induced transcriptional changes between different studies it is important to note that the amount and developmental time period (i.e. prenatal, pubertal or adulthood) of exposure may result in different gene signatures. When transcriptional analysis of mammary glands from BPA (250 µg/kg/bw/d) prenatally (GD 9- PND 1) exposed rats were performed only a small number of gene changes were observed at PND 4 (alpha-lactalbumin), that were unchanged at PND 21. It was not until PND 50 that most the gene changes were found, and they included genes involved in cell cycle regulation [71]. Similarly, Moral et al. [42] showed that when rats were exposed to BPA (25 or 250 µg/kg) between GD 10 and 21 that a small set of genes from female offspring mammary glands were altered at PND 21, and less at PND 35, but many gene changes from both dose groups were observed at PND 50, and in the highest dose at PND 100. However, increased TEBs were observed as early as PND 21. These data suggest that while morphological changes occur very early during puberty that possibly the greatest effects don't occur until well after these changes have occurred. Very few gene changes were observed in the mammary gland at PND 34 following BPAF exposure even when the FDR was increased and even fewer changes were observed in a microarray that was completed using mammary tissue from littermates at PND 20. Therefore, it is quite possible that if we had performed transcriptional analysis at time points later than PND 34, and closer to the times we saw phenotypic responses that we may have observed additional changes. Future transcriptional studies should be performed at later time-points.

This first study of the gene changes induced in the mammary gland of prenatally BPAF- and BPS-exposed mice during early life stages indicates some of the latent health effects that were detected in previous work (Chapter 3). Many of the cellular functions include changes in cell cycle and differentiation, as well as changes that are critical in breast cancer development (circadian rhythm and mitochondrial dysfunction), and as a result may serve as a precursor for abnormal mammary growth. Although only one time-point was assessed by microarray, PCR confirmed that some changes were sustained over time and could persist into later age. Further studies at different time-points will be necessary to confirm this, but many of these findings have also been observed following early life exposures to BPA and should warrant concern regarding human exposure and health outcomes.

Table 5-1: Gene Primer List

Gene	IDT Primers
Per3	Mm.PT.58.12973804
Nr1d1	Mm.PT.58.17472803
Arntl	Mm.PT.58.11121936
Pi3k	Mm.PT.58.5285167
Map2k7	Mm.PT.58.28392891
c-jun	Mm.PT.58.32691984.g
Uqcrc1	Mm.PT.58.42202784
Aco2	Mm.PT.58.6843105
Rpl19	Mm.PT.58.12385796
Bax	Mm.PT.58.14012210
Bc12	Mm.PT.58.7362966
Erra	Mm.PT.58.29487425
Errg	Mm.PT.58.28487522
Esr1	Mm.PT.58.8025728
Esr2	Mm.PT.58.43122172
Il-6	Mm.PT.58.10005566

Gene	Forward Primer	Reverse Primer
H2-Eb1	CAGATGTGGCAGGACAATAGA	CCTTGAGCAGGCTGATTTAGA
H2-Q4	CAGATCCTCCAAAGGCACAT	CAGGATGATGTTAGCAGGGTAG

Table 5-2: Top 10 up and down regulated gene expression changes in mammary gland following BPS exposure

Molecule FDR 0.10	Gene Name	Location	Δ Fold
GSN	gelsolin	extracellular	190.79
DBP	D-box binding PAR bZIP transcription factor	nucleus	3.360
BHLHE40	Basic helix-loop-helix family member e40	nucleus	2.414
ASPG	asparaginase	cytoplasm	2.385
TEF	TEF, PAR bZIP transcription factor	nucleus	1.860
PER3	Period circadian clock	nucleus	1.843
NR1D1	Nuclear receptor subfamily 1 group D member 1	nucleus	1.672
NR1D2	Nuclear receptor subfamily 1 group D member 2	nucleus	1.669
ACO2	Aconitase 2	cytoplasm	1.638
PIM1	Pim-1 proto-oncogene, serine/threonine kinase	cytoplasm	1.554
ZNF397	Zinc finger protein 397	nucleus	-2.348
XIST	X inactive specific transcript (non-protein coding)	nucleus	-2.223
Ear3	Eosinophil-associated, ribonuclease A family, member 3	other	-2.195
Kctd12b	Potassium channel tetramerisation domain containing 12b	Plasma membrane	-2.032
CREBBP	CREB binding protein	nucleus	-1.989
APC	APC, WNT signaling pathway regulator	nucleus	-1.978
ARID4B	AT-rich interaction domain 4B	nucleus	-1.973
HDLBP	High density lipoprotein binding protein	nucleus	-1.947
UTRN	Utrophin	Plasma membrane	-1.940
LPP	LIM domain containing preferred translocation partner in lipoma	nucleus	-1.928

BPS data was filtered with an FDR of 0.10.

Table 5-2: Top 10 up and down regulated gene expression changes in mammary gland following BPS exposure (cont.)

Molecule FDR 0.20	Gene Name	Location	Δ Fold
GSN	Gelsolin	extracellular	190.79
UCP1	Uncoupling protein 1	cytoplasm	4.526
Zfp945	Zinc finger protein 945	nucleus	3.443
CIDEA	Cell death inducing DFFA like effector A	cytoplasm	3.412
DBP	D-box binding PAR bZip transcription factor	nucleus	3.360
COX7A1	Cytochrome c oxidase subunit 7A1	cytoplasm	3.218
CPT1B	Carnitine palmitoyltransferase 1B	cytoplasm	2.988
SLC27A2	Solute carrier family 27 member 2	cytoplasm	2.540
BHLHE40	Basic helix-loop-helix family member e40	nucleus	2.414
ASPG	Asparaginase	cytoplasm	2.385
CP	Ceruloplasmin	extracellular	-3.373
RARRES1	Retinoic acid receptor responder 1	plasma membrane	-3.086
Rn18s	18s ribosomal RNA	other	-2.424
ZNF397	Zinc finger protein 397	nucleus	-2.348
Ear2	Eosin-affiliated, ribonuclease A family, member 2	cytoplasm	-2.334
RAPGEF4	Rap guanine nucleotide exchange factor 4	cytoplasm	-2.328
XIST	X- inactive specific transcript (non-protein coding)	nucleus	-2.223
EAR3	Eosin-affiliated, ribonuclease A family, member 3	cytoplasm	-2.195
BPTF	Bromodomain PHD finger transcription factor	nucleus	-2.191
Kctd12b	Potassium channel tetramerisation domain containing 12b	nucleus	-2.032

BPS data was filtered with an FDR of 0.20.

Table 5-3: Top Cellular and Molecular Functions for BPS-exposed mammary tissue

BPS (0.10 FDR)	**# Molecules**	**BPS (0.20 FDR)**	**# Molecules**
Cell Cycle	27	Energy Production	15
Cellular Movement	14	Lipid Metabolism	40
DNA Replication, Recombination, and Repair	19	Small Biochemistry	54
Gene Expression	35	RNA Post Transcriptional Modification	20
Lipid Metabolism	9	Cell Cycle	68

The top molecular and cellular functions are based on p-values. FDR 0.10 (p-value range 2.85E-04 - 2.31E-02) and FDR 0.20 (P-value range = 6.04E-05 –2.80E-02)

Table 5-4: Predicted Upstream Regulators following BPS prenatal exposure

BPS (FDR 0.10)	Molecule Role	Activation z-score	p-value overlap
TP53	Transcription regulator	0.479	3.15E-04
NUPR1	Transcription regulator	1.00	1.62E-03
ARNTL	Transcription regulator	ND	2.19E-03
Sos	Group	ND	2.71E-03
PER2	Transcription regulator	ND	4.28E-03
BPS (FDR 0.20)	Molecular Role	Activation z-score	p-value overlap
KDM5A	Transcription regulator	-3.05	3.27E-05
ARNTL	Transcription regulator	ND	2.19E-03
Sos	group	ND	2.71E-03
ESRRA	Ligand-dependent nuclear receptor	ND	2.62E-03
TP53	Transcription regulator	1.47	1.16E-02
PER2	Transcription regulator	ND	4.28E-03
PPARGC1A	Transcription regulator	2.46	4.67E-03
PER1	Transcription regulator	ND	1.89E-02
PPARA	Ligand-dependent nuclear receptor	2.80	1.73E-02

Selected predicted upstream regulators identified by IPA. Activation score ≤ 2 predicts inhibition and ≥ 2 predicts activation. ND = no activation thus z-score could not be determined. Note: Predicted regulators are genes involved in cancer, circadian rhythm, estrogen receptor and the peroxisome proliferator family.

Table 5-5: Quantitative PCR of Candidate Genes (BPS)

	PND 20	PND 35	PND 55
Aco	-1.11 ± 0.31	1.90 ± 0.21**	1.19 ± 0.25
Uqcrc1	1.11 ± 0.21	1.53 ± 0.12**	1.06 ± 0.20
Arntl	-1.82 ± 0.26 **	-1.13 ± 0.12	1.79 ± 0.13
NR1D1	1.38 ± 0.11	2.93 ± 0.06**	2.26 ± 0.13**
Per3	1.56 ± 0.17	2.32 ± 0.30**	1.38 ± 0.32
c-jun	-1.48 ± 0.20*	-1.35 ± 0.29	-1.34 ± 0.20
Map2k7	-1.14 ± 0.22	1.08 ± 0.13	1.03 ± 0.09
Pi3k	-1.04 ± 0.18	1.49 ± 0.22*	-1.10 ± 0.12
Evi5	-1.00 ± 0.12	1.34 ± 0.09**	1.00 ± 0.11
RNF168	1.05 ± 0.19	-1.00 ± 0.09	-1.15 ± 0.16
Erra	-1.12 ± 0.29	1.21 ± 0.12	1.40 ± 0.19
Errg	-1.16 ± 0.07	1.23 ± 0.10*	-1.18 ± 0.27
Esr1	-1.32 ± 0.29	1.16 ± 0.15	1.06 ± 0.19
Il-6	-1.21 ± 0.28	1.49 ± 0.29	-1.34 ± 0.06
Bax	1.02 ± 0.14	-1.08 ± 0.20	1.41 ± 0.15
Bcl2	-1.04 ± 0.17	-1.02 ± 0.06	1.64 ± 0.26

Data are represented as mean fold induction ± SEM. N= 3-5 samples/treatment per time-point. A student's t-test was performed to determine statistical significance. † $p = 0.06$, * $p< 0.05$ and **$p< 0.01$.

Table 5-6: Top 10 up and down regulated gene expression changes in mammary gland following BPAF exposure

Molecule	Gene Name	Location	Δ Fold
FDR 0.10			
SL35E2B	Solute carrier family 35 membrane E2B	Other	1.204
HLA-DRB5	Major histocompatibility complex, class II, DR beta 5	Plasma membrane	-6.853
SFRP5	Secreted frizzled related protein 5	Plasma membrane	-2.374
TSC22D1	TSC22 domain family member 1	Nucleus	-1.950
B3GALT5	Beta-1,3-galactosyltransferase 5	Cytoplasm	-1.281
SNAPIN	SNAP associated protein	Plasma membrane	-1.216

BPS data was filtered with an FDR of 0.10.

Table 5-6: Top 10 up and down regulated gene expression changes in mammary gland following BPAF exposure (cont.)

Molecule	Gene Name	Location	Δ Fold
FDR 0.20			
*HLA-A**	Major histocompatibility complex, class I, A	Plasma membrane	4.607
ELOVL3	ELOVL fatty acid elongase 3		3.439
*Zfp945**	Zinc finger protein 945		3.063
Tmsb4x	Thymosin, beta 4, X chromosome		2.085
ASPG	Asparaginase		1.779
*Zfp40**	Zinc finger protein 40		1.726
SLC35E2B	Solute carrier family 35 member E2B		1.204
SIN3B	SIN3 transcription regulator family	Nucleus	1.179
*PPM1B**	Protein phosphatase Mg2+/Mn2+ dependent 1B		1.131
*AKT3**	AKT serine/threonine kinase 3	Cytoplasm	1.118
HLA-DRB5	Major histocompatibility complex, class II, DR beta 5	Plasma membrane	-6.853
HLA-DMB	Major histocompatibility complex, class II, DM beta	Plasma membrane	-3.250
RARRES1	Retinoic acid receptor responder 1		-2.952
8030497I03Rik	Riken cDNA *8030497I03 gene*		-2789
*Zfp989/Znf41-ps**	ZNF41		-2.714
*ITCH**	Itchy E3 ubiquitin protein ligase		-2.464
*ZNF616**	Zinc finger protein 616		-2.458
*SFRP5**	Secreted frizzled related protein 5	Plasma membrane	-2.374
*HLA-E**	Major histocompatibility complex, class I, E	Plasma membrane	-2.258
*NRIP1**	Nuclear receptor interacting protein 1	nucleus	-2.192

BPS data was filtered with an FDR of 0.20.

Table 5-7: Top Cellular and Molecular Functions for BPAF-exposed mammary tissue

BPAF (0.10 FDR)	**# Molecules**	**BPAF (0.20 FDR)**	**# Molecules**
Cell Death and Survival	1	Cellular Growth and Proliferation	18
Cell Cycle	1	Energy Production	4
Cellular Development	3	Cell Death and Survival	17
Cellular Assembly and Organization	1	Cell Cycle	5
Cell-To-Cell Signaling	2	Cell Morphology	12

The top molecular and cellular functions are based on p-values. FDR 0.10 (p-value range = 2.94E-03 -3.68E-04) and FDR 0.20 (p-value range = 4.96E-02 – 4.13E-04)

Table 5-8: Predicted Upstream Regulators following BPAF prenatal exposure

BPAF (0.10 FDR)	Molecule Role	Activation z-score	p-value overlap
B3GALT1	enzyme	ND	3.68E-04
COPS4	other	ND	7.35E-04
oxazepam	chemical drug	ND	7.35E-04
AFP	transporter	ND	2.94E-04
Map3k7	kinase	ND	2.01E-02
Beta-estradiol	chemical	ND	1.45E-02
BPAF (0.20 FDR)	Molecule Role	Activation z-score	p-value overlap
B2M	Transmembrane receptor	ND	1.61E-05
POR	enzyme	ND	3.96E-05
MYD88	other	-2.16	8.95E-05
Beta-estradiol	Chemical	ND	5.45E-04
ERBB4	Kinase	ND	8.41E-04
ERRB2	Kinase	0.152	2.25E-03
EGFR	Kinase	ND	3.07E-03
IL10RA	Transmembrane receptor	-1.98	4.32E-03
IL6	cytokine	-1.36	1.05E-02

Selected predicted upstream regulators identified by IPA. Activation score ≤ 2 predicts inhibition and ≥ 2 predicts activation. ND = no activation z-score determine.

Table 5-9: Quantitative PCR of Candidate Genes (BPAF)

	PND 20	PND 35	PND 55
H2-Eb1(HLA-DRB5)	-2.50 ± 0.41††	-1.75 ± 0.40	-1.71± 0.23†
H2-Q4 (HLA-A)	1.40 ± 0.50	1.54 ± 0.27	1.10 ± 0.25
B3galt5	NA	-1.43 ± 0.68	NA
Tsc22d1	1.24 ± 0.03**	-1.16 ± 0.09	-1.44 ± 0.16††
Erra	-1.58 ± 0.19	-1.04 ± 0.13	-1.27 ± 0.21
Errg	-1.54 ± 0.22	1.20 ± 0.14*	-1.20 ± 0.23
Esr1	-1.31 ± 0.16	-1.03 ± 0.08	-1.13 ± 0.25
Esr2	-1.23 ± 0.17	-1.51 ± 0.19	-1.01 ± 0.01
Il-6	-4.04 ± 0.62†	-1.38 ± 0.43	-1.61 ± 0.49
Bax	-1.14 ± 0.16	-1.05 ± 0.11	-1.27 ± 0.11
Bcl2	-1.29 ± 0.10	-1.06 ± 0.10	1.08 ± 0.28

Data are represented as mean fold induction ± SEM. N= 3-5 samples/treatment per time-point. NA= Not analyzed. Genes with NA had low expression levels. A student's t-test was performed to determine statistical significance. †† $p = 0.07$, † $p = 0.06$, * $p < 0.05$, and ** $p < 0.001$.

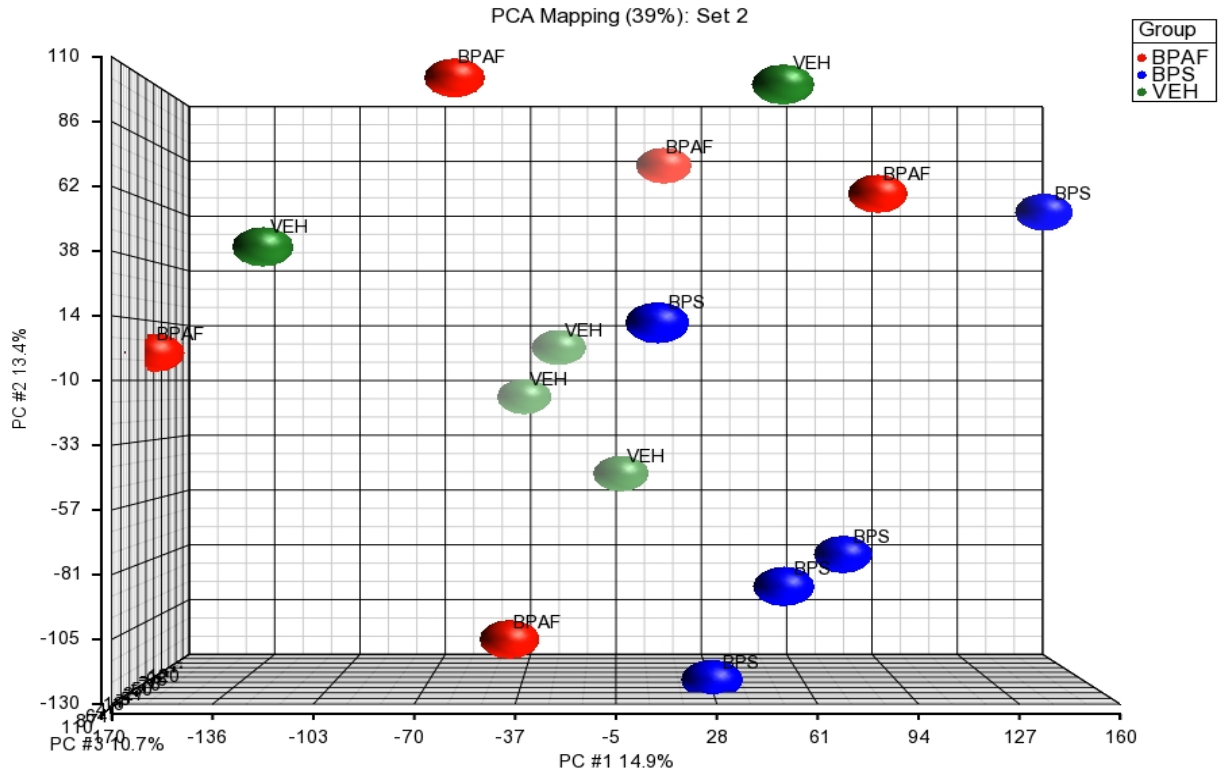

Figure 5-1: Principle component analysis of vehicle, 5 mg/kg BPAF and 0.5 mg/kg BPAF mammary gland gene expression changes at PND 34. Green dots = vehicle, red dots = BPAF and blue dots = BPS. N= 5 glands/treatment/time-point.

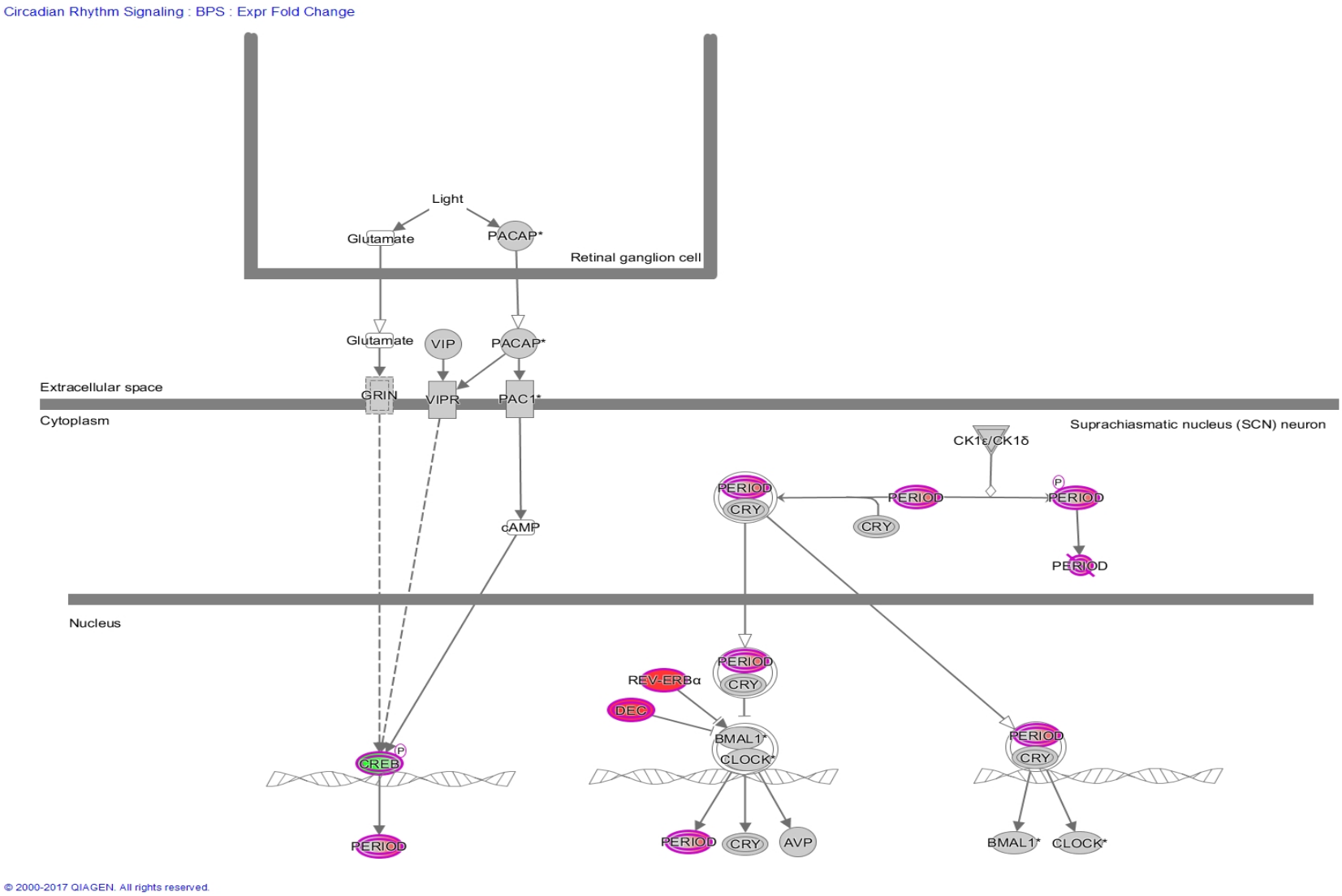

Figure 5-2: Circadian rhythm canonical pathway identified by IPA (BPS). All genes were upregulated except for *CREB*. Red = upregulated genes; Green = downregulated genes. Ingenuity Pathway Analysis (www. Ingenuity.com).

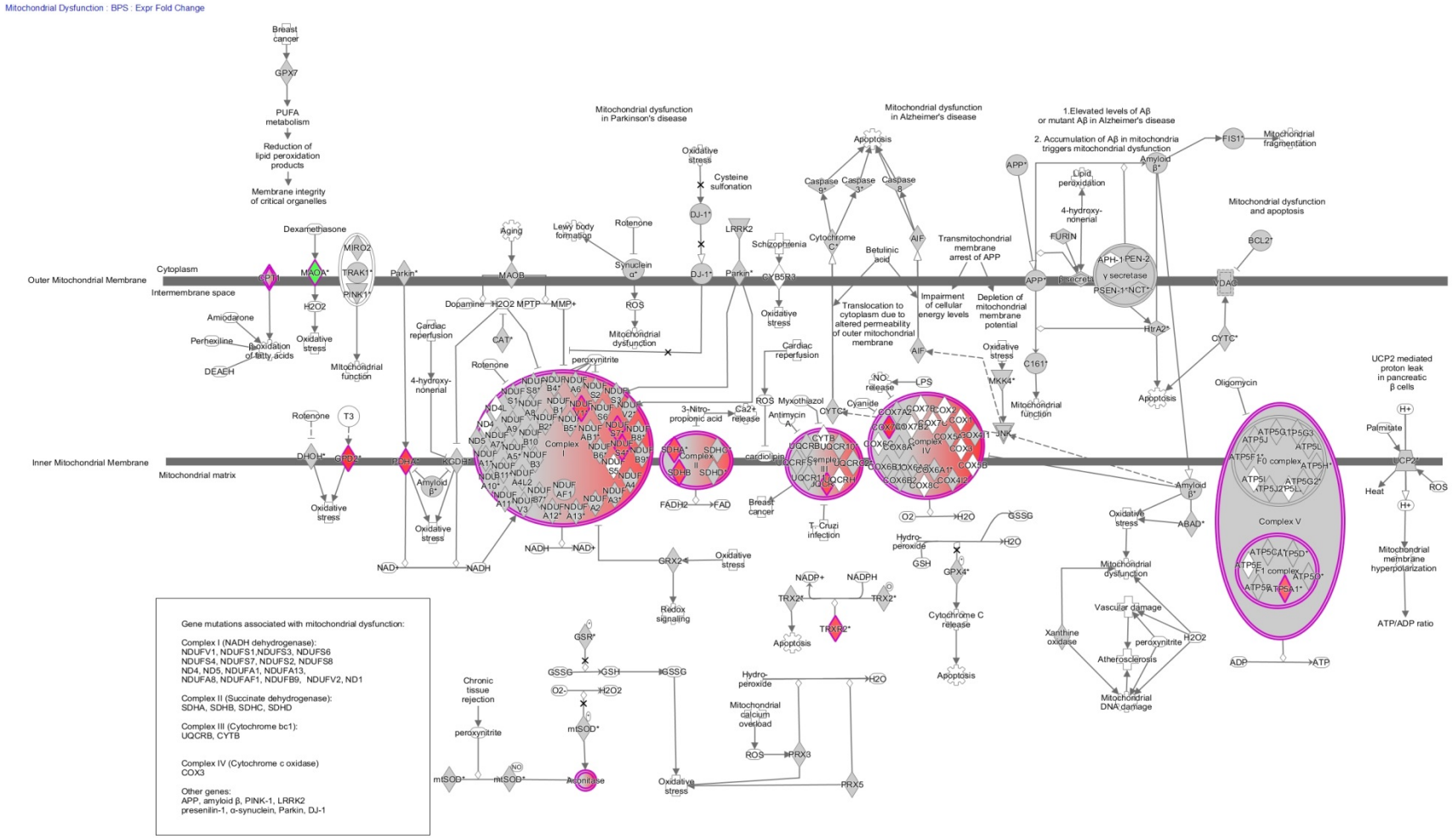

Figure 5-3: Mitochondrial Dysfunction canonical pathway identified by IPA (BPS). All genes were upregulated. Red = upregulated genes; Green = downregulated genes. Ingenuity Pathway Analysis (www. Ingenuity.com).

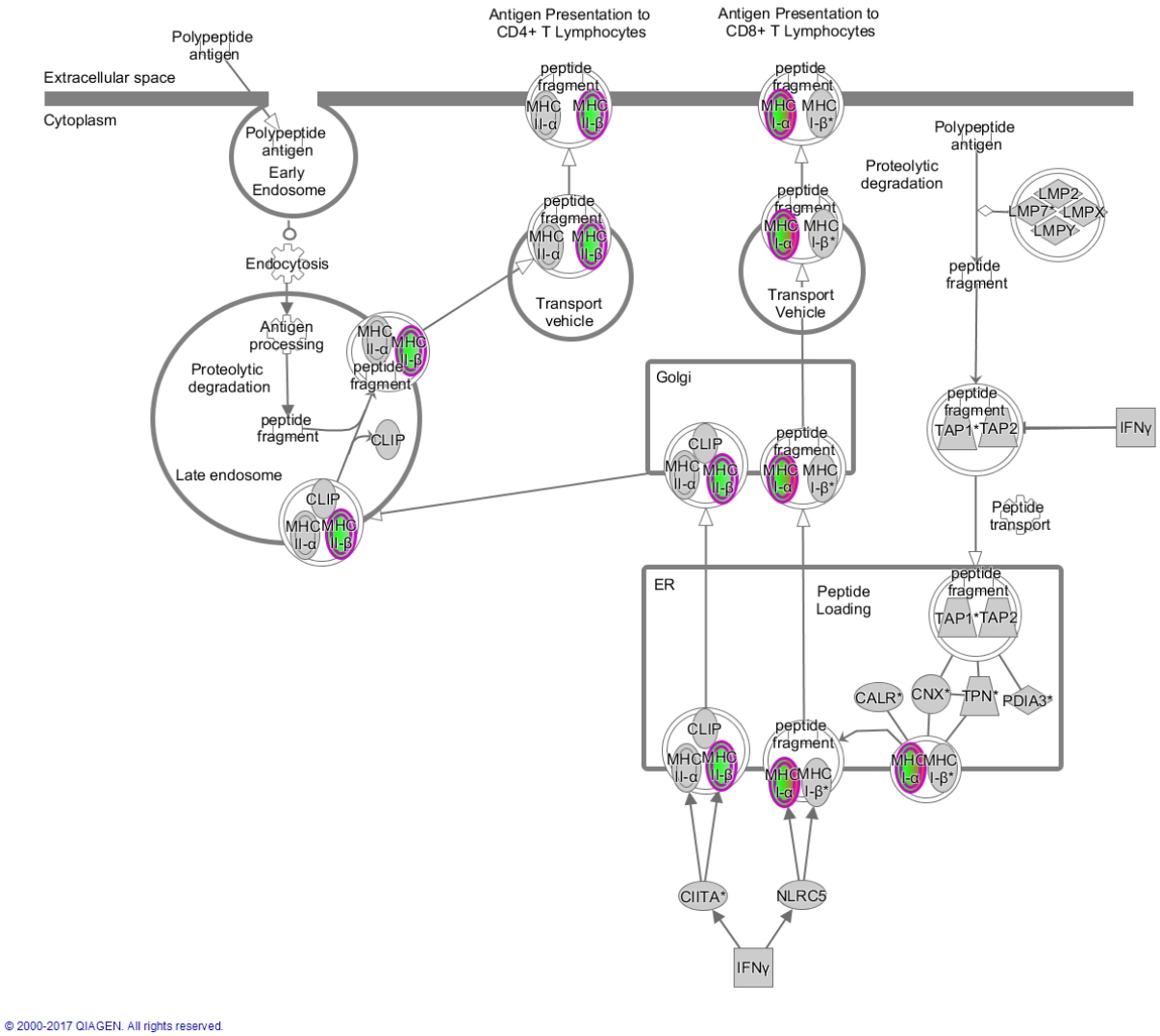

Figure 5-4: Antigen Presenting Pathway canonical pathway identified by IPA (BPS). All genes were downregulated. Green = downregulated genes. Ingenuity Pathway Analysis (www. Ingenuity.com).

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CHAPTER 6

Conclusions and Future Perspectives

SUMMARY

The research from this dissertation serves as an initial step to address the current data gaps associated with bisphenol analogue exposures, specifically BPAF and BPS, and their effects on the mammary gland. These data describe the disposition and pharmacokinetics of these chemicals in a pregnant mouse model and confirm fetal bisphenol exposure, a known critical window of mammary gland development. This work is also the first to detail accelerated pubertal mammary gland development and progression to increased mammary hyperplasia, inflammation, and in some cases, neoplasia in the prenatally exposed offspring.

Further investigation of the pubertal mammary gland shed light on potential molecular and cellular markers that were responsible for its advanced morphological development early in life, and sought to determine if they served as early predictors of disease formation. BPAF and BPS had little effect on stem and luminal cell counts and MRU frequency. These discoveries and the lack of changes in endocrine related genes led to the discovery of changes in genes involved in immunity, mitochondrial dysfunction, and circadian rhythm. BPA remains the most widely studied bisphenol because of its extensive use in many products and its inevitable exposure to humans and potential health effects. However, with the emergence of BPS, BPAF, and several other structurally similar chemicals being used in everyday products, undertaking this research was essential to inform the scientific and lay communities of their effects.

In order to put this research into context of bisphenol exposure and human health impact it was necessary to use doses that were within human relevant levels. Serum BPS has only been reported in one study [1] and urinary data only serves as a biomarker of exposure. Therefore, bisphenol concentrations were chosen based on BPA risk assessment data. The current LOAEL for BPA is 50 mg/kg and therefore the recommended daily intake (RDI) for human exposure has been set at 50 µg/kg/bw/day. We chose a dose range of ≤ 50 mg/kg for BPA and a dose range of ≤ 5 mg/kg for BPAF and BPS since we anticipated human exposure to these chemicals to be less than BPA.

Neonatal tissue measurements have confirmed BPA placental transfer [2, 3] and the effects associated with fetal exposure to BPA have been well documented in rodent studies [4-8]. Prior to beginning my thesis work, there were no studies that addressed BPAF and BPS transfer to the fetus, and to date there remains none. Therefore, before the effects of BPAF and BPS on the mammary gland could be determined, it was first necessary to confirm that the offspring were being exposed. Following a single dose exposure, BPA, BPAF, and BPS were all measured by mass spectrometry and were above the LOD in the amniotic fluid inferring placental transfer and fetal exposure. Following birth, male and female offspring had measurable levels of each administered chemical in their serum that continuously decreased between PND 3 and 5 and was often ≤ LOD in BPAF animals. Lack of decreases or an increase of chemical in serum over time would have indicated that at this early age that metabolizing enzymes necessary to clear the chemicals had little to no activity. One study showed the UGT2B1 and UGT1AG gene expression in livers from male and female C57Bl/6 neonates were much lower compared to their mothers [9]. When liver microsomes of neonates were exposed to BPAF (0.2 mM), BPA (0.2 mM), or BPF (0.2 mM) the glucuronidation activity was significantly lower in both male and

females at GD 18.5 and did not reach maternal activity until PND 21. These findings may further explain data that were obtained in my study [10]. We confirmed that serum and urinary half-lives in all chemically exposed dams were < 24 hrs. This finding has been noted in human exposures regardless of route and amount of exposure, and in pregnant and non-pregnant disposition studies involving BPA [11-13]. Due to ethical reasons, we could not place pregnant dams into metabolic cages and therefore excreta were only collected at designated time points. Metabolic caging would have allowed collection of 24 hr fecal samples (for a more precise measurement), but possible contamination from urine would have confounded our measurements. BPA, BPAF, and BPS were recovered in dam urine and feces. Recovery of both BPAF and BPS were highest in the urine. Although cumulative excreta was not collected and calculated, a similar trend to what we reported was also noted in earlier rat and mouse disposition studies [14], and human urine measurements over a 14 year span [15]. The low recovery of BPAF in my study inferred either increased rapid excretion or distribution to target organs, including the mammary gland; a question left unanswered and of interest in future studies. Collectively, these findings proposed a twice daily dosing to eliminate the occurrence of episodic circulating exposures, and were adopted for all developmental studies.

In Chapter 3, potential BPA, BPAF, and BPS-induced mammary gland effects were assessed using an extended dosing schedule at concentrations which were not materno- or fetotoxic (Chapter 2). Prenatal exposure to BPA, BPAF, or BPS caused increased TEBs, branching density, and longitudinal growth of mammary epithelia during puberty (PND 20-56). All of these changes have previously been reported in prenatally BPA-exposed mammary glands [7, 16, 17]. Interestingly, TEBs were still present during early adulthood (3 mo.) in our mice, a time when they should be mostly differentiated. In a study reported at the 2016 EDC Gordon

Conference, chemically exposed mammary glands thought to exhibit TEB structures for longer than usual were sectioned, and the TEB were actually hyperplastic lesions (L Vandenberg, personal communication). Further histopathological evaluation of the glands in our mice, using a method developed to prepare H&E stains from whole mounts (Appendix I) [18], determined no epithelial abnormalities. However, the increased presence of these structures poses a concern and may increase susceptibility to developing proliferative lesions in later life; TEB contain the most mitotically active and carcinogen-sensitive cells in the gland. Between 3 and 14 mo., mammary glands from all treated groups demonstrated an increased incidence of inflammation and epithelial hyperplasia. Mammary adenocarcinomas were detected as early as 11 mo. and other tumors including carcinoma, squamous cell carcinoma and papillary carcinoma were apparent by 14 mo. in BPAF 5 mg/kg and BPS 0.5 mg/kg mice. It should be noted that we did not anticipate observing adenocarcinomas; these tumors rarely occur spontaneously in CD-1 mice, especially at this young age (mid-life). While we saw significant changes in proliferative epithelial lesions and inflammation, had the study been powered with more animals to detect mammary tumors then we may have seen significant effects on cancer, also.

Indices of pubertal maturation including timing of vaginal opening, first estrus, and cyclity patterning were all unchanged. Mixed findings on these measurements have been reported with BPA in other studies, depending on timing, route, and amount of exposure [19, 20]. We observed increased serum estradiol and progesterone levels during early puberty which would provide an explanation for the accelerated development of the mammary gland. However, testosterone and DHEA was decreased between late puberty and early adulthood; a finding that often is associated with an increased risk to developing breast cancer in women [21]. Serum steroid hormone levels and gene expression of *Esr1, Pgr, Gper1,* and *Ar* were all unchanged at

14 mo., compared to control mice. Taken together, the morphological changes and progression to cancer formation, with the concomitant lack of changes in pubertal timing, serum hormone levels, and gene expression of classical nuclear receptors later in life, suggest a role for indirect endocrine signaling or changes in other pathways. It also suggests that BPA, BPAF, and BPS are causing permanent disruption in pathways during early mammary development.

To gain a better understanding of how bisphenol exposure accelerated pubertal mammary development and incidence of proliferative lesions, changes within specific mammary cell types were examined (Chapter 4). The mammary gland is comprised of several cell types including epithelial, adipocytes, immune, endothelial and fibroblasts, however, metastatic tumors arise from epithelial cells that comprise the ducts and lobules. Therefore, for this study, only mammary stem and luminal epithelial cells were examined; theorizing that increases in these cell populations may have been early markers of later life effects and may contain altered cells that go on to form tumors. Prenatal exposures to BPAF 5 mg/kg and BPS 0.5 mg/kg were chosen for these studies since they invoked significant deleterious mammary phenotypes over time in previous studies (Chapter 3).

Neither BPAF nor BPS altered total epithelial counts or individual luminal and stromal counts. Notably, stem cell counts were greatest just prior to vaginal opening and first estrus at PND 22, the same period that TEBs, branching density, and serum estradiol levels were significantly increased. Throughout the remainder of puberty, this population was lower than PND 22 counts and was statistically decreased at PND 35 suggesting that BPAF and BPS further decrease the stem cell population with increased pubertal age. This decreased stem cell recovery likely attributed to my observed lack of gland reconstitution in the treated groups. We would suspect that since the same cell concentrations for each chemical were used during

transplantation that the reconstitution frequency would be similar. However, as previously mentioned, an overall decrease in basal cells would also be reflected in a decrease in MRU-cells since they are a sub-population of the basal cells. MRU frequencies were greatest at PND 22 (control>BPAF) apart from BPS stem cells that could not re-constitute a gland at any pubertal time-point. Prior studies have shown that BPA exposure transiently alters the stem cell population and increases PR expression in luminal cells and tissues [22].There were approximately 3-7 weeks in between the final administered dose and when cell counts and transplants were initiated, and therefore we could have missed the critical window when many of these changes were taking place. We chose these times assuming permanent effects on the stem cell populations, and specifically thought that the wave of increased stem cells in puberty may drive the latent phenotypes previously seen (Chapter 3). More recent data has implicated the fetal mammary stem cell as the population that may endure the greatest changes following chemical exposure [23]. Still, this data helped define the pubertal MRU frequency range and defined the window for the stem cell wave of puberty in the CD-1 mouse, and expands on the knowledge that is already known in other strains [24].

The final goal of this research was to narrow down the possible mechanism(s) for BPAF and BPS effects on the mammary gland (Chapter 5). The same concentration and time points used in Chapter 4 were used to address this research question. Glands from mice exposed prenatally were collected on PNDs 20 and 35 in Chapter 3 studies, and were evaluated by whole genome microarray to compare molecular changes. However, treated glands from PND 20 were not statistically different from control animals in microarray analyses using an FDR of up to 0.4, and were therefore not used for this comparison. PND 35 analyses provided more useful information. BPAF altered a handful of genes compared to BPS at FDR of 0.1 and 0.2, and all

changes involved genes that were associated with antigen presentation and B cell development. HLA-DRB5, a transmembrane receptor that presents extracellular proteins to CD4+ T cells, was downregulated which could promote an environment for tumor growth. In contrast HLA-A, which presents cytosolic proteins to CD8+ T cells, was upregulated. HLA-A has been associated with tumor metastasis, however, several alleles of this gene exist and some have been shown to have a protective effect against tumor formation [25]. Mouse homologs for both genes (*H2-Eb1* and *H2-Q4*) were evaluated using PCR, but neither were significantly changed vs controls. Interestingly, at PND 20 and 55, *H2-Eb1* (*HLA-DRB5*) expression in bisphenol exposed mammary tissue approached significance but was unchanged at PND 35. BPS produced numerous changes in pathways involved in mitochondrial dysfunction, circadian rhythm, and Rac signaling; pathways shown to play a role in cancer formation or risk.

Mitochondria are the energy source of the cell and alterations in its function have been implicated as a mechanism for several chemical toxicants [26, 27]. *Aco* and *Uqcrc1* were increased in both the microarray and confirmatory PCR. We also selected genes from the list of top up and down regulated altered genes from both treatments and evaluated them by PCR. Although none of these genes were linked to a specific canonical pathway, several were chosen based on their role in epithelial cancers, neoplasia and carcinoma/adenocarcinoma formation, since this was one phenotype that we observed following prenatal exposure to BPAF and BPS. *Rnf168, Evi5, B3galt5* and *Tsc22d1* are all regulated by cytokines and chemokines and are involved in apoptosis, cell differentiation and proliferation. *Rnf168* and *Evi5* were downregulated by BPS whereas BPAF caused downregulation of *B3galt5* and *Tsc22d1*. Only one gene from each chemical was significantly altered when assessed by PCR (*Evi5* and *Tsc22d1*) and interestingly both genes were upregulated in contrast to the microarray. Because we did not

observe any nuclear receptor changes in the adult mammary gland, we wanted to determine if receptor changes in younger glands existed as a possible mediator of increased estrogen sensitivity, as all of these bisphenols possess estrogenic activity in vitro. Neither BPAF nor BPS altered *Erra*, *Esr1*, or *Esr2* between PND 20 and 55, but *Errg* was significantly upregulated by BPAF at PND 35.

Immunomodulatory genes were also examined as potential mediators of effect in the mammary gland following bisphenols exposure, as they have been reported as modulators of BPA effect in the mammary gland [28], and we previously observed significant increases in inflammatory cells in mammary tissue following BPS and BPAF exposures (Chapter 3). *Il-6*, *Bax* and *Bcl2* were unchanged after treatment with BPS and BPAF. This is an interesting observation, considering *IL-6*, *IL1RN*, *IL1B*, *IL1A*, and *IFNG* were all predicted upstream regulators for BPS. Future validation of the *IL-1* cytokine family will be necessary to confirm these predictions. Taken together, these data suggest that there are likely several mechanisms that are causing BPAF and BPS induced mammary gland changes.

One major limitation in these studies is that we used an outbred mouse strain that presented more variability than we could compensate for in our study design. These studies were designed to evaluate early life outcomes such as pup number, growth, and pubertal outcomes and we chose this strain knowing that it had been previously used to demonstrate significant effects of BPA in the mammary gland in other labs [REFs]. The study progressed to late life evaluation and cancer outcomes because of the significant changes seen in the pubertal mammary gland and the retention of TEB at 3 mo. Had we known some of these findings, we would have a priori increased the animal numbers and more carefully selected the time points for evaluation, focusing on fetal stem cell populations and late life outcomes in a well powered cancer study.

Also, we feel that the variability in this mouse line led to few gene changes seen in microarray studies and some of our ongoing analyses will assess for outliers in our data sets. Further studies should use an estrogen responsive inbred mouse line and more samples per treatment group in gene analyses, or possibly a more sensitive way to measure gene differences between the groups (other transcriptomic approaches).

IMPACT OF FINDINGS

In my opinion, the bisphenol replacements BPAF and BPS are not as safe as previously considered, but the fields of research for these chemicals are still in their infancy and require additional animal studies to increase the weight of evidence for their removal from marketplace products. In 2008, the NTP nominated BPAF for further assessment, but has only published one report since that time. My work has addressed data gaps that exist in the current literature and knowledge of BPAF and BPS, and their effects on the developing mammary gland, and has validated previous BPA findings. Although developmental abnormalities have been observed in rodents at doses lower than the BPA NOAEL and LOAEL [29, 30], the FDA has noted that the decision to ban the use of BPA in baby bottles and formula packaging was attributed to abandonment and not safety [31]. The data presented here should be used by regulatory and risk assessment agencies to set BPAF and BPS exposure guidelines. Many of the established breast cancer risk factors include early exposure to estrogenic compounds and the early onset of puberty. BPA exposure as a risk factor for breast cancer is still heavily debated in the field [32, 33]. Similar to BPA, both BPAF and BPS induced adverse morphological mammary changes well after the chemical insult was removed. Therefore, this data implies that following the first "estrogenic hit" the accelerated mammary phenotype manifested as a result of a second estrogenic hit from increased estradiol and progesterone serum levels either prior to puberty or

due to the estrous cycle. Decreased DHEA levels and changes in immune and mitochondrial functions likely compounded these risks. In the grand scheme of things, the experimental design and unique methods to generate this data will be useful to assess other bisphenol analogues as well as other suspected mammary toxicants.

FUTURE PERSPECTIVES

As mentioned throughout this work the mammary gland is composed of many cell types that signal through paracrine, endocrine and autocrine factors. While many studies have focused on the effects of BPA on the mammary epithelium, it has also been shown to alter the stroma, fat pad and immune cells. Prenatal BPA exposure in mice caused a decrease in stromal BrdU incorporation by PND 30 [7], but was increased at 6 mo. of age in a different study [6]. Mammary glands from female offspring exposed to BPA (250 ng/kg) experienced decreased fat pad density, increased Bax-positive cells, and increased number of adipocytes per 1 mm distance of epithelium [34]. Genes associated with the fatty acid beta oxidation 1 pathway were significantly increased (*ACAA2, ECHS1, HADHA,* and *SLC2742*) in BPS-exposed mammary glands according to the microarray data in my studies. Several of these genes are regulated by the peroxisome proliferator activated receptor isoforms and many of these isoforms were predicted as upstream regulators in my Ingenuity Pathway Analyses (*PPAR α, δ, γ,* and *PPARGC1A*). In mouse liver samples in on-going studies in our lab, changes in these pathways were linked to adverse changes in mitochondrial morphology, gene expression, and activity (S. Fenton, personal communication). Fatty acids are found within various tissues and are broken down by oxidation to produce cellular energy. A study of triple-negative breast cancer showed that fatty acid oxidation upregulation in a mouse model that overexpressed MYC was required

for tumor growth [35]. This leads us to believe that BPAF and BPS alters the mammary fat pad and in turn gland development. To test this theory in on-going work, BPAF and BPS treated mammary glands from PND 9 were collected from the littermates of animals from Chapter 5. This early time-point represents an important period when epithelial structures have not completely invaded the fat pad and ER expression is likely higher in stroma than the epithelium. Therefore, sections of adipose tissue and epithelial tissue will be used to test for expression pattern differences.

The effects of BPA on epigenetic modulation has been observed in several tissues but has only been reported for the mammary gland in two studies [36, 37]. The mammary glands of Wistar-Furth rats were examined at PND 4, 21 and 50 with BPA concentrations found to induce ductal carcinoma situ (250 ug/kg); results showed that pubertal mammary glands exhibited an altered epigenome [37]. Mammary glands from prenatally BPA exposed female Wistar Furth rats displayed widespread changes in DNA methylation at PND 4 and PND 21. Both hypo and hypermethylated loci were detected, especially at PND 21. However, the methylation pattern changes between control and BPA did not always remain similar for each time point. NCTR Sprague Dawley rats exposed to low (2.5-2700 µg/kg bw/day) and high (100,000 and 300,000 µg/kg bw/day) BPA and EE2 (0.5 and 5.0 µg/kg bw/day) were also assessed for changes in global genomic DNA methylation in mammary tissue at PND 90, but no significant changes were detected [36, 38].

Microarray analyses from Chapter 5 showed that DNA methylation and transcriptional repression signaling was a canonical pathway of interest. DNA methyltransferase 3A (*DNMT3A*), chromodomain helicase (*CHD4*), and AT-rich interaction domain (*ARID4B*) were all downregulated, and methyl Cpg binding (*MECP2*) was upregulated following BPS exposure.

DNA methyltransferases are ubiquitously expressed in normal human tissue but overexpressed in many cancers including the breast [39]. *DNMT3A* is overexpressed in approximately 3% of breast cancer cases [40] and sex specific differences in gene expression in the prefrontal cortex and hypothalamus following prenatal BPA exposure [41]. *CHD4* is a part of the NuRD complex and can be regulated by ESR1. ARID4B is also known as breast cancer associated antigen gene 1 and contains a similar antigen epitope to retinoblastoma binding protein 1 (Rb-1) an anti-oncogene that binds to E2F to repress transcription of genes involved in the cell cycle [42]. The downregulation seen in our analyses could indicate that genes were signaled to continue proliferating. MECP2 is highly expressed in the brain and altered expression is associated with neurodevelopmental disorders and was increased by BPA in primary embryonic cortical neuron [43]. Contralateral glands from Chapter 5 have been preserved to analyze DNA methylation status in the near future.

Because this data is novel and potentially useful for regulatory bodies, it will be necessary to repeat these studies to determine their effects in other strains/species. The OECD 421 Reproductive and Development Toxicity screening tests, suggest that rats are the recommended species and mice can also be used [44]. Our CD-1 disposition data (Chapter 2) was very similar to the disposition in B6/F1 mouse and Sprague Dawley rats reported by the NTP [14]. Therefore, we believe that these and other rodent models will be good models to test the effects of BPAF and BPS on mammary gland development. Currently our lab is undertaking a BPAF transcriptomic study from Harlan Sprague Dawley dam and offspring exposed between GD 6 and during lactation to PND21. Multiple endocrine related tissues have been collected and we anticipate that this data will help to identify and explore important pathways of interest.

The overarching goal of this project was to understand the chemical toxicity of BPAF and BPS in the mammary gland. Cancer development was not an expected outcome, and demonstrates the importance or further assessing these chemicals for latent disease susceptibility. Exposure to these chemicals, combined with well-established non-heritable breast cancer risks that include breast density, age at first period, and the emerging obesity epidemic in younger children, may further increase one's susceptibility for developing cancer. It has already been reported that women working in canning facilities, that use resins to line the cans, are at an increased risk for breast cancer [45]. Making these connections to the bisphenols will require more epidemiological studies that will be able to track development over a lifetime, but also determine the serum concentrations in human populations to design better human relevant animal studies. We anticipate that BPAF and BPS effects will be brought to the forefront of bisphenol research and these data will influence the undertaking of similar studies with other bisphenol analogues. We anticipate these studies will be influential for regulatory and risk assessment decisions.

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APPENDIX I

Preparation of high quality hematoxylin and eosin-stained sections from rodent mammary gland whole mounts for histopathologic review[1]

OVERVIEW

Identifying environmental exposures that cause adverse mammary gland outcomes in rodents is a first step in disease prevention in humans and domestic pets. 'Whole mounts' are an easy and inexpensive tissue preparation method that can elucidate typical or abnormal mammary gland morphology in rodent studies. Here we propose procedures to facilitate the use of whole mounts for histological identification of grossly noted tissue alterations. We noted lesions in mammary whole mounts from 14-month old CD-1 mice that were not found in the contralateral gland hematoxylin and eosin (H&E)-stained section. Whole mounts were removed from the slide and carefully processed to produce high quality histological sections that mirrored the quality of the original H&E-stained section in order to properly diagnose the unidentified gross abnormalities. Incorporation of this method into testing protocols which focus on low-dose human relevant chemicals and endocrine disruptors will increase the chances of identifying lesions in the gland and reduce the risk of false negative findings. This method can be especially invaluable when lesions are not always palpable during the course of the study or visible at necropsy, or when a single cross-section of the mammary gland is otherwise used for detecting lesions.

INTRODUCTION

Whole mounts are an invaluable tool used primarily in rodent studies to illustrate the progression of normal or chemically-altered mammary gland epithelial growth, development,

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function, and disease. When collected during various stages of development, the whole mount provides information regarding 1) early development of mammary tissue before endogenous hormone changes, 2) pre- and peripubertal epithelial alterations in offspring as a consequence of chemical transfer through placental/lactational exposure 3) early signs of lactational impairment in dams when evaluated during mid-pregnancy, 4) influences of circulating endogenous hormone or endocrine disrupting chemical (EDC) exposures, and 5) gross pre- and neoplastic lesions that can be verified and properly diagnosed using histopathology [1]. The latter examples often involve tissues collected in adulthood at final necropsy in studies involving chemicals with known or suspected endocrine disrupting activities, although mammary tissue can and should be collected at other life stages.

Historically, rodent mammary glands were prepared and evaluated as a representative cross-section that included the skin and very little mammary epithelial tissue. Current recommendations [2] include using coronal or longitudinal sections to improve the ability to observe any abnormalities; the whole mount also provides an image of the entire gland and therefore is another acceptable technique of choice for histopathological evaluation of the mammary gland. The mammary whole mount is typically prepared from the inguinal 4^{th} and 5^{th} glands on one side of the animal. Glands from this region are easier to access compared to the thoracic glands thus preserving the glands natural architecture when spread onto a charged slide. One set of inguinal glands are mounted onto the slide, fixed, stained and covered with a coverslip, or sealed in a bag of methyl salicylate for temporary or permanent storage, and the contralateral gland may be processed for histology [2].

When using this approach, a comparative visualization of a whole mount along with H&E-stained sections prepared for histologic examination can provide an excellent appreciation

of the morphological and cellular alterations occurring in the tissue, especially in cases where a palpable tumor is not observed. However, acquiring and evaluating glands from both sides of the animal may not always be feasible (i.e. tissue required for RNA or protein analysis or due to insufficient funding), or gross abnormalities may occur asymmetrically. The latter was the case during the analysis of a current study where the abnormal findings in the whole mounts were disproportionately higher compared to the contralateral histopathology findings. In response, we developed an inexpensive and streamlined protocol to examine H&E-stained paraffin sections of the original mammary gland whole mount using brightfield microscopy. The development of a standardized protocol will facilitate tissue comparison between labs and may limit the need for additional tissues originally processed for H&E staining.

MATERIALS AND METHODS

Whole Mount and Contralateral Gland Preparation

Whole mounts were prepared by excising the 4^{th} and 5^{th} inguinal mammary glands from adult female Crl: CD1 (ICR) mice (CD-1; Charles River Laboratory, Raleigh, NC) in an on-going study (collected at 14 mo of age) or from animals that failed to deliver healthy litters (for practicing sectioning and technique specifics). The whole mounted gland was sandwiched between parafilm (Bemis, Neenah, WI) and another glass slide with slight pressure applied to flatten the gland and increase the surface area for fixation and staining. Fixation (Carnoy's), staining (carmine alum) and defatting (xylene) were performed as previously described in detail in Davis and Fenton [2].

The contralateral gland was removed and fixed in 10% neutral buffered formalin (Fisher Scientific, Fairlawn, NJ) for 48 hours and dehydrated in 70% ethanol to minimize adipose

distortion that can occur during sectioning. Within 48 hr. following fixation, samples were embedded in paraffin, sectioned at 5µm, and stained with H&E on an automated platform (Leica ST2050 Multistainer Workstation, Buffalo Grove, IL). H&E-stained sections were visualized on an Olympus BX41 (Olympus Scientific Solutions Americas Corp., Waltham, MA), digitally captured on an Olympus DP70 camera and examined by a board certified veterinary pathologist (S.H.B.).

Whole Mount to H&E Preparation

We recommend that before the sectioned tissue stages of this protocol are started, that high quality images of the intact whole mounts be taken for any future analyses. The first step in preparing mammary whole mounts for sectioning was removal of the glass coverslip. Each whole mount was placed in a glass Coplin jar and immersed overnight in xylene, followed by two additional fresh xylene soaks; 6 hr. and overnight, respectively. Covers fall off during this process. Slides were held perpendicular to the bottom of a glass petri dish containing xylene and the tissue was scraped from the slide with a sharp disposable razor blade beginning at the top of the slide. To ensure the tissue was removed intact, the blade was slowly and carefully pulled down the slide, parallel to the tissue. This allowed the gland to fall into the xylene at the bottom of the glass petri dish, minimizing air drying of the sample. The tissue was then placed in a histology cassette for processing. When necessary, larger glands were halved at the midline where the pad narrows and the tissues were placed into two separate cassettes, one labeled for the top half and the other for the bottom half. Since the tissue was thin, we do not advise placing both halves in the same cassette. The cassettes were held in xylene for up to 2 hr. before being placed on the tissue processor (Leica Microsystems TP1020, Chicago, IL). Glands were processed in xylene for 30 min. This step was repeated and followed by immersion in a 1:1

xylene: molten paraffin (30 min.). The cassettes were then transferred to the next station, which consisted of molten paraffin (1 hr.), followed by another incubation in molten paraffin (2 hr.). Processor settings included a temperature of 60°C and the vacuum on for all stations. See Table AI-1 for a summary of the tissue processing schedule. Finally, the mammary pad was embedded in paraffin with the flat surface down (side adjacent to the glass slide). Prior to sectioning, the paraffin blocks were incubated at -20°C for 1 hr. This step aided in obtaining quality sections for histopathology. Glands from animals not included in our study (small litters or gestation timing off the group average by more than 12 hr.) were sectioned at 4 µm (superior quality vs. a thicker section (6 µm); Figure AI-1) using a low profile blade (Sakura, Finetek, Inc. USA, Torrance, CA). All slides were incubated overnight at 37°C prior to an automated staining with H&E, as described above for the contralateral gland. Tissue de-staining was unnecessary, since very little of the whole mount carmine alum staining remained in the tissue sections.

Scanning

High quality archival whole mount images were recorded to preserve intact structures for future assessments. Images were prepared by scanning the whole mount mammary gland glass slide on a flatbed scanner (Epson Perfection V750 Pro, Epson America, Long Beach, CA).

Digital images of H&E-stained slides were captured on the Aperio AT2™ slide scanner (Leica) using Imagescope™ software (v.12.1, Leica). White balance correction and image resizing were completed using Adobe Photoshop™ (Adobe Photoshop Creative Cloud 2014.0.0).

RESULTS

It is important to note that the intact mammary gland whole mount, displaced with xylene from the glass slide, is not optimized for sectioning in the traditional histologic sense. In the current situation, the whole mount was permeated with a heavy layer of mounting media, such as Permount (Sigma Aldrich), so that a coverslip may be placed over the thick mammary pad without the introduction of air bubbles. In developing this protocol, the whole mount paraffin blocks from animals not included in our study were originally sectioned at 6 μm. The sections appeared thick, piling up of cells was evident, and specific cellular detail was insufficient (Figure AI-1A). Tissue blocks sectioned at 4 μm yielded superior results with cellular features and stromal area infiltrates more easily discernable (Figure AI-1B).

This modification in tissue block sectioning is being used for a large on-going study. The histopathology findings of the H&E-stained longitudinal sections from the contralateral gland of 14 month old female CD-1 mice in our study were inconsistent with whole mounts collected from those same animals, and were found to contain mammary lesions. Two tissue samples with conflicting diagnoses were chosen to illustrate the effectiveness of this method; however, multiple abnormalities were identified and will be described elsewhere in detail. Histopathologic evaluation of the original contralateral H&E-stained tissue section revealed a normal gland with no histologic findings. Normal mammary gland architecture consisted of ducts lined by a single layer of simple cuboidal epithelial cells and supported by a layer of myoepithelial cells. Ductal lumens were evident and the adipocytes were uniform throughout the sections (Two examples; Figure AI-2A and AI-3A). In contrast, gross visualization of the contralateral whole mount revealed abnormal gland architecture (Figures AI-2B and AI-3B; boxed area) characterized by increased opacity around ducts and stromal structures of the mammary gland. It was not possible

to determine whether these areas of opacity represented inflammatory, hyperplastic, or neoplastic changes without an examination of a representative H&E-stained section of the gland.

Results from examination of the sectioned, H&E-stained whole mounts are shown in Figures AI-2C, AI-2D, AI-3C, and AI-3D. Samples from one 14 mo old animal (Figure AI-2C and AI-2D) contained lesions diagnosed as perivascular inflammation; characterized by an increased number of perivascular lymphocytes that extended into the adjacent adipose tissue. A portion of the whole mount of a second virgin 14 mo old animal (Figure AI-3B) was morphologically similar to the appearance of the mammary gland during pregnancy (mid-gestation) or to the mammary gland of male rats (Filgo et al., submitted), characterized by extensive lobular budding. A full evaluation of H&E-stained sections from the whole mount (Figure AI-3C and AI-3D) revealed lobular alveolar hyperplasia characterized by focal to multifocal enlarged lobules comprised of increased numbers of relatively normal alveolar epithelial cells. Alveolar epithelial cells were well differentiated, round, often vacuolated and formed a single concentric layer around ducts that typically contained proteinaceous fluid. Ducts were lined by a single layer of well-differentiated columnar epithelial cells. Alveoli and ducts were normally distributed and there was no evidence of adipocyte compression or cellular atypia.

DISCUSSION

Histopathological evaluation plays an essential role in identifying abnormalities in the mammary gland that are not grossly obvious. This is especially important when evaluating low-dose exposure to EDCs and chemical mixtures that approximate human exposure levels. Whole mammary gland mounts depict the intact morphological landscape and allows an accurate assessment of where and how many abnormalities are occurring. Use of both whole mount and

H&E-stained section evaluation is ideal, but not always practical; however, sole reliance on one method can potentially result in missing significant findings. This is especially true when a single H&E-stained mammary gland section is used for histopathological evaluation.

This sectioning method highlights the dual utility and benefits of the mammary gland whole mount. In the two examples discussed, inflammation and lobular hyperplasia were identified in the H&E-stained representative whole gland mount section, but not in the H&E-stained representative from the contralateral mammary gland. This technique is applicable and effective at identifying and quantifying a range of abnormalities, especially in mammary glands of chemically treated or genetically modified rodents. The regular evaluation of mammary tissue from mice and rats during various life stages of development (i.e. wean, puberty and early pregnancy) is highly recommended, due to its small tissue size and relatively low epithelial density. We anticipate that this method will 1) serve as a standardized protocol to reduce inter-lab variability, 2) increase awareness within the scientific community of the ease with which the mammary gland can be incorporated into studies at minimal added cost [3], and 3) stimulate collection of the mammary gland into chemical test guideline studies, which may or may not include evaluations (OECD TG443 and NTP) [4, 5], fail to focus on specific mammary gland developmental endpoints [6]or are not collected at all (OPPTS 890.1450, US EPA 2009 and OPPTS 890.1500 2009b) [7, 8].

Although it is probable that other histochemical and immunohistochemical staining may be applied to sections derived from whole mounts, it was beyond the scope of this work and further testing would be necessary to make these determinations. Furthermore, it may be possible to prepare smaller sections of abnormal tissue (i.e., punch-biopsy samples) in the same fashion as the whole gland; however, those technical variables were not tested and would require further

efforts. It is hoped that evaluation of the entire mammary gland using a combination of whole mounts and their representative H&E-stained sections will minimize the possibility of false negatives in a rodent study, and lead to increased awareness of environmental factors that adversely modify mammary development or function.

Table AI-1: Mammary Whole Mount Tissue Processing Schedule

Process	Temperature (°C)	Time (min)
Xylene	37°	30
1:1 xylene: molten paraffin	60°	30
Molten paraffin	60°	60
Molten paraffin	60°	120

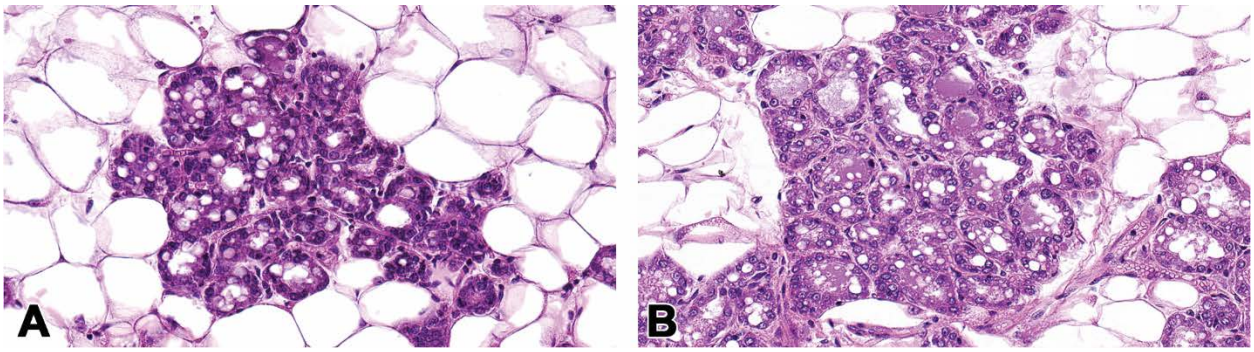

Figure AI-1: Determining an optimal thickness to prepare mammary gland sections. Mammary gland sections of (A) 6 μm and (B) 4 μm thickness. Due to the increased clarity and cellular distinction in (B), the 4 μm thickness is preferred. Magnification 40x. Mammary tissues were obtained from animals otherwise destined for euthanization.

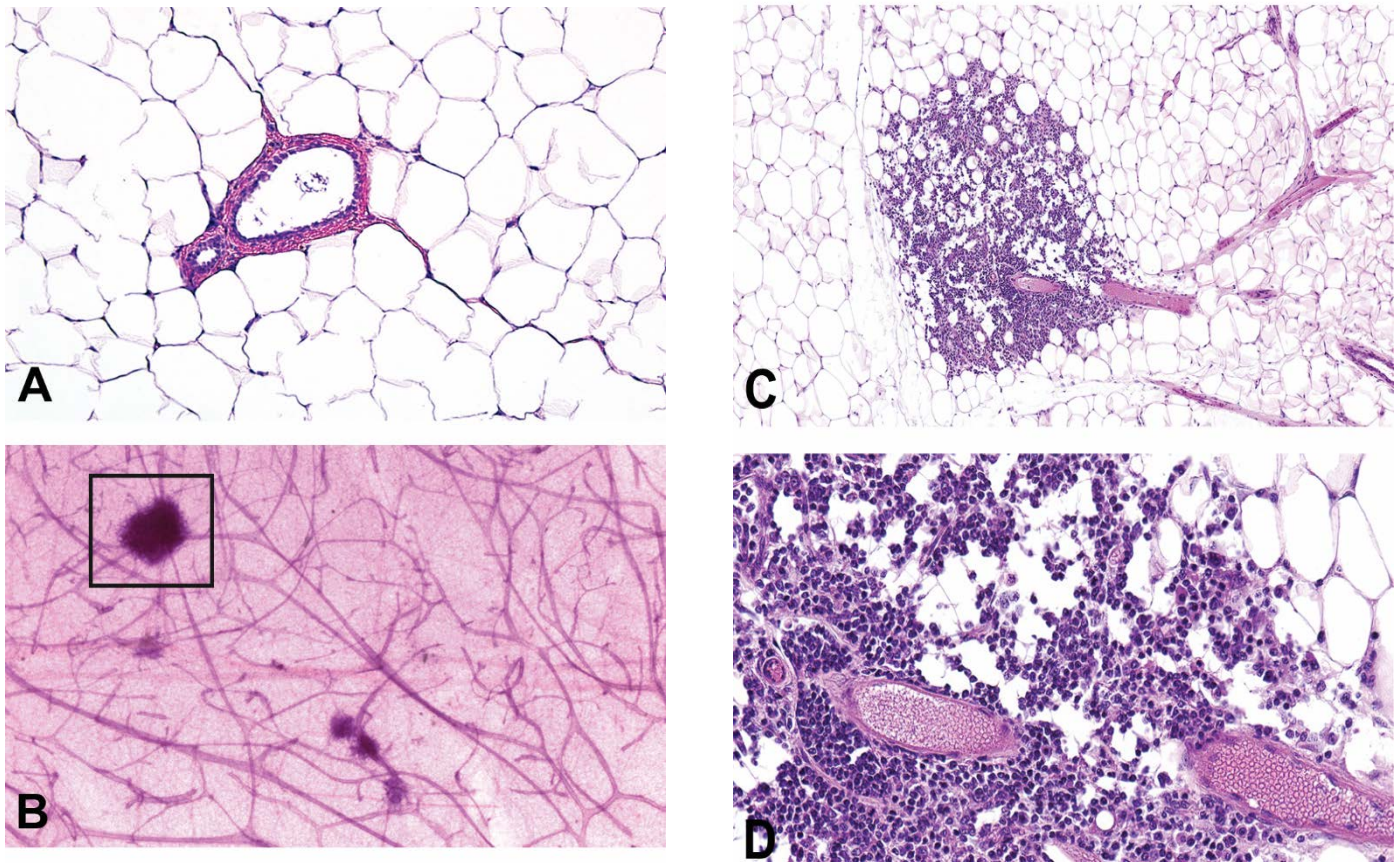

Figure AI-2: Mammary gland section of perivascular inflammation using the whole mount method. Normal mammary gland section with no histopathologic findings, formalin-fixed H&E-stained, magnification 10x (A); Increased opacity around ducts and stromal structures (box) in the contralateral carmine-stained mammary whole mount (B); At low magnification, in the contralateral mammary gland H&E prepared from the whole mount (boxed area shown), there are clusters of mononuclear cells around the blood vessel and extending into the adjacent adipose tissue magnification 20x (C); Higher magnification illustrated that the inflammation is composed of perivascular lymphocytes; magnification 40x (D). Tissue samples are from a 14 mo. old female CD-1 mouse.

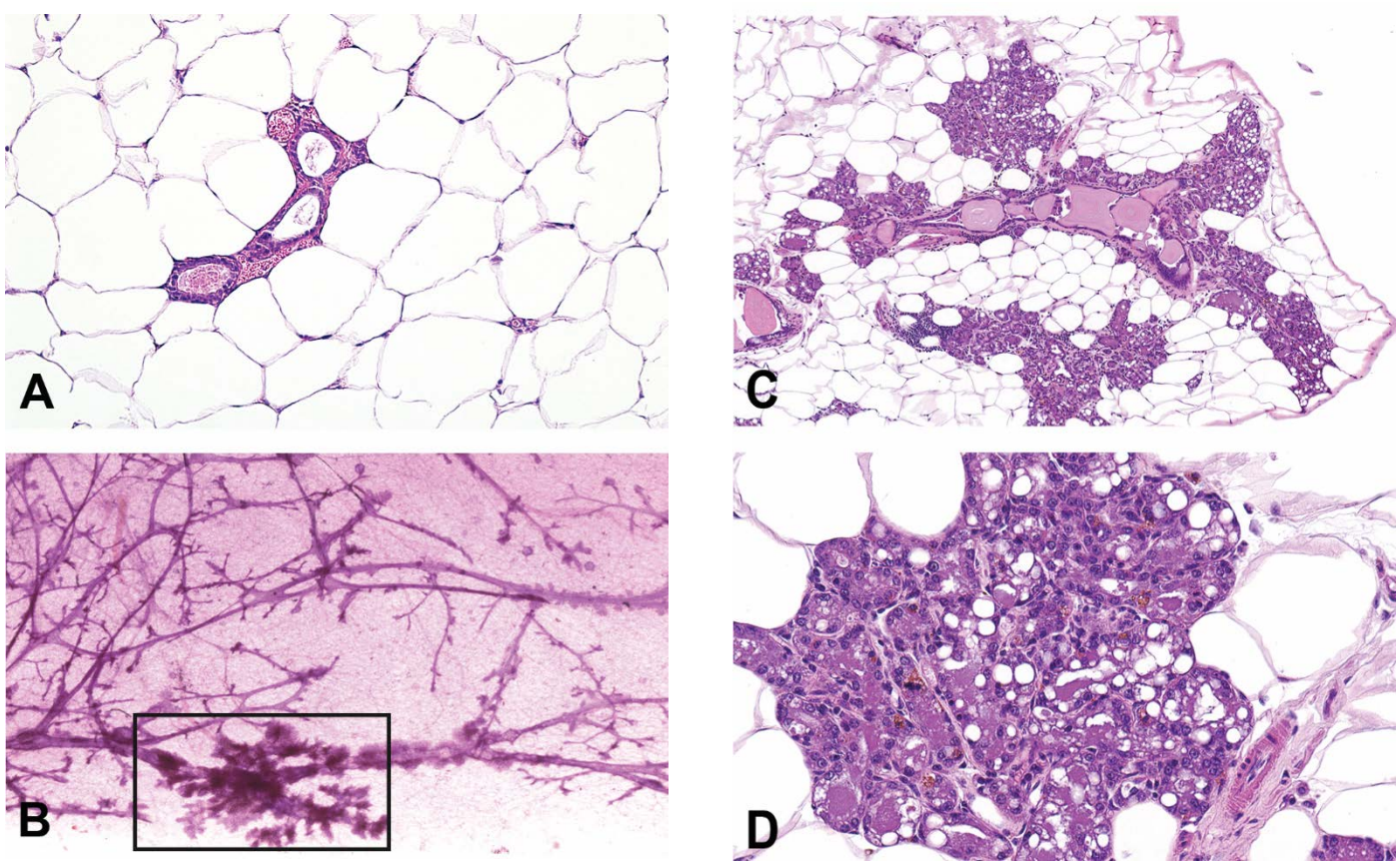

Figure AI-3: Mammary gland section of lobular hyperplasia using the whole mount method. Normal mammary gland section with no histopathologic findings, formalin fixed an H&E-stained, magnification 10x (A); Increased opacity around ducts and stromal structures (box) in the contralateral carmine-stained mammary whole mount. (B); At low magnification, in the contralateral mammary gland prepared from the whole mount (boxed area), there is enlargement of the lobule due to an increase in the number and size of cells (lobular alveolar hyperplasia); magnification 10x (C); Higher magnification illustrated enlarged lobules consisting of increased numbers of normal alveolar epithelial cells which were well differentiated and often vacuolated. Alveolar lumens often contained proteinaceous fluid; magnification 40x (D). Tissue samples are from a second individual 14 mo. old female CD-1 mouse.

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APPENDIX II

The mammary gland is a sensitive pubertal target in CD-1 and C57Bl/6 mice following perinatal perfluorooctanoic acid (PFOA) exposure[1]

OVERVIEW

Perfluorooctanoic acid (PFOA) is a known developmental toxicant in mice, with varied strain outcomes depending on dose and period of exposure. The impact of PFOA on female mouse pubertal development at low doses (≤1 mg/kg), however, has yet to be determined. Therefore, female offspring from CD-1 and C57Bl/6 dams exposed to PFOA, creating serum concentrations similar to humans, were examined for pubertal onset, including mammary gland development. Mouse pups demonstrated a shorter PFOA elimination half-life than that reported for adult mice. Prenatal exposure to PFOA caused significant mammary developmental delays in exposed female offspring in both strains. Delays started during puberty and persisted into young adulthood; severity was dose-dependent. In contrast, an evaluation of serum hormone levels and pubertal timing onset in the same offspring revealed no effects of PFOA compared to controls in either strain. Therefore, our data suggest that the mammary gland is more sensitive to the effects of early low level PFOA exposures compared to other pubertal endpoints, regardless of strain.

INTRODUCTION

Perfluorooctanoic acid (PFOA) is an eight carbon member of the perfluoroalkyl acid family. Its use as a surfactant in various industrial and consumer products, as well as its ability to resist further degradation under extreme temperatures, has made it both persistent and ubiquitous within the environment resulting in the inevitable exposure to both humans and wildlife.

[1]This chapter was previously published in Reproductive Toxicology. Tucker, D.K., Macon, M.B., Strynar, J.M., Dagnino, S., Anderson, E. and Fenton, S.E. (2015). The mammary gland is a sensitive pubertal target in CD-1 and C57Bl/6 mice following perinatal perfluorooctanoic acid (PFOA) exposure. Reproductive Toxicology 54, 26-36.

Serum PFOA levels in the general U.S. population have declined from 5.21 ng/mL (1999-2000) to 3.07 ng/mL over a decade (2009-2010) [1], potentially due to consumer awareness and a gradual phase out of PFOA production in the U.S. However, residents living in areas of elevated PFOA exposure (sites near manufacturing facilities) have serum levels 10-100 times higher than the national average, and children aged 2-5 years demonstrated a geometric mean serum concentration of 600 ng/ml (as of 2006) [2]. Pubertal aged children from this area also experienced higher total geometric mean serum levels (<12 years, 77.6 ng/ml and 12-19 years, 59.9 ng/ml) [3]. Exposure at these ages is mostly related to drinking water sources but may also be contributed to by the ingestion of contaminated dust, food sources and hand to mouth contact. PFOA exposure may also occur during gestation or shortly after birth, as PFOA was detected in human and rodent milk, rodent serum, urine, amniotic fluid and at relatively low levels in human cord blood [4-6]. These findings suggest that PFOA is not only capable of being transferred from mother to offspring due to its long half-life (3.8-4.4 years in humans [7]), but that the longer the exposure, the higher the potential is for increased serum PFOA levels in offspring. Parental exposures prior to conception may compromise embryonic development and continued placental and lactational [8] exposures may have deleterious effects on the developing fetus, newborn and infant. Early life exposures to PFOA may induce alterations in the epigenome, such as altered DNA methylation [9, 10], that have the potential to alter normal development and lead to adverse health outcomes in later life.

High dose exposures in adult rodents (≥10 mg/kg PFOA) have resulted in hepatotoxicity, tumors of the liver and pancreas and in pregnant adult mice, neonatal toxicity and mortality of their offspring [11-13]. Lower perinatal doses, however, have resulted in an array of reproductive

and developmental alterations in mouse offspring that have included advanced preputial separation in males, sex specific neurobehavioral differences [14] and impaired mammary gland development [15]. The mammary gland is a unique organ in that the majority of its development occurs postnatally. Although differentiation is not complete until late pregnancy, puberty demarks an exponential growth phase that is facilitated by various paracrine and endocrine factors including estrogen and progesterone and includes an increased mammary stem cell population [16-18]. Additionally, the highly proliferative terminal end buds (TEBs) lead the migration of the mammary epithelium into the fat pad resulting in the extensive ductal branching patterns of the gland. Exposures to endocrine disrupting chemicals (EDCs) during gestation or early life are especially of concern because they may affect the normal progression of mammary development that may not become apparent until puberty or later life. EDCs can disrupt normal functions by mimicking naturally occurring hormones, inhibiting hormone receptor binding or causing an abnormal inhibitory or stimulatory endocrine response [19]. High dose PFOA exposure has demonstrated these properties in the mammary gland by stimulating growth in the glands of peripubertal exposed C57Bl/6 female mice [20]. These effects were only apparent when the ovaries were present and also resulted in the upregulation of estrogen receptor alpha (ERα) expression within the gland. However, the mechanism for prenatal PFOA exposure on mammary gland development has yet to be determined, but it is evident that early exposures set in motion key alterations that may be exacerbated by circulating endogenous hormones. This could potentially further target the proliferating TEBs resulting in the increased potential for cellular transformations that may make the gland more susceptible to developing later life diseases, such as breast cancer [21, 22].

Full and late gestational exposure to low dose PFOA has been shown to induce stunted mammary epithelial growth in CD-1 female offspring, an abnormality that persisted into adulthood, which can begin as early as postnatal day (PND) 56 in some mouse strains. Macon *et al.* [15] reported adverse changes in female CD-1 mouse mammary gland development from 0.01 to 1 mg/kg PFOA following a late gestation exposure, which is defined as a one-time daily oral gavage exposure between gestational days 10-17. Multigenerational effects have also been observed in CD-1 mice administered separate and combined oral gavage and drinking water exposure to 5 mg/L PFOA. All PFOA treated F_1 females demonstrated reduced mammary development that continued until 63 days of age (young adulthood) [23]. Mammary development was also attenuated at weaning in F_2 females that were never exposed to PFOA via placental transfer. Much higher oral exposures appear to be required for mammary effects in other strains, using peripubertal exposures. Yang *et al.* [24] reported striking differences among C57Bl/6 and Balb/c mice peripubertally exposed to ≥ 5 mg/kg PFOA. Peripubertal PFOA exposure significantly inhibited mammary growth at 5 and 10 mg/kg in Balb/c animals, whereas in C57Bl/6 mice stimulatory effects were reported at 5 mg/kg, but inhibitory effects predominated at 10 mg/kg. Mammary gland development was not altered in either strain following peripubertal exposures at 1.0 mg/kg suggesting that some mouse strains are more sensitive than others to PFOA effects and/or the effects in the mammary gland are highly dependent upon the timing of exposure (peripubertal vs. *in utero*).

PFOA has also been associated with changes in other pubertal developmental landmarks. Girls living in close proximity to PFOA-polluted areas have self-reported delays in menarche that were associated with increased serum PFOA levels [25]. Rodent models have also been assessed for similar hallmarks that include timing of first estrus, vaginal opening (VO) and

estrous cyclity, as the onset of cyclity occurs after VO and first estrus; VO and first estrus in prenatally exposed pups were slightly delayed at dose levels outside those that humans would be exposed to (20 mg/kg) and unchanged at the lowest dose tested (1.0 mg/kg) [13]. EDCs have been reported to elicit non-monotonic responses, wherein lower doses may produce changes not observed at higher doses [26]. To date, no study has examined the low-dose effects of PFOA on all pubertal events in prenatally exposed female mice nor determined how the mouse strain may influence any potential effects. Our goal was to provide a thorough evaluation of the low dose effects of PFOA exposure on the timing of critical pubertal events in the CD-1 and C57Bl/6 female mouse including mammary gland development, VO and first estrus, in addition to the evaluation of serum steroid hormone and PFOA levels. These assessments may provide valuable information as to the most sensitive pubertal targets influenced by prenatal PFOA exposure, as well as indicate pathways that may be perturbed by other related toxicants during pubertal onset.

MATERIALS AND METHODS

PFOA

Perfluorooctanoic acid (PFOA, ammonium salt; >98% pure) was obtained from Fluka Chemical (Steinheim, Switzerland) and 1, 2-$^{13}C_2$-perfluoroctanoic acid ($^{13}C_2$-PFOA) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). PFOA dosing solutions were dissolved in de-ionized water (DI water) and freshly prepared daily.

Animals

Timed pregnant CD-1 and C57Bl/6 mice were obtained from Charles River Laboratories (Raleigh, NC) on gestational day 0 (GD 0) when they were confirmed sperm positive. Upon

arrival, each dam was weighed and randomly assigned to one of five treatment groups, equalizing the mean starting body weight in each group. Pregnant dams were individually housed in polypropylene cages with microbarrier lids and received chow (LabDiet 5001, PMI Nutrition International LLC, Brentwood, MO) and tap water *ad libitum*. Animal facilities were maintained on 12:12h light-dark cycle, at a controlled temperature (20-24 ^{o}C), with 40-60% relative humidity. All animals were treated humanely and in accordance with the National Institute of Environmental Health Sciences Animal Care and Use Committee (ACUC).

Experimental Design

For space reasons, the studies in CD-1 mice were completed in three blocks (block 1 n=97, block 2 n= 40, and block 3 n=26). Studies with the C57Bl/6 animals were completed in one block (n=41). For each experimental block, timed pregnant dams were orally gavaged daily with vehicle (DI water), 0.01, 0.1, 0.3 or 1.0 mg PFOA/kg/body weight (bw) between GD 1-17. A full gestation exposure design was chosen to recapitulate murine serum levels that have been established in previous experiments and were found to overlap with PFOA serum levels in residents living in heavily contaminated areas in the U.S. Dams were weighed daily to administer a dose volume of 10 µl/g bw. Parturition normally occurred on the eve of GD 18 and PND 1 was defined as the first day following parturition (usually > 0.5 days old). On PND 3, pup sex was determined and CD-1 litters were equalized to 10 pups per dam. If numbers permitted, each dam received 6-7 females and 3-4 males. Pregnancy rates in CD-1 females were >60% (block 1 =78% block 2 =67% and block 3 = 88%); however, the C57Bl/6 block yielded a much lower rate of approximately 27% (41/152). Regardless of treatment, C57Bl/6 dams produced litter sizes of between 5-8 pups. Any litters with an n<5 pups were excluded from these studies. On PND 21,

pups of both strains were weaned and all female pups were retained and housed 3-5 mice per cage in an effort to keep litter mates together. Male weanlings from controls were retained in order to circulate pheromones into the room that are known to assist in normalizing female cycling [27, 28]. Additionally, every 3-4 days (at cage changes) a small sample of bedding from the males was placed into each female cage.

Necropsy

With the exception of PND 21 (sexually immature), vaginal lavage was performed on all females prior to necropsy to determine their stage of the estrous cycle. We attempted to necropsy females during the first day of estrus in order to minimize morphological and hormonal fluctuations caused by normal changes in the estrous cycle. Vaginal smears were acquired between 6 and 8 AM on the day of sacrifice and necropsied shortly thereafter (9 AM-12 PM) [29]. On PND 21, 35, and 56 for CD-1 mice (n=4-7 litters/treatment), and PND 21(n=2-6 litters/treatment) and 61 (n= 3-9litters/treatment) for C57Bl/6 mice (fewer time points due to this strain historically having fewer pups per live birth and low pregnancy rates following plug identification) female pups were first weighed and then sacrificed using swift decapitation to obtain trunk blood for PFOA and hormone analysis. Mammary glands were collected from the fourth and fifth inguinal glands and prepared as carmine stained whole mounts. Body and liver weights were also recorded.

Pubertal Endpoints

Pubertal maturation in females was assessed using VO and vaginal cytology to determine timing to first estrus in the estrous cycle. Visual assessment for VO started on PND 18 in CD-1

mice and on PND 23 in C57Bl/6 mice, as VO has been reported to begin as late as PND 30 in that strain [30]. Once VO occurred, daily vaginal lavage was performed using 1X phosphate buffered saline and a sterile eyedropper to obtain cells to determine the presence of cornified epithelial cells, the first indication of estrus [13, 31]. All samples were evaluated fresh daily on a Leica DM2000 light-microscope (Leica Microsystems). Body weights were taken daily and recorded on the day of VO and first estrus. Pubertal endpoints were only measured in CD-1 block 1 (n= 7-8/treatment) and 2 (n=4-5/treatment) litters. Three to seven C57Bl/6 pups were evaluated per litter.

Mammary Whole Mount Preparation and Analysis

The entire fourth and fifth mammary gland from each mouse was placed on a charged slide and flattened to the natural surface area. Each whole mount was fixed in Carnoy's solution (EtOH, acetic acid and chloroform), stained in carmine alum and de-fatted in xylene [32]. For each collection, all samples were compared to age-matched animals within the same group and across other treatment groups [32]. Each gland was assessed and assigned a qualitative score on a scale of 1-4 (1=poor development and 4=best development) using a Leica Z16APO and DFC295 light microscope and camera (Leica Microsystems Inc., Buffalo Grove, IL), respectively. Qualitative scoring criteria is based on, but not limited to, lateral and longitudinal epithelial growth, branching density, changes in epithelial growth, appearance of budding from ductal tree, number of differentiating duct ends and the presence or absence of terminal end buds. Each gland was scored by two individuals without knowledge of treatment and averaged to obtain a final score [15]. Approximately, 4-7 CD-1 females/treatment/block and 2-10 C57Bl/6 offspring/treatment were scored.

PFOA Serum

Serum concentrations of PFOA were obtained using the methods reported by Reiner *et al.* [33]. Briefly, 25-50 µL of serum from each individual was transferred to its own 15 ml propylene tube. A pre-determined amount of internal standard ($^{13}C_2$-PFOA) was added to approximate the midpoint of the calibration curve for the anticipated sample range. 0.1 M Formic acid was added to the sample to denature the proteins and it was vortexed. Cold acetonitrile (-20C) was added to precipitate the proteins followed by vortexing and centrifugation at 2000 x g for 3 min. Supernatant from the acetonitrile mixture were placed in liquid chromatography vials containing ammonium acetate buffer (pH 6.5) (1:1). All samples were analyzed using a Waters Acquity Ultra Performance Liquid Chromatograph interfaced with a Waters Quattro Premier XE triple quadropole mass spectrometer (Waters Corporation, Milford, MA). Blank matrices were obtained using Pel-Freeze Biologicals CD-1 control mouse serum (Rogers, AR). At least six standards were used to generate the calibration curve with the coefficients of determination at 0.99 or higher. Calibration curves were obtained by plotting the ratio of PFOA peak area to ^{13}C2-PFOA peak area vs. concentration and were fitted to a linear regression equation with 1/x weighting. The limit of quantitation (LOQ) was defined as the lowest point on the standard curve at which analysis may be reported with confidence. Quality control (QC) samples were interspersed throughout the analytical run and were run in duplicates for accuracy determination. Method accuracy and precision were determined by analyzing the QC samples repeatedly. Accuracy was calculated as the percentage of the concentration found compared with the theoretical concentration and the precision was calculated using the average relative standard deviation of the replicate analysis of the QC materials. Additionally, approximately 10% of all unknown samples were analyzed in duplicate for precision measurements. Each sample batch

run contained a calibration standard and a matrix and methanol blank that were prepared under the same conditions as the unknown samples. The LOQ for CD-1 serum samples ranged from 5-100 ng/ml (Control=5 ng/ml, 0.01mg/kg=10 ng/ml, 0.1, 0.3 and 1.0 mg/kg=100 ng/ml). The LOQ for C57Bl/6 animals was 10 ng/ml. Samples that were below the LOQ were reported as the LOQ /√2 for statistical purposes. Serum PFOA was measured from at least one pup from each litter per treatment group in the CD-1 (n=5-10) and C57Bl/6 (n= 2-6) studies.

Serum Estradiol and Progesterone Analysis

Serum estradiol and progesterone levels were measured using a commercially available assay kit (Meso Scale Discovery, Gaithersburg, MD). Coefficients of variation ≤ 20% were considered acceptable. Assays were performed in a multiplex format in 96-well, 4 spot plates that were pre-coated with estradiol and progesterone capture antibodies and 50 µl serum samples. Sandwich immunoassays were conducted by adding test serum followed by conjugated detection antibodies (anti-estradiol and anti-progesterone) containing an electrochemiluminescent compound. Electrochemiluminescence was detected using a SECTOR Imager 2400. Quantitation of estradiol or progesterone was based on the intensity of the emitted light. A rat serum sample of known concentration was also tested for QC purposes. Data from the Sector Imager was transferred to Excel worksheets for further analysis. The limit of detection (LOD) for estradiol was 5 pg/ml and 0.07 ng/ml for progesterone.

All unknown serum samples were analyzed in duplicate and standard curves for estradiol and progesterone were generated using calibrators supplied with the kit. Estradiol concentrations between 0 and 4 ng/ml and progesterone concentrations between 0 and 50 ng/ml were fitted to a sigmoid dose response curve and used to quantify the level of each hormone in test samples.

Serum samples from each treatment group were stratified across plates so that not all of one dose group was assayed on the same plate.

Statistical Analysis

For all studies, dams or litters were considered the unit of measurement. Therefore, if a dam was represented by more than one pup, their values were averaged. Outlier values were determined by bodyweight and were calculated using GraphPad's QuickCalc Grubb's test (http://graphpad.com/quickcalcs/grubbs1/). Outliers were only removed when the animal presented as being moribund. When an individual animal was found to be an outlier within a specific end point, the data for that individual was removed from all data sets that it was contained in. Replicated experiments were treated as blocks and were analyzed using two-way analysis of variance (ANOVA) to determine block and treatment effects using Tukey's post hoc test ($p<0.05$). A linear regression test was performed on the mammary glands to determine if any dose related effects existed. Serum PFOA, estradiol and progesterone levels were assessed using ANOVA followed by a Dunnett's post hoc test. Graphs and tables were created using GraphPad Prism 6 (La Jolla, CA) and Microsoft Excel. All data are represented as the mean ± SEM.

RESULTS

Internal PFOA burdens and liver enlargement. Serum PFOA concentrations in exposed female offspring were measured to determine the internal concentrations at which pubertal effects were being observed. PFOA levels were detectable in all exposed female offspring from both strains at PND 21 (Table AII-1). PFOA was undetectable in control mice of both strains. Serum PFOA concentrations increased in a dose dependent manner and were significantly

different at the two highest doses in the CD-1 females at PND 21 and 35 compared with control females. Detectable levels were measured in all treated animals at PND 35, however, by PND 56 all animals experienced a 4-5 fold decrease in serum levels compared to littermates collected at PND 35, a decrease that was larger than expected, given the reported 15.6-21.7 d half-life of PFOA in adult CD-1 mice [34] following a single oral adulthood exposure [34]. By PND 56, serum PFOA levels were below LOQ in the 0.01mg/kg group and only statistically increased from control levels at 1.0 mg/kg. The same dose dependent trend observed at PND 21 in CD-1 mice was also shown in the C57Bl/6 pups; however, differences were not significant, more than likely reflecting the fair amount of variability between mice in a small litter size, rather than treatment. Additionally, treatment with the same PFOA exposures in C57Bl/6 mice resulted in serum levels that were lower on average compared to those observed in the CD-1 strain of the same age. By PND 61 the C57Bl/6 animals in the three highest dose groups had measurable levels that were still above the LOQ threshold, however, these levels were ~10-100 fold reduced from those observed in their PND 21 litter mates. Taken together, these data suggest a faster rate of clearance of PFOA from the C57Bl/6 offspring and potentially a faster clearance in rapidly growing offspring than the 15.6-21.7 d half-life previously reported in adult CD-1 mice [34]. Based on our 1.0 mg PFOA/kg data and serum data collected previously [15], we estimated half-life clearance in the CD-1 animals between PND 21 to PND 56 to be 7.3-8.9 days and C57Bl/6 to be 6.0 days between PND 21 and PND 61.

Full gestation PFOA exposure (0.01 to 1 mg/kg) did not affect absolute body weight measurements in either CD-1 or C57Bl/6 female offspring (Tables AII- 2 and AII-3, respectively). Net body weight was defined as the liver weight subtracted from the body weight and was found to be significantly reduced in the highest dose group in CD-1 females (1.0 mg/kg)

at PND 21 and 35. This effect had little biological significance, as it resolved and net body weights were comparable to the control levels by PND 56. These finding are in agreement with the body weights observed at post weaning ages following a full gestation exposure to PFOA levels ≤ 1.0 mg/kg [13, 15]. No significant effects for net body weight were observed in the C57Bl/6 females.

Absolute liver weight differences in both strains were not significantly altered in comparison to their respective controls (Tables AII-2 and AII-3). CD-1 females in the 1.0 mg/kg dose group exhibited significantly elevated relative liver weights at PND 21 that recovered to normal levels by PND 35. As relative liver weights had returned to values similar to controls by PND 28 following a full gestational exposure in a previous study, our transient finding is thought to be biologically irrelevant [15]. C57Bl/6 mice did not demonstrate any significant relative liver weight changes at PND 21 or PND 61. Together these data confirm that liver effects resulting from early and low exposures are transient and may begin to dissipate during early puberty. This appears to correlate well with the higher internal body burdens in CD-1 mice that showed increased liver hypertrophy (relative liver weight) in 1 mg PFOA/kg bw weanlings compared to no effects seen in C57Bl/6 pups (Tables AII-2 and AII-3).

Pubertal Assessment. Pubertal onset in the female mouse is associated with an increased production of the steroid hormones estradiol and progesterone as a result of increased signaling from the hypothalamus and pituitary gland to the ovaries. Both hormones are critical for ovulation and uterine changes, as well as normal mammary gland development and sexual maturation [35, 36]. To understand the effects of prenatal PFOA on circulating steroids, serum was obtained from females in the stage of estrus, except at PND 21 where all hormone analysis

samples were from females that had not undergone VO. Figure AII-1A illustrates that estradiol levels in both strains were similar between PFOA treated and control mice. A similar lack of statistical difference for treatment effect was shown for progesterone (Figure AII-1B), however; progesterone levels were overall elevated in CD-1 animals at PND 56, in all exposure groups, compared to levels seen at PND 21 and 35. Progesterone levels in PFOA-exposed C57Bl/6 females were comparable to control at both PND 21 and 61.

An assessment of the effects of PFOA on VO and first estrus are summarized in Table AII-4. Females from only two of the three CD-1 blocks were measured. Although all assessments were completed in an identical manner, it was necessary to assess each block separately due to block effect differences. VO occurred ~4 days later in block 2 compared to block 1, however, comparisons between the control and treatment groups within each block revealed no significant differences due to treatment. The reason for the block difference could not be determined. Timing to first estrus was also unchanged within each block. However, block 1 females appeared to undergo their average first estrus 2-3 days following VO whereas it almost immediately followed VO in block 2 females, with the exception of the 1.0 mg/kg group (~2.5 days timing to first estrus). Neither body weight at VO nor first estrus timing was changed by PFOA treatment in either block. The age of VO, day of first estrus and body weights at these times was also unchanged in the C57Bl/6 animals. The fact that no treatment related effects were observed in either strain implies that the differences in the normal timing of these events in each strain are likely a result of strain differences rather than PFOA exposure.

The effects of PFOA on mammary gland development were assessed by whole mount using factors that include lateral and longitudinal branching, the presence or absence of terminal end buds and branching density. However, these criteria were dependent upon the age of

development. Both CD-1 and C57Bl/6 strains exhibited developmental delays that were apparent at the lowest dose (0.01 mg/kg) and were significantly different from controls in CD-1 mice. As early as PND 21 we observed a dose dependent decrease in developmental scores in the CD-1 animals with the highest dose group scoring ~1.2 points lower than control (Table AII-5). Score reductions were significant at PND 21 in the three highest dose groups (0.1-1.0 mg/kg); however, by PND 35 and 56 growth delays were obvious in all CD-1 treatment groups resulting in significantly lower scores. When a trend analysis was performed on the CD-1 glands the mammary scores were found to be inversely related to the dose ($p<0.001$), indicating that with increased dose the greater the developmental delays observed. Representative morphological evaluations of the CD-1 glands are illustrated in Figure AII-2. TEBs, lateral and longitudinal branching and secondary branching were all decreased with increased PFOA dose, resulting in a much smaller gland (Figure AII-2). By PND 35, in addition to the growth defects already described, PFOA caused a delay in the fourth and fifth glands growing together. Although, our evaluation of pubertal timing in both strains has established that C57Bl/6 animals reach sexual maturation at a later point than CD-1 mice, mammary glands were still evaluated on PND 21 to remain consistent throughout each block. We did note that control scores from both strains were very similar; it should be noted that scoring is based on the level of development compared to controls and may be based on entirely different criteria that can still result in similar scores across strains.

In the C57Bl/6 strain, only the 0.3 and 1.0 mg/kg developmental scores were significantly reduced from that of controls (Table AII-5), however a trend analysis also confirmed very similar trend as those seen in the CD-1 animals in which at higher doses greater delays were seen. The presence of TEBs was minimal at PND 21, considering that estradiol levels in both

strains at this time point were within the same range. However, significant defects in branching density due to PFOA exposure were visible in the 0.3 and 1.0 mg/kg PFOA groups at both PND 21 and 61 (Table AII-5) . C57Bl/6 PFOA treated animals exhibited, at most, minimal budding and secondary branching with little to no lateral and longitudinal branching (Figure AII-3). At PND 61, the C57Bl/6 glands at the highest dose still had TEBs and internal budding throughout the fat pad; whereas at the same time point control glands were almost fully matured. This demonstrates that there are significant differences in the natural rate of mammary gland development in two different strains. Also, the impact of PFOA treatment on C57Bl/6 females was not as severe as that seen in CD-1 females and is likely due to differences in body burden, as serum PFOA levels were lower in C57Bl/6 than CD-1 across dose groups at the same ages. Regardless of those strain differences, the effects of PFOA on mammary gland development persisted in both strains through early adulthood.

DISCUSSION

The objective of these studies was to determine the pubertal development effects of early life PFOA exposures, which produce serum concentrations in the CD-1 and C57Bl/6 female mouse that coincide with reported human blood PFOA concentrations in contaminated area, specifically evaluating for dose and strain-related effects. Perinatal low dose exposures to PFOA resulted in greater alterations in the pubertal mammary gland for both strains compared to all other measured pubertal endpoints. Mammary gland developmental delays were visible as early as PND 21 in both strains and were significantly different from controls at concentrations as low as 0.01 mg/kg in the CD-1 mouse. Our study suggests that at these low doses the strain

differences, in response to PFOA, are dose dependent. Given identical oral doses during the exact same window of exposure, both strains experienced delayed mammary development at 0.3 and 1.0 mg/kg PFOA and lacked changes in the timing of other pubertal events. The CD-1 mouse responded to lower doses of PFOA, demonstrating effects on mammary development at 0.01 and 0.1 mg/kg, along with the higher doses. This is likely a reflection of the higher and longer circulating PFOA levels within the blood of the CD-1 mice. Although an increase in relative liver weight was evident in CD-1 mice and not the C57Bl/6 at 1.0 mg/kg PFOA exposure, we are unclear whether this observation was biologically relevant without having measurements from earlier time-points. From these data, we suggest that it is the peak serum PFOA concentration that regulates these effects (may have occurred between birth and within the first two weeks of life), rather than the serum PFOA level at the time of evaluation. This is further supported by earlier findings from Macon *et al.* [15] in which serum collected from a full gestation 1.0 mg/kg PFOA revealed that peak concentrations occurred at PND 14, rather than PND 7. To date, serum levels between PND 0 and PND 6 have yet to be reported for a full gestation study. In one study, where PFOA was administered once during late gestation (GD 17), pup serum concentrations peaked at PND 1 for all concentrations [5]. Therefore, the observed effects are likely a result of the *in utero* exposure, followed by exacerbation of effect from the exposure during lactation.

In these studies, PFOA induced abnormal mammary gland development in both strains. This suggests that either the other pubertal end points measured have a much different dose response threshold or that the hypothalamic ovarian axes that govern the various processes differ in their response to PFOA. Full gestation (GD1-17) PFOA exposure studies have previously reported delayed mammary development at concentrations $\leq$ 3.0 mg/kg in the CD-1 mouse [15]. Our

current findings fully support and extend those previous observations with reduced developmental scores at the lowest dose of 0.3 mg/kg, and with an n that is 3-4 times that in the previous experiments. Previous changes were evident beginning on PND 7 and persisted until the last collection on PND 84. We confirm here the persistent mammary effects at lower doses and also determined through trend analysis that the severity of the effects increased with higher does in both strains ($p < 0.001$). Macon and colleagues [15] also observed reduced mammary gland scores at PND 21 following a late gestation exposure (GD10-17) to PFOA in all concentrations including the 0.01 mg/kg dose. While they noted reduced mammary developmental scores following PFOA exposure, longitudinal growth measurements were only changed in the 1.0 mg/kg group and the number of TEBs were significantly reduced in the 0.1 and 1.0 mg/kg groups. All other quantitative measurements, however, were similar to age matched controls. Studies comparing the effects of peri-pubertal PFOA exposure in C57Bl/6 and Balb/c have also shown differences by strain in mammary gland development [24, 37]. Although they found no significant effects at 1.0 mg/kg in either strain compared to control for ductal length, number of TEBs or stimulated terminal ducts, C57Bl/6 animals had an increased number of TEBs compared to the Balb/c at PND 56. Many of the glands from our C57Bl/6 highest dose group (1.0 mg/kg) also exhibited poor branching and differentiation; TEBs were very apparent at PND 61, in addition to the substantial number of long ducts with little to no branching. Zhao *et al.* [37] also evaluated serum PFOA in C57Bl/6 and Balb/c pups, and as concentrations increased, C57Bl/6 mice were shown to have much lower serum PFOA levels compared to Balb/c females (≥ 5 mg/kg). While we are aware of the fact that concentration plays a major role in the depth of effects seen, our data further supports that the excretion rate for PFOA varies amongst mouse strain, and plays a significant role in whether or not PFOA affects the mammary gland at the

lowest doses tested. The fact that we saw adverse mammary changes in the CD-1 mice at 0.01 and 0.1 mg/kg suggests that this strain is the most sensitive to prenatal PFOA exposures for mammary gland developmental delays.

Puberty demarks a pivotal window of susceptibility for environmental exposures and the development of endocrine regulated tissues in both humans and rodents. During this period of development, upstream pulsatile increases from gonadotropin releasing hormone trigger sex hormones, such as estrogen and progesterone, to circulate at higher concentrations. In turn, other downstream cues are prompted that result in the occurrence of VO (rodents) or first menarche (humans), sexual maturation and rapid mammary gland development. Early exposure to EDCs, however, can alter the timing at which these processes occur or alter the morphological development altogether resulting in the increased risk of development of adulthood diseases, such as breast cancer [21, 22]. Since the onset of puberty is highly influenced by sex hormones, we were also interested in measuring circulating estradiol and progesterone to determine whether PFOA contributed to altered levels. Progesterone is involved in developmental branching [15, 16, 20] and estradiol has both paracrine and endocrine roles in mammary development [15, 16, 20]. We collected all post-pubertal animals on the same stage of the estrous cycle (first day of estrus) to minimize variability and found no difference in serum estradiol or progesterone in either strain in response to PFOA exposure. These findings are both supported and contrasted by the work of Zhao *et al.* [20]. C57Bl/6 mice exposed to 5 mg/kg during adolescence/puberty had no change in serum estradiol, yet were reported to have increased levels of progesterone. In a follow up study using PFOA doses of 2.5 mg/kg in Balb/c or 7.5 mg/kg in C57Bl/6 the same group of researchers were unable to see significant changes in progesterone due to an inadequate number of controls in each stage of the estrous cycle; however, they reported a significant

decrease in ovarian protein levels of StAR, CYP11A1, HSD3β1, HSD17β1, aromatase and PPARα, which are all involved in the biosynthesis pathway. Taken together, it appears that circulating levels of hormones have little, if any, effect on PFOA-induced decreases in mammary branching density. Thus, while other endocrine disruptors have been shown to alter estradiol and progesterone levels at much lower doses, we postulate that PFOA may alter mammary branching density through other endocrine related mechanisms that are specific to the mammary gland.

Evaluation of vaginal cytology is also a valid tool to identify the effects of toxicants, toxins or xenobiotics on the hypothalamic-pituitary-ovarian axis in rodents. In mice, VO is thought to denote the onset of puberty and is heavily influenced by increases in serum estradiol; however, it doesn't necessarily reflect the onset of sexual maturity. In the untreated CD-1 mouse VO typically occurs around PND 23, although normal timing to this event can vary in other mouse strains. Neither strain in our study exhibited changes in timing of either VO or first estrus following prenatal exposure to PFOA. Body weights were unchanged at the time of these events, as well (Table AII-4). Lau *et al.* [13] have shown a 3 day delay for VO and first estrus at 10 mg/kg following prenatal exposure, and consistent with our findings, the 1.0 mg/kg group showed no effect. Serum levels were not reported in their study but it was implied that increased PFOA serum levels in rodents result in greater pubertal delays. Although no differences were observed between treated and control animals, there were marked differences between the timing of VO and first estrus in CD-1 controls compared to C57Bl/6 controls. This observation has been previously noted [29]. Inbred strain comparisons at puberty showed that C3H and DBA animals began VO at approximately PND 22-24, whereas C57Bl/6 mice were around PND 27 [29]. C57Bl/6 mice were also the last to exhibit cornification in that 3-strain comparison. Our data supports these findings and reinforces the idea that even in non-treated animals there appears to

be differences in genetic profiles between strains that govern the timing of these occurrences. This innate difference may also influence windows of sensitivity to chemicals. Variability within the outbred CD-1 strain was also apparent within our vaginal cytology data and thus the reason for representing it in two separate blocks. The different seasons during which these blocks were tested could have contributed to these differences, as it has been noted that VO tends to occur earlier in rodents born during the summer months compared to those born during winter [38]. Our animals in block 1 were born in mid-summer whereas block 2 females were born during early winter. Body weights at VO in both blocks were similar indicating that our second block required additional time to achieve the body weight necessary to begin these events.

Serum PFOA levels were measured in our studies to determine the internal doses required for pubertal changes, such as mammary gland development, and also confirmed our lab's previous findings that internal exposures were within the range of serum PFOA levels reported in humans living in contaminated areas of the U.S. Children in the Ohio River Valley have been reported to have geometric mean (GM) serum PFOA levels as high as 600 ng/ml [2] and more recently, reports demonstrated levels of PFOA in children aged 12-19 even within the general population (GM: 1.81 ng/ml) that are comparable to those found in adults aged ≥20 (GM 2.12) [39]. Most noteworthy from these findings are the fact that our PND 21 serum concentrations from the 0.01 mg/kg treated groups in both strains fell within the serum range reported in male and female participants aged <12 years old from the C8 Science panel (GM 34.8 ng/ml) [3]. PFOA levels were elevated in a dose dependent manner and were within the same range at PND 21 in both strains, and were detectable in the three highest treatment groups on the final collection date in each strain.

Interesting correlations between potential rodent and adolescent health impact may be realized if the half-life of PFOA following exposure during gestation is compared. With a half-life of about 3 years in humans, by the time prenatal exposure had gone through three half-lives, a child would be around 9 or 10 years old (average age for Tanner Stage II; [40]). Similarly, in the prenatally exposed mouse pup with a PFOA half-life of 6-7 days (as calculated from our data), three half-lives would equate to PND 18-21 days old, which is also the time that the mammary tissue begins to develop. While there haven't been any reports that have directly linked PFOA exposure to breast developmental timing in girls, new evidence suggests that there may be a correlation. Higher serum PFOA levels were associated with the length of time that 6-8 year old girls had been breastfed [41] and their source of water and serum PFOA concentrations were highly correlated. In another study [42], breastfed girl's experienced delayed onset of breast development in comparison to formula fed girls and was even more exacerbated by the length of time that they were breastfed.

Girls living in the Ohio River Valley area reported increased delays (self-reported) to menarche within the measured concentration ranges that were observed in our 0.01 and 0.1 mg/kg group. This may indicate a lack of concordance between rodents and humans for other pubertal indicator endpoints following PFOA exposure. It has been well documented that the mode of action for these pubertal endpoints [43] are fairly different between rodents and girls and that VO and time to first estrus in rodents may not be translatable indicators for puberty timing in girls.

While most of organogenesis occurs during embryonic development, the mammary gland undergoes most of its growth during puberty. Still, exposure to excessive amounts of hormones or EDCs too early in life can have a permanent effect on the developing gland. Because

mammary alterations were documented following early low dose PFOA exposures in our study and by others [23], without altering other pubertal endpoints or liver: body weight ratios, implies that the mammary gland may be the most sensitive tissue to the prenatal effects of PFOA. This can also be said for other chemicals in which the mammary gland has been studied. Early exposure to human relevant levels of endocrine disruptors in rodents has led to both accelerated (Bisphenol A) and delayed mammary gland growth (TCDD and atrazine) that have presented in the form of an extended presence of TEBs [44], increased stromal and epithelial tissue and sensitization to estradiol [45-47]. Some of those chemicals, as well as PFOA [23, 48], have adversely affected the lactational capacity of rodents to nourish their litters. Dams [48] and their F1 offspring [23] demonstrate abnormal lactating gland morphology, decreased pup weight, and/or altered milk protein gene expression following exposure to PFOA during pregnancy. Studies using slightly higher exposures of PFOA [49] have also shown that the developmental effects of prenatal exposure had long-lasting effects; increased stromal hyperplasia and disorganized, scant mammary epithelium were reported in 18 month old PFOA-exposed mice.

The hypothesized mode of action for some of these chemicals involves either direct or indirect alterations to key receptors, such as ERα, or their corresponding signaling pathways. At least within the mammary gland PFOA has been shown to not directly bind to the ERα, nor cause a stimulatory effect in ovariectomized animals [20]. PPARα knock-out mice exhibited mammary effects following a peripubertal exposure indicating that PPARα may not play a predominate role in the mammary gland changes observed after PFOA exposure as those seen in rodent livers [20]. Work by Macon and colleagues (in press) suggest that post transcriptional changes may play a role in the observed mammary changes [50]. Pparα and γ expression were decreased in microarray validation studies, however, only Pparγ protein levels were increased.

Additionally, Pparγ molecular weight shifts were also found in Westerns, leading the authors to propose phosphorylating modifications. The stroma may play an important role, as lipid metabolism genes were also found to be altered. Therefore, we conclude that during early PFOA exposures post- transcriptional modifications in the gland itself may be responsible for the altered epithelium and microenvironment. Also, since PFOA was still present in the serum 5 weeks following weaning, as a result of a lactational exposure, the effects may have been compounded and the peripubertal mechanism suggested by Zhao *et al.* [20] may have also taken effect. Regardless of the mechanism behind the outcome, each of these chemicals, including PFOA, has the potential to significantly alter the glands normal development, thereby increasing the gland's risk to further environmental insults.

The few studies that have tried to correlate breast cancer risks to perfluorinated chemicals (PFCs) found that in a Greenland Inuit cohort the PFC levels were linear to the cancer risks [51], while a Danish cohort reported weak significance [52]. Whether PFOA directly causes cancer still requires additional evaluation, however, the delayed phenotype that it creates in the rodent ultimately leaves the gland more susceptible to other harmful exposures. Because of the potential for increased lifetime susceptibility to disease or dysfunction, pubertal assessments of chemical effect performed in rodent models should include an evaluation of the mammary gland. This suggestion has recently been echoed by the scientific community [53, 54]. It will also be necessary to evaluate for changes in breast development based on early life or cord blood PFOA levels in future epidemiological studies.

Although PFOA production and use in the USA is scheduled to be phased out by 2015 [55], its lasting effects may continue to be seen well after this time due to its persistence within the environment and long half-life in humans. Because PFOA exposure occurs throughout the

lifetime in humans, it may be necessary to continue monitoring heavily exposed populations to determine if later life diseases arise. These studies confirm that the mammary gland is highly sensitive to low dose prenatal PFOA exposure. Our findings suggest that measures should be put in place to limit PFOA exposures in sensitive populations, especially in pregnant women and prepubescent females. Decreased exposure could potentially minimize early life effects as well as those that may persist throughout life to reduce the mammary glands susceptibility for later life diseases and dysfunction.

Table AII-1: PFOA Serum Concentrations in pubertal CD-1 and C57Bl/6 mice

	Control (n)	**0.01 mg/kg (n)**	**0.1 mg/kg (n)**	**0.3 mg/kg (n)**	**1.0 mg/kg (n)**
CD-1					
PND 21	<LOQ (6)	74.8 ± 16.9 (10)	457.3± 91.0 (9)	904.8 ± 131.5 (10) *	3119.0 ± 396.4 (5) **
PND 35	<LOQ (11)	14.3 ± 2.3 (12)	61.7 ± 6.5 (9)	200.9 ± 32.2 (11) *	889.8 ± 117.5 (11) **
PND 56	< LOQ (5)	ND	15.0 ± 5.0 (4)	46.2 ± 7.5 (6)	200.2 ± 21.1 (7)**
C57Bl/6					
PND 21	<LOQ (2)	26.1 ± 19.0 (1)	247.1 ± 11.4 (2)	891.3 ± 528.7 (3)	2141.67 ± 666.8 (2)
PND 61	<LOQ (6)	<LOQ (2)	27.7 ± 10.4 (3) *	9.3± 2.2 (5)	22.0 ± 7.6 (5)

Data presented as the mean ± SEM
The LOQ for all C57Bl/6 animals were <10 ng/ml. Significance observed in comparison to control; *p≤0.05.
CD-1 n= 4-12
C57Bl/6 n= 2-6

Table AII-2: Body and Liver Weights of Pubertal female CD-1 mice

	Control (n)	0.01 mg/kg (n)	0.1 mg/kg (n)	0.3 mg/kg (n)	1.0 mg/kg (n)
Body Weight (g)					
PND 21	11.9 ± 0.2 (20)	12.1 ± 0.2 (22)	12.5 ± 0.3 (22)	11.6 ± 0.3 (22)	10.9 ± 0.2 (21)
PND 35	23.1 ± 0.3 (17)	22.9 ± 0.5 (16)	23.0 ± 0.4 (14)	22.2 ± 0.3 (17)	21.8 ± 0.4 (16)
PND 56	26.6 ± 0.8 (9)	27.7 ± 0.7 (14)	27.6 ± 0.2 (8)	25.69 ± 0.83 (4)	28.5 ± 0.7 (9)
Net Body Weight (g)					
PND 21	11.3 ± 0.2 (19)	11.5 ± 0.2 (22)	11.9 ± 0.3 (22)	11.1 ± 0.3 (22)	10.3 ± 0.2 (21) *
PND 35	22.0 ± 0.3 (17)	21.7 ± 0.4 (16)	21.9 ± 0.4 (14)	21.1 ± 0.3 (17)	20.7 ± 0.3 (16) *
PND 56	25.2 ± 0.8 (9)	26.3 ± 0.7 (13)	26.3 ± 0.2 (8)	25.6 ± 0.5 (10)	27.1 ± 0.6 (9)
Absolute Liver Weight (g)					
PND 21	0.60 ± 0.02 (19)	0.62 ± 0.02 (22)	0.61 ± 0.02 (22)	0.59 ± 0.02 (22)	0.62 ± 0.02 (21)
PND 35	1.16 ± 0.03 (17)	1.14 ± 0.04 (16)	1.13 ± 0.04 (14)	1.13 ± 0.02 (17)	1.13 ± 0.04 (16)
PND 56	1.36 ± 0.05 (9)	1.35 ± 0.04 (13)	1.29 ± 0.03 (8)	1.22 ± 0.02 (10)	1.36 ± 0.07 (9)
Relative Liver					
PND 21	0.051 ± 0.002 (19)	0.051 ± 0.001 (22)	0.049 ± 0.001 (22)	0.051 ± 0.001 (22)	0.057 ± 0.001 (21) *
PND 35	0.050 ± 0.001 (17)	0.050 ± 0.002 (16)	0.049 ± 0.002 (14)	0.051 ± 0.001 (17)	0.051 ± 0.001 (16)
PND 56	0.052 ± 0.002 (9)	0.048 ± 0.003 (13)	0.047 ± 0.001 (8)	0.046 ± 0.001 (10) *	0.048 ± 0.001 (9)

Data are represented as Mean ± SEM
Net Body Weight= Body weight (g) – Liver Weight (g)
Relative Liver weight = Body weight (g)/Liver weight (g)
Significance observed in comparison to control; *p≤0.05
n= 8-22

Table AII-3: Body and Liver Weights of Pubertal female C57Bl/6 mice

	Control (n)	0.01 mg/kg (n)	0.1 mg/kg (n)	0.3 mg/kg (n)	1.0 mg/kg (n)
Body Weight (g)					
PND 21	8.4 ± 0.4 (6)	8.6 ± 0.1 (4)	9.5 ± 0.9 (2)	8.2 ± 0.7 (5)	7.5 ± 0.3 (5)
PND 61	19.1 ± 0.3 (9)	19.8 ± 0.3 (5)	20.1 ± 0.5 (3)	20.1 ± 0.4 (9)	19.9 ± 0.5 (8)
Net Body Weight (g)					
PND 21	8.0 ± 0.4 (6)	8.3 ± 0.03 (4)	9.1 ± 0.9 (2)	7.9 ± 0.6 (5)	7.1 ± 0.3 (5)
PND 61	18.2 ± 0.2 (9)	18.8 ± 0.3 (5)	19.2 ± 0.5 (3)	19.2 ± 0.3 (9)	19.0 ± 0.4 (8)
Absolute Liver Weight (g)					
PND 21	0.37 ± 0.03 (6)	0.43 ± 0.04 (4)	0.45 ± 0.03 (3)	0.38 ± 0.03 (6)	0.39 ± 0.01 (5)
PND 61	0.93 ± 0.02 (9)	0.97 ± 0.03 (5)	0.90 ± 0.05 (3)	0.95 ± 0.05 (9)	0.89 ± 0.03 (8)
Relative Liver					
PND 21	0.044 ± 0.002 (6)	0.049 ± 0.004 (4)	0.048 ± 0.001 (2)	0.045 ± 0.001 (5)	0.052 ± 0.001 (5)
PND 61	0.048 ± 0.001 (9)	0.049 ± 0.001 (5)	0.045 ± 0.002 (3)	0.047 ± 0.002 (9)	0.045 ± 0.002 (8)

Data are represented as Mean ± SEM
Net Body Weight= Body weight (g) – Liver Weight (g)
Relative Liver weight = Body weight (g)/Liver weight (g)
N= 2-9

Table AII-4: Assessment of Pubertal Events following prenatal PFOA exposures

	Control	0.01 mg/kg (n)	0.1 mg/kg (n)	0.3 mg/kg (n)	1.0 mg/kg (n)
CD-1 (Block 1)					
Vaginal Opening	22.8 ± 0.7 (8)	24.1 ± 0.7 (11)	23.8 ± 0.9 (7)	25.7 ± 0.6 (7)	23.9 ± 0.7 (10)
First Estrus	25.1 ± 0.6 (8)	26.7 ± 0.7 (11)	26.1 ± 0.9 (7)	28.2 ± 0.6 (7)	26.3 ± 0.6 (10)
CD-1 (Block 2)					
Vaginal Opening	28.2 ± 1.4 (4)	27.7 ± 1.1 (5)	28.8 ± 0.4 (5)	28.1 ± 1.4 (5)	26.6 ± 0.9 (4)
First Estrus	28.8 ± 1.2 (4)	29.8 ± 1.2 (5)	28.9 ± 0.4 (5)	29.2 ± 1.4 (5)	29.1 ± 0.4 (4)
C57Bl/6					
Vaginal Opening	31.7 ± 0.8 (7)	31.2 ± 0.4 (3)	29.9 ± 0.3 (3)	31.3 ± 0.7 (5)	31.6 ± 0.6 (5)
First Estrus	33.6 ± 0.9 (7)	32.9 ± 0.1 (3)	31.9 ± 1.0 (3)	31.7 ± 0.7 (5)	32.4 ± 0.8 (5)

Data are represented as Mean ± SEM
CD-1 n= 4-11
C57Bl/6 n=3-7

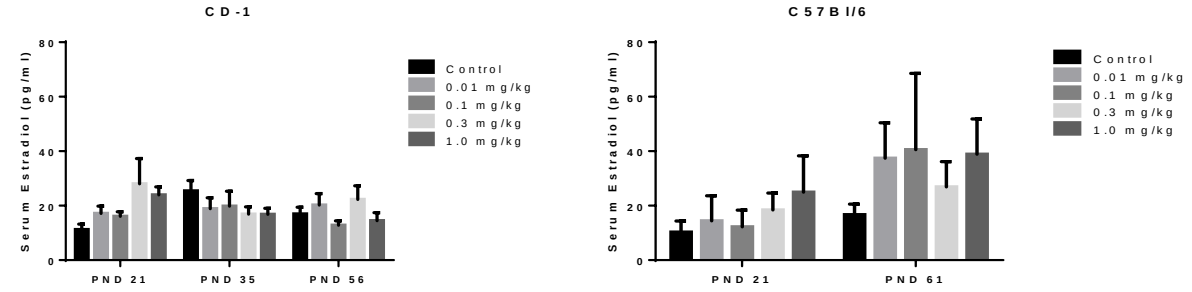
Table AII-5: Mammary Gland Developmental Scores

	Control (n)	0.01 mg/kg (n)	0.1 mg/kg (n)	0.3 mg/kg (n)	1.0 mg/kg (n)
CD-1					
PND 21	2.9 ± 0.1 (19)	2.4 ± 0.1 (22)	2.3 ± 0.1 (22)**	2.0 ± 0.1 (21)***	1.7 ± 0.1 (21)****
PND 35	3.1 ± 0.1 (16)	2.3 ± 0.2 (17)**	2.2 ± 0.2 (14)**	2.3 ± 0.1 (16)**	1.9 ± 0.2 (14)****
PND 56	3.3 ± 0.1 (9)	2.3 ± 0.2 (13)**	2.5 ± 0.2 (8)*	2.2 ± 0.1 (10)**	1.9 ± 0.2 (9)****
C57Bl/6					
PND 21	2.9 ± 0.2 (7)	2.5 ± 0.4 (5)	2.1 ± 0.7 (2)	1.8 ± 0.3 (6)*	1.8 ± 0.2 (5)*
PND 61	2.8 ± 0.2 (10)	2.2 ± 0.2 (5)	2.6 ± 0.1 (3)	2.1 ± 0.1 (10)*	1.7 ± 0.1 (8)***

Data are represented as Mean ± SEM

Significance observed in comparison to control; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. CD-1 n= 8-19 and C57Bl/6 n=2-10

A.



B.

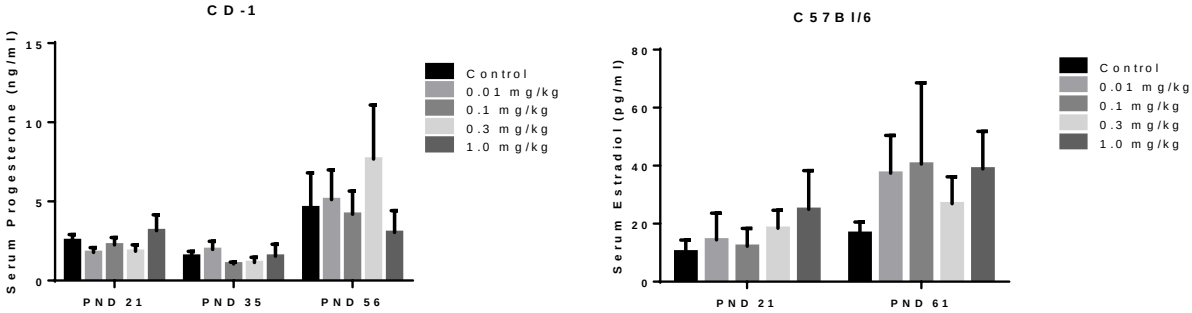


Figure AII-1: Effects of PFOA treatment on serum hormone levels. A) estradiol B) progesterone levels. All animals were sacrificed during estrus, with the exception of PND 21 when vaginal opening had not occurred. CD-1 litter/treatment group n=12-15 (PND 21), n=9-10 (PND 35) and n=8-10 (PND 56). C57Bl/6 litter n= 3-4 (PND 21) and n=3-9 (PND 61). Data presented as the mean ± SEM.

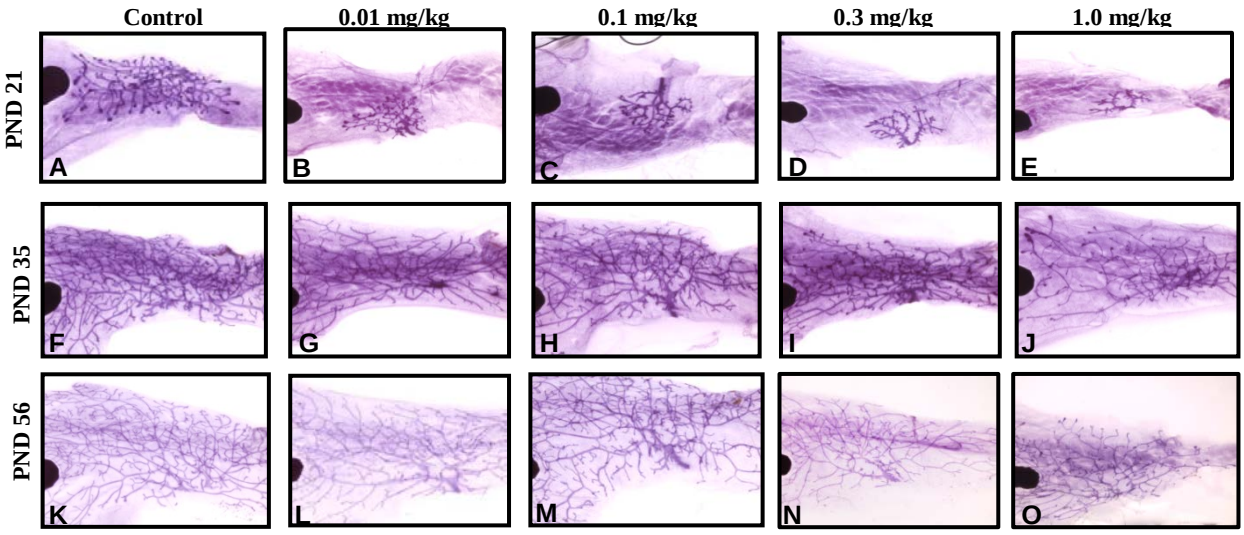


Figure AII-2: Whole mount assessment of early and late pubertal glands in CD-1 offspring. Representative image of control A) PND 21, F) PND 35, and K) PND 56, 0.01 mg/kg B) PND 21, G) PND 35 and L) PND 56, 0.1 mg/kg C) PND 21, H) PND 35 and M) PND 56 0.3 mg/kg D) PND 21, I) PND 35 and N) PND56 and 1.0 mg/kg E) PND 21, J) PND 35and O)PND 56. CD-1 n= 4-11 litters/treatment group.

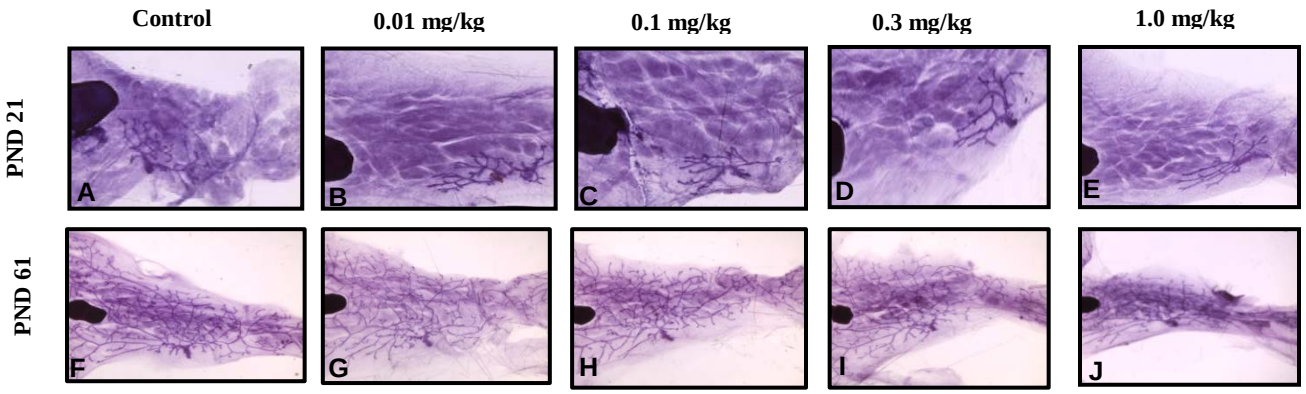


Figure AII-3: Whole mount assessment of early and late pubertal glands in C57Bl/6 offspring. Representative image of control A) PND 21 and F) PND 61, 0.01 mg/kg B)PND 21 and G) PND 61, 0.1 mg/kg C) PND 21 and H) PND 61, 0.3 mg/kg D) PND 21 I) PND 61 and 1.0 mg/kg E) PND 21 and J) PND 61. C57Bl/6 n=2-10 litters/treatment group.

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APPENDIX III

Table AIII-1. Premature animal deaths

DOB	ID	Chemical	Dose (mg/kg)	Date Found	Age	Necropsy Findings/Cause of Death
8/16/2013	8253	BPS	5	9/20/2014	13 mos.	
8/16/2013	8257	BPS	0.5	9/19/2014	13 mos.	
8/16/2013	8211	BPA	50	8/21/2014	12 mos.	
8/16/2013	BPA 5 mg	BPA	5	8/21/2014	12 mos.	
8/16/2013	8266L	BPS	0.05	1/13/2014	5 mos.	
8/16/2013	8229 or 8231	BPAF	5	1/12/2014	5 mos.	
8/16/2013	8206	Vehicle	0	5/30/2014	9 mos.	
8/16/2013	8215	BPA	5	7/25/2014	11 mos.	Opaque calcification throughout abdominal cavity
8/16/2013	8226	BPA	0.5	7/15/2014	11 mos.	
8/16/2013	8205 or 8206	Vehicle	0	7/15/2014	11 mos.	
DOB	**ID**	**Chemical**	**Dose (mg/kg)**	**Date Found**	**Age**	
9/6/2013	8336R	BPAF	0.5	9/24/2014	12 mos.	
9/6/2013	8334L	BPAF	5	9/26/2014	12 mos.	
9/6/2013	8338L	BPAF	0.5	8/15/2014	11 mos.	
9/6/2013	8335	BPAF	5	8/25/2014	11 mos.	
9/6/2013	No Info	-	-	9/26/2014	12 mos.	
9/6/2013	8217	BPA	5	4/23/2014	7 mos.	
9/6/2013	No Info	-	-	12/28/2013	3 mos.	Fighting
9/6/2013	No Info	-	-	4/1/2014	6 mos.	
9/6/2013	8321B	BPA	5	1/21/2014	4 mos.	
9/6/2013	8362	BPS	0.5	8/7/2014	11 mos.	
9/6/2013	8359RR	BPS	0.5	3/14/2014	6 mos.	Hepatosplenomegaly

During the course of the study some animals from each block prematurely died and were not necropsied in time to determine cause of death or retrieve tissue due autolysis. These animals were not used in analysis involving the mammary gland, hormone, or hormone levels. They may have been used in pubertal timing endpoints (VO, estrus and cyclity). (-) no information was provided in animal health report.