MECHANISTIC PATHWAYS UNDERLYING LOW-DOSE PERFLUOROOCTANOIC ACID (PFOA) EFFECTS IN MURINE MAMMARY TISSUES

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Toxicology in the School of Medicine.

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A**BSTRACT**

Madisa Macon: Mechanistic pathways underlying low-dose perfluorooctanoic acid (PFOA) effects in mouse mammary tissues
(Under the direction of Suzanne E. Fenton)

Perfluorooctanoic acid (PFOA) is a synthetic surfactant that is as a prominent environmental toxicant. Previous studies have characterized the morphological effects of prenatal PFOA exposure on the mammary gland at 5mg/kg/day PFOA. The goal of this project was to identify major signaling pathways involved in this effect using a mouse model at dosing exposures that overlap with human serum levels. To minimize the overt developmental toxicity of PFOA, prenatal levels were reduced and/or exposures were abbreviated to a critical window of mammary gland organogenesis, gestational days 10-17. A systems biology approach was utilized to characterize the morphological and molecular changes using microarray, RT-PCR, Western blots, immunohistochemistry, histology, whole mount analysis, mammary epithelial transplant recombination, and serum hormone analysis. Following low-dose (0.01-1.0 mg/kg/day) and abbreviated (GD 10-17) PFOA exposures, mammary glands of treated mice displayed characteristics of delayed development which persisted into adulthood. These adult morphological alterations were characterized by misdirected growth patterns, thicker collagen density, increased active TEBs, and reduced side branching of the ductal tree. Genome-wide microarray analysis of young mammary tissues revealed PFOA altered RNA post-transcriptional modification, lipid metabolism and cholesterol biosynthesis; peroxisome proliferator-activated receptor (*Ppar*), *Wnt*, and endocrine related signaling were identified as targeted signaling pathways and confirmed by RT-PCR. The majority of RNA expression changes occurred early in life yet PFOA altered protein levels of

candidate gene across multiple time-points. In whole cell lysates, at PND 7 PPARγ and ERα levels were increased; at PND 21 ERα protein levels was reduced; at PND 56 PPARα and PPARγ levels were severely reduced. Results from blots coincided with IHC stained sections for ERα which were reduced at PND 21 and 56 in treated glands. Circulating testosterone levels were reduced at PND 21 and DHEA was reduced at PND 56. Altered steroid levels, differences in steroid receptor populations, and dense breasts in women are associated with increased breast cancer risk, and similar to effects observed in mouse mammary glands following PFOA exposure. Collectively, these data show that prenatal PFOA exposure alters endocrine disruption and steroid receptor expression leading to phenotypic features associated with increased breast cancer risk. Importantly, these effects were observed in mice as PFOA serum concentrations approached background levels and overlapped with reported human serum levels.

DEDICATION

This is dedicated to my dad and mom.

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LIST OF ABBREVIATIONS

Acox1	Acyl-coactivator oxidase 1, palmitoyl
ACS	American Cancer Society
Acvr1	Activin A receptor 1
Adip	Adiponectin
Adipr	Adiponectin Receptor
ANOVA	Analysis of Variance
Aqp7	Aquaporin 7
Ar	Androgen Receptor
Bnc1	Basonuclin 1
BW	Body Weight
Ccnd1	Cyclin D 1
CDC	Centers for Disease Control and Prevention
Cebpβ	CCAATT enhancer binding protein beta
Ctnnβ1	Beta Catenin
EPA	Environmental Protection Agency
ErbB2	V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog
ErbB2ip	ErbB 2 interacting protein
Esr1/Erα	Estrogen receptor alpha
Errγ	Estrogen related receptor gamma
Fabp3	Fatty acid binding protein 3
Fatp	Fatty acid binding transporter protein
Fos	FBJ Murine osteosarcoma viral oncogene homolog
Frzd2	Frizzled 2

GD	Gestational Day
Gpr30	G-coupled protein receptor 30
H&E	Hematoxylin and esosin
Hmgcs1	3-hydroxy-3-methylglutaryl-CoA synthase 1
Hsd11β1	Hydroxysteroid dehydrogenase 11 beta 1
Hsd17β11	Hydroxysteroid dehydrogenase 17 beta 11
Hsd17β12	Hydroxysteroid dehydrogenase 17 beta 12
IHC	Immunohistochemistry/immunohistochemical
Insr	Insulin receptor
Irs1	Insulin receptor substrate 1
Kg	kilogram
KO	Knock-Out
Lef1	Lymphocyte binding enhancing factor
Lep	Leptin
Lgals7	lectin, galactoside-binding soluble 7
Ltf	Lactoferrin
LOD	Limit of Detection
LOQ	Limit of Quantification
Lpl	Lipoprotein lipase
Lxr	Liver x receptor
Lypd3	LY6/PLAUR domain containing 3
mg	milligram
µg	microgram
MOA	Mode of Action

Myc	V-Myc avian myelocytomatosis viral oncogene homolog
Myod1	Myogenic differentiation 1
NCSAB	North Carolina Scientific Advisory Board
NHANES	National Health and Nutritional Examination Survey
NIEHS	National Institute of Environmental Health Sciences
Nfkb	Nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor
ng	nanogram
NMR	Nuclear Magnetic Resonance
Nr3c1	Glucocorticoid receptor
p53	Tumor protein p53
PCR	Polymerase Chain Reaction
ppb	parts per billion
PFOA	Perfluorooctanoic Acid
pg	picogram
Pgr	Progesterone receptor
PND	Postnatal Day
PPAR	Peroxisome-Proliferator Activated Receptor
PPARα	Peroxisome-proliferator activated receptor alpha
PpargCoa1	Peroxisome-proliferator activated receptor gamma coactivator 1
PPARγ	Peroxisome-proliferator activated receptor gamma
PPARβδ	Peroxisome-proliferator activated receptor beta/delta
Pxr	Pregnane x receptor
QC	Quality control
RT-PCR	Real Time-Polymerase Chain Reaction

Rxrα	Retinoid x receptor alpha
SAS	Statistical Analysis System
Scap	Sterol regulatory element binding chaperone
Serpinb5	Serpin peptidase inhibitor, clade B, member 5
Sp3	Sp3 transcription factor
Srebf1	Sterol regulatory element binding transcription factor 1
Stat5a	Signal transducer and activator of ranscription 5 A
Tbp	TATA binding box
TDLU	Terminal Ductal Lobular Units
TEB	Terminal End Buds
Tgfβ3	Transforming growth factor beta 3
Tgfβ3r	Transforming growth factor beta 3 receptor
Tnf	Tumor necrosis factor
Ucp1	Uncoupling protein 1
Wif1	Wnt inhibitory factor 2
Wnt2	Wingless-type MMTV integration site family, Member 2
WT	Wild type

CHAPTER 1

INTRODUCTION[1]

Breast cancer is the second most common type of cancer among women in the United States (second to skin cancers) and the second leading cause of death due to cancer, after lung cancer (*1*). In fact, the most recent US statistics estimate that 1 in 8 women will be diagnosed with breast cancer in their lifetime (*2*), a number that has not declined even following a dramatic decrease in the use of postmenopausal hormone treatments shown to be associated with increased breast cancer risk (*2*). Breast cancer also affects men, but to a much lesser degree, with only 1% of all new cases in the US being attributed to men (*3*). As with other cancers the formation of breast tumors has a heritable link; between 5 to 27 % of all breast cancers are attributed to factors such as specific gene mutations, certain tissue traits (i.e., dense breasts, fibrocystic disease), and metabolic issues (*4*). The environmental and lifestyle factors that contribute the other 70–95 % of breast cancer risk are largely unknown. Some general risk factors for breast cancer include: sex, age, parity, age at menarche, and age at menopause. The importance of environmental influences and exogenous exposures on factors such as parity and age at menarche have been demonstrated (*5*-*7*), but we have only begun to understand how exogenous chemicals that disrupt the function of the endocrine system may increase susceptibility to breast cancer and other diseases.

Normal growth of the mammary gland involves endocrine signaling from the hypothalamic-pituitary-gonadal axis. Autocrine and paracrine hormones and growth factors also play critical roles in development and regulation of mammary gland growth. Some of the many endocrine hormones and

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growth factors known to modulate mammary gland development include: growth hormone, prolactin, oxytocin, epidermal growth factor, insulin, insulin-like growth factors, adrenal corticosteroids, transforming growth factors, thyroxine, estrogen, progesterone, activin, and inhibin. These growth factors influence signaling between the 4 main cell types of the mammary gland: epithelial, adipocytes, collagen forming fibroblasts, and immune cells (*8, 9*). Alterations to normal mammary growth can lead to altered breast development during puberty, the inability to nurse, modulated immune profiles in milk, as well as disease such as breast cancer. Aberrant mammary gland development, whether caused by inheritable mutations or exogenous insults, can lead to the development of disease.

Perfluorooctanoic acid (PFOA) is a synthetic compound utilized for its physiochemical properties in industrial applications. Prenatal PFOA exposure has been shown to alter mammary gland development and maturation. As the mode of action (MOA) for this effect remains uncharacterized, the overarching goal of this dissertation project was to characterize PFOA-induced effects in the mammary gland and to elucidate signaling pathways involved in this toxicity in an attempt to assess its relevancy to human health. To understand the basis for this project, background information is provided for PFOA, the PPAR signaling family, and the mammary gland.

BACKGROUND

Perfluorooctanoic acid (PFOA)

Perfluorooctanoic acid is a fully fluorinated 8-carbon carboxylic acid. It is a synthetic surfactant that is used in the production of fluorotelomers. PFOA has a wide variety of industrial applications and also a wide range of toxicities. PFOA poses a health concern particularly in the US due to mass contamination from consumer products and primary manufacturing sites in New Jersey, North Carolina, and most notably West Virginia and Ohio. Due to industrial pollution, residents near the WV plant are the recipients of one of the largest class action settlements against a US industrial company (*10*). As part

of the settlement, the C8 Science Panel was formed to assess the health consequences of environmentally high PFOA exposures in this community. Numerous occupational, epidemiological, and animal studies have been conducted to determine PFOA toxicity and its potential health risk to humans. Although PFOA toxicity has been extensively studied, the mode of action (MOA) for several of its target tissues has yet to be characterized.

Chemical Properties

PFOA is aliphatic as it has a hydrophobic and hydrophilic end and is a heat stable compound due to its physiochemical properties. PFOA can be found in both linear and branched forms. Its chemical structure is similar to octanoic acid as shown in Figure 1-1, also known as Caprylic acid, a natural component of coconut oil and breast milk. PFOA is synthesized by electrochemical fluorination where fluorine atoms are added to octanoic acid, or by teleomerization where two smaller chained fluorinated carbons are combined to form PFOA and other longer chained perfluorinated compounds (*11*). Fluorine is a halogen and one of the most electronegative elements and forms strong, stable bonds with carbon atoms. As a result, this perfluorinated carboxylic acid is extremely stable and is resistant to heat and enzymatic breakdown. These properties make PFOA an ideal surfactant and, consequently due to contamination, a persistent environmental compound.

Exposure

PFOA is a ubiquitous environmental contaminant that is found in the air, soil, water, and in the blood of animals and humans globally. Emissions from industrial plants that manufactured PFOA have largely contributed to environmental contamination. Humans are thought to be exposed to PFOA mostly through contaminated drinking water, yet exposures can come from a variety of other sources such as household dust and consumer products. PFOA is most commonly associated with non-stick cookware, i.e. Teflon™. As a surfactant, PFOA serves as a great repellant of water and oil and is often included in

food packaging such as popcorn microwave bags, french fry boxes, and pizza boxes (*12*). PFOA is also found in water and stain-resistant fabrics, firefighter foam and flame retardant products, ski and surf board wax, paint and lacquer, electrical wiring, and biosolids used as fertilizers (*13-15*). Many of these consumer products do not include PFOA itself, but contain fluorotelemors that subsequently break-down into PFOA after degradation. Reports indicate levels of PFOA in consumer products have increased from 2007-2011 (*16*). As PFOA is found in water sources, fish and aquatic animals, particularly crustaceans, have been reported to have PFOA in their blood (*17, 18*). PFOA is also found in breast milk, amniotic fluid, and cord serum, and thus is available for placental transfer to offspring. As PFOA is not readily metabolized, the half-life for PFOA in humans is relatively long, at approximately 3.8 years (*19*).

From these sources of exposure, the general adult US population has an average 3.1 ng/ml PFOA in their serum according to the latest report from the Centers for Disease Control and Prevention (CDC) which is based on data collected by the National Health and Nutritional Examination Survey (NHANES, 2013)(*20*). Infants and children tend to have higher circulating levels of PFOA, ~ 6.1 ng/kg (2001-2002 data)(*21*). Indeed, a study by Mondal and colleagues (2012) reported children had higher levels of PFOA in comparison to their mothers until 12 years of age. Serum PFOA concentrations in the US have gradually decreased over time; conversely, levels have increased in Asian countries (*23*). As stated earlier, there is an area near the Washington Works DuPont plant in Parkersburg, West Virginia where residents have high levels of PFOA due to contamination of their water source. PFOA serum concentrations of residents are ~32 ng/ml, and concentrations have been reported to be >10 times higher than those (*24*).

Toxicity

Health concerns regarding PFOA exposure have been raised due to its wide range of toxicity. Numerous studies have been conducted to assess the hazard and risk of PFOA exposure. From *in vitro* and animal studies, PFOA is considered a non-genotoxic carcinogen, although there is evidence that it

has indirect mutagenic properties by induction of reactive oxygen species (*25*) and has cytotoxic properties (*26*). In rodent studies, adult high dose PFOA exposure is known to cause a common tumor triad of hepatocellular carcinoma, pancreatic acinar tumors, and Leydig cell tumor (*27*). Adult rodent exposures result in toxicities of the liver, kidney, cardiovasculature, and immune system (*28*). In male monkeys, adult sub-chronic exposures to PFOA led to changes in all dose groups investigated and included reduced body weights and increased relative liver weights (*29*). PFOA is also a reproductive and developmental toxicant. Gestational PFOA exposures in mice increased prenatal mortality, full litter resorptions, gestational length, postnatal mortality of pups, and increased developmental abnormalities in offspring (*30-32*). Prenatal PFOA exposure in mice resulted in reduced body weights, delayed eye opening, neurodevelopmental delays, elevated relative liver weights, skeletal malformations, and delayed female and accelerated male sexual maturation (*11, 32, 33*). *In vitro* studies in chicken and zebrafish embryos reported that developmental PFOA exposures led to cardiac toxicity (*34, 35*). Low-dose prenatal PFOA exposure in mice was reported to increase body weights in mid-life compared with controls (*36*). A comprehensive review of these effects can be found in Lau 2007 (*11*) and Post et al (2013)(*37*).

Occupational and Epidemiological Evidence

There is a great deal of epidemiological evidence regarding the toxicity of PFOA that has resulted from the works of the C8 Science Panel. This panel was created, as a part of a class-action legal settlement, to assess the health effects of PFOA exposure in a community known to have high contamination and consequently high exposure levels. This group has linked PFOA exposure to the development of testicular, prostate, ovarian, liver, and kidney cancer, leukemia, non-Hodgkin's lymphomas, ulcerative colitis, high cholesterol, thyroid disease, and pregnancy-induced hypertension (*38-40*). Occupational exposure to PFOA or related perfluorinated chemicals (PFCs) has shown positive correlations with increased kidney cancer mortality (*41*). High PFOA exposures are also linked to liver

and kidney toxicity, and increased estradiol levels in men. Some studies have found positive associations between PFOA exposure and low birth weight and/or developmental delays (*42, 43*), while others have not (*44-46*). For later life effects, a prospective study has found positive association between PFOA and increased body weight gain at early adulthood (*47*) which parallels effects that were observed in mice by Hines and colleagues (2009)(*36*).

Regulatory Measures

Due to its ubiquity in the environment, slow breakdown, and wide range of toxicities, many regulatory measures have been taken to reduce PFOA environmental exposures. PFOA is considered a "probable human carcinogen according to the US Environmental Protection Agency (USEPA)"(*48*). Based on toxicity data from *in vitro*, animal, occupational, and epidemiological studies, the USEPA crafted a risk assessment for PFOA human health effects and set a recommended drinking water limit at 0.4 µg/L. In 2006, many of the top industrial manufacturers of PFOA in the US signed the PFOA Stewardship Program, an agreement with the USEPA to voluntarily reduce their emission by 2010 and phase out production and use of PFOA by 2015. However, PFOA is a final break-down product of fluorotelomers and can still be detected at high levels near these manufacturing sites; in addition, these companies are beginning to replace PFOA with poly-fluorinated hydrocarbons. On the international level, in 2013, the Norwegian Environmental Protection Agency (NEA) banned products containing PFOA to be sold. In addition, the International Agency for Research on Cancer (IARC) will review PFOA for human relevant carcinogenicity in June 2014.

In order to effectively determine human health relevance from laboratory studies, a mode or mechanism of action (MOA) for each outcome is needed. PFOA-induced liver toxicity has been extensively studied in adult-exposed rodents and is most commonly associated with activation of peroxisome proliferator-activated receptors alpha (*Pparα*) and as a result, the majority of PFOA-toxicity

studies have focused on the influence of this receptor. Consequently, a growing body of evidence suggests that PFOA-induced liver toxicity, and thus other toxicities, may rely, in part, on other pathways (*49*). A brief description of relevant PPAR-associated toxicity is provided.

PPAR

PPARs are ligand-activated nuclear receptors and transcription factors that mediate glucose homeostasis, fatty acid oxidation, lipid metabolism, and adipocyte differentiation (*50*). PPARs form heterodimers with Retinoid X receptor (RXR), translocate to the nucleus and bind to peroxisome proliferator response elements (PPRE) on DNA to regulate transcription of target genes (*51*). There are three subtypes of PPAR that have been identified thus far: alpha, beta/delta, and gamma. PPAR subtypes have overlapping transcriptional targets but also have specific targets and functions.

PPAR subtype alpha (PPARα) is highly expressed in metabolic tissues, including the liver, kidney, and heart, in rodents and humans (*52, 53*). Due to its high expression in the liver, most studies have characterized the signaling pathway of PPARα in this tissue. However colleagues of the Nuclear Receptor Signaling Atlas have recently reported that *Pparα* in rodents is more highly expressed in brown adipocytes then in the liver (*54*). According to the KEGG pathway, PPARα signaling is associated with ketogenesis and fatty acid beta-oxidation.

Administration of PPARα agonists such as fibrates causes hepatomegaly, hepatocellular hypertrophy, and hepatocellular carcinoma in rodents. This is due to increased production of peroxisomes in liver tissues and cells, an effect only observed in rodents. Although PPARα is highly expressed in livers of humans compared to other tissues, human PPARα liver expression is much lower in comparison with rodent *Pparα* liver expression. In addition, activation of PPARα in humans does not induce peroxisome proliferation, a common effect of Pparα activation in rodents (*51*). As a result, hepatocellular carcinoma is not considered a relevant health outcome in humans following PFOA exposure. However, recent studies in fish and rodents have shown liver toxicity and increased liver

tumors in the absence of peroxisome proliferation, suggesting that other signaling pathways may exist that result in PFOA-induced liver toxicity (*55*). PPARα knockout (KO) mice generally develop normally. However, constitutively active PPARα has deleterious health effects on skin and cardiac function (*56*). PPARα is highly expressed in the mammary gland during development but is not required for mammary gland development (*57*). Expression of PPARα is decreased during pregnancy and lactation and activation of this receptor during pregnancy impairs milk production and output (*58*).

PPAR subtype gamma (PPARγ) has several isoforms; currently 8 have been identified in humans and rodents. Most information has been acquired on the first 2 isoforms. PPARγ is mostly associated with adipocytes and immune cell function in comparison to the other PPAR subtypes. PPARγ is required for placental development as well as adipocyte differentiation and modulation of inflammatory responses. Agonists for PPARγ such as thiazolidinediones (TZDs) have been used to treat diabetes. Like PPARα, PPARγ is reported to be expressed at high levels during early mammary development then to decrease during pregnancy and lactation (*57*).

PPAR subtype beta (PPARβ, aka PPAR delta) is the least studied of the 3 subtypes. This subtype is associated with ubiquitination and gluconeogenesis. PPARβ is highly expressed in the intestines, kidney, stomach, intestines and lung in rodents (*52, 54*). PPARβ is ubiquitously expressed in the mammary gland throughout early development, pregnancy and lactation (*57*). Although the effects of PPARβ activation are more similar to that of PPARα, PPARβ KO studies in mice reveal the importance of this subtype in placental development and adipogenesis, which are commonly associated with PPARγ (*59{Peters, 2000 #2724*)}. KO studies also reveal PPARβ integral role in the development of the brain and skin (*60*).

Mammary Gland

Normal mammary gland growth requires intricate crosstalk between the epithelium and the surrounding stroma in order to balance proper proliferation/apoptosis and for remodeling the gland at the different stages of life. Rapid mammary gland development occurs in three distinctive life stages: fetal, peri-pubertal, and pregnancy (*61*). In girls, the fetal mammary bud begins to form late in the first trimester of pregnancy. During the last few weeks of pregnancy, the nipple and the primary epithelial ducts form, and ducts branch outward into the stroma. Relatively little epithelial growth is observed until around the time of puberty when the gland growth is influenced by the release of pituitary and ovarian hormones. During thelarche, one of the earliest signs of puberty, female breast tissue enlarges and grows outward, making it noticeable. At the same time the mammary fat pad enlarges in size, and the rapidly extending epithelium form bulbous or club-like structures at the duct ends, termed terminal ductal lobular units (TDLU). The TDLU are structurally similar to terminal end buds (TEB) in rats and mice, present during the same life stage. These structures are undifferentiated and highly proliferative and, as such, are sensitive to the effects of carcinogens and other chemicals. The breast reaches a static stage some-time after first menstruation, changing slightly with each additional menstrual cycle. Thereafter, the gland remains in a fairly static stage throughout life, until a pregnancy occurs. At this time, morphological maturation is achieved and the gland continues to branch and fill with lobulo-alveoli to support the production and release of milk. [D]isruption of normal development at any and all of these stages can cause permanent developmental abnormalities, impaired lactation, and influence risk for the development of breast cancer.

In women, it is estimated that 40% of all cancers are hormonally regulated (*9*) and thus there are many potential modes or mechanisms by which environmental exposures could modify cancer risk. Many factors that influence breast cancer are endocrine regulated processes (e.g., age at menarche, age at 1st pregnancy, and age at menopause) and may be modified by endocrine disrupting compounds

(EDCs). EDCs that do not affect DNA are proposed to influence tumorigenesis in a variety of ways, including impaired or altered 1) epithelial growth rate, 2) stromal composition of the gland, 3) immune response, 4) response to endogenous hormones, 5) terminal end bud presence, or 6) cell-cell communication, to name a few. Yet, it is important to note that exposure to EDCs may influence tumorigenesis through both DNA-mediated and non DNA-mediated mechanisms.

Although the ductal or lobular epithelia are often the sites of tumor production, mammary stroma growth patterns can also influence breast cancer risk. The communication of the multiple cell types in the mammary gland, i.e. stromal-epithelial interaction, is critical for normal development throughout life (*9*). Studies have linked increased stromal density in the breast to later development of epithelial-based mammary tumors (*62*). When mammary tumor tissues were separated into epithelial and stromal compartments, recombination of normal epithelium with tumor stroma resulted in the growth of epithelial tumors while recombination of tumor epithelium with normal stroma resulted in the growth of normal tissue, indicating that stromal tissues have strong influences on overall gland growth and tumor status (*63*).

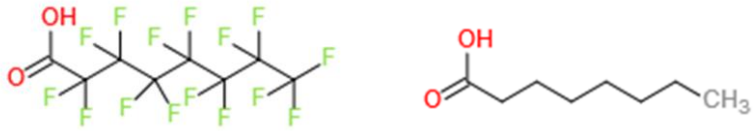
Deleterious modifications to stromal tissue may accompany accelerated or delayed overall mammary gland growth and is another way that EDC exposure can influence the development of mammary tumors (*64-66*). In fact, thickening of the stromal compartment surrounding the epithelium has been found in the case of prenatal EDC exposure in rats (*65*). As expected, exposure to EDCs that alter mammary development may also cause an array of detrimental health effects in other organs.

PFOA and the Mammary Gland

PFOA has been reported to have endocrine disrupting properties and affect various tissues in that system, including the mammary gland. Gestational PFOA exposures at 5 mg/kg in mice reduced mammary gland function by lowering milk production and altering milk quality (*67*). Prenatal PFOA exposure at 5mg/kg results in severely stunted mammary gland of female offspring (*67, 68*). Growth of

PFOA-treated mammary glands remained abnormal and resulted in mammary glands with increased collagen density and potential areas of epithelial hyperplasia by 18 months. Developmental effects in the mammary gland have been shown to be trans-generational at 5mg/kg (*69*). Recently, peri-pubertal studies reported dose and strain-specific effects on mammary gland differentiation following PFOA exposure; growth delays were observed in Balb/C mice at 5-10 mg/kg; growth advancements (5 mg/kg) and delays (7.5 mg/kg) were observed in C57Bl/6 mice.

Studies in Pparα KO and WT mice have not produced definitive results concerning the role of Pparα in mediating PFOA mammary gland effects. However, data suggest that endocrine disruption plays a role (*70, 71*). Therefore, the studies described for this project were designed to elucidate the major signaling pathways involved in prenatal-PFOA-induced mammary gland delays, building on the previous work done in our laboratory. To hone in on the MOA for this specific developmental effect, PFOA exposure levels were lowered and/or exposure lengths were abbreviated to minimize the influence of toxicities from other tissues. From these projects, the morphological and molecular effects of low-dose prenatal PFOA exposure on the mammary gland were characterized and the role of *Pparα* in these effects was addressed with *Pparα* WT and KO mice.



Perfluorooctanoic Acid Caprylic Acid

Figure I-1. Chemical Structures of PFOA and Caprylic Acid. Created in Chem Doodle.

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CHAPTER 2

Prenatal Perfluorooctanoic Acid Exposure in CD-1 Mice: Low-Dose Developmental Effects and Internal Dosimetry[2]

OVERVIEW

Perfluorooctanoic acid (PFOA) is an environmental contaminant that causes adverse developmental effects in laboratory animals. To investigate the low-dose effects of PFOA on offspring, timed-pregnant CD-1 mice were gavage dosed with PFOA for all or half of gestation. In the full-gestation study, mice were administered 0, 0.3, 1.0, and 3.0 mg PFOA/kg body weight (BW)/day from gestation days (GD) 1-17. In the late-gestation study, mice were administered 0, 0.01, 0.1, and 1.0 mg PFOA/kg BW/day from GD 10-17. Exposure to PFOA significantly ($p < 0.05$) increased offspring relative liver weights in all treatment groups in the full-gestation study and in the 1.0 mg PFOA/kg group in the late-gestation study. In both studies, the offspring of all PFOA-treated dams exhibited significantly stunted mammary epithelial growth as assessed by developmental scoring. At postnatal day 21, mammary glands from the 1.0 mg/kg GD 10-17 group had significantly less longitudinal epithelial growth and fewer terminal end buds compared with controls ($p < 0.05$). Evaluation of internal dosimetry in offspring revealed that PFOA concentrations remained elevated in liver and serum for up to 6 weeks and that brain concentrations were low and undetectable after 4 weeks. These data indicate that PFOA-induced effects on mammary tissue (i) occur at lower doses than effects on liver weight in CD-1 mice, an observation that may be strain specific, and (ii) persist until 12 weeks of age following full-gestational

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exposure. Due to the low-dose sensitivity of mammary glands to PFOA in CD-1 mice, a no observable adverse effect level for mammary developmental delays was not identified in these studies.

INTRODUCTION

Perfluorooctanoic acid (PFOA) is a synthetic eight-carbon perfluorinated compound that is commonly used in the production of fluorotelomers due to its physicochemical properties. PFOA has widespread industrial applications, is persistent in the environment, and is also the final breakdown product of several fluorotelomers (*1*). This surfactant is ubiquitous in the environment and has been detected in sera of animals and humans (*2, 3*). For these reasons, PFOA has become a heavily studied environmental contaminant.

Humans are thought to be exposed to PFOA mainly through ingestion of contaminated water or food products (*4-6*) and also through residues found in residential dust (*5, 7*). PFOA is found in cord blood, human, and rodent milk and, as such, can be passed to developing offspring (*8-11*). The most recent U.S. geometric mean serum PFOA concentration is 3.9 ng/ml (2003–2004, (*12*)) according to population-based biomonitoring studies; yet, in young children, serum PFOA concentrations are slightly higher (6.1–7.6 ng/ml, least square mean estimates, 2001–2002; (*13*)). PFOA is a persistent compound as it is slowly eliminated from the body, with an estimated half-life in adult humans of 3.8 years (*14*). Due to the risk for developmental exposure, its ubiquitous nature, and the presence of this compound at relatively high levels in certain communities (*15*), developmental exposure studies were initiated in the United States and other countries to determine potential adverse health effects of PFOA exposure (*8, 16-22*). The significance of the findings from many of these studies has been summarized in a recent review article (*23*).

From previous adult exposure studies in rats, it has been shown that PFOA causes hepatomegaly and a common tumor triad of hepatocellular adenoma, Leydig cell tumors, and pancreatic acinar cell

tumors (*24, 25*). PFOA has also been reported to modulate the immune system of adult mice (*26-28*). Exposure to PFOA causes an array of developmental toxicities in mice. In CD-1 mice, gestational exposure ≥ 10 mg/kg PFOA resulted in increased prenatal loss and prolonged gestational length in dams, whereas offspring exhibited reduced birth weight, delayed eye opening, increased postnatal mortality, delayed sexual maturation in females, and precocious sexual maturation in males (*29*). Some of these developmental effects have been reported at lower doses (≥ 0.1 mg/kg) in 129S1/Sv1mj mice (*30*). Increased body weight (BW) gain accompanied by elevated serum leptin and insulin was reported in adult CD-1 mice prenatally exposed to low doses of PFOA (0.01–1.0 mg/kg BW; (*31*)). PFOA exposure may also alter neurodevelopment, as changes in motor function have been observed in early life (*32*) and in adulthood (*33*).

Female rats rapidly eliminate PFOA ($t_{1/2}$ = 2–4 h vs. 4–6 days in males; (*34*). This hyperexcretion leads to episodic fetal and neonatal body burdens in developmental exposure studies (*10*). However, gender differences in elimination of PFOA are not observed in humans or mice, and thus, mice have been utilized as the more appropriate rodent model in PFOA studies, particularly in studies involving developmental or female-specific endpoints. As it pertains to the mammary gland, both rats and mice are known to develop in a morphologically similar pattern to the human breast (reviewed in (*35*)). Due to the similarities in relative elimination rates of PFOA and mammary gland development compared with humans, the mouse has been used in our laboratory as the most appropriate rodent model.

The mammary gland appears to be a sensitive tissue to developmental PFOA exposure, although sensitivity may vary across mouse strains. Prenatal exposure to 5 mg PFOA/kg severely stunted mammary gland development in female CD-1 offspring (*36*), and the current lowest observable adverse effect level (LOAEL) for abnormal mammary gland development in this mouse strain is 3 mg/kg (*37*). Recent studies by another laboratory have demonstrated that peripubertal exposure to 5 mg/kg or higher PFOA delays mammary gland development in inbred Balb/C mice and either deters (at 10 mg/kg)

or accelerates (at 5 mg/kg) mammary development in C57Bl/6 mice, depending on dose (*38*). No significant effects on mammary growth were detected at 0.1 mg/kg in Balb/C or 1 mg/kg in either strain (*38*). Thus, even though cross-foster studies suggest that doses lower than 3 mg/kg may alter mammary gland development in CD-1 mice (*37*), studies in other strains, under different exposure conditions, do not necessarily support this LOAEL.

Herein, two studies were conducted to further address the issue of low-dose effects of PFOA in the mammary gland of CD-1 mice and the internal dosimetry corresponding to these effects. Specifically, we tested the hypothesis that PFOA exposures that result in human relevant internal doses are also associated with perturbed mammary development in mice. In the first study, with a maximum dose of 3 mg/kg, PFOA was administered to pregnant mice for the entire gestational period (GD 1–17), and we determined effects from birth until adulthood. In a subsequent study, utilizing overlapping and lower doses, PFOA was administered to pregnant mice for only the latter portion of gestation (GD 10–17), as this period of gestation was previously identified as a window of susceptibility for mammary gland developmental abnormalities (*36*). In the late-gestation study, we evaluated early life time points to better assess the internal dosimetry for these effects in mice. The overarching goal of these two studies was to establish a LOAEL and a no observable adverse effect level (NOAEL) for mammary gland effects following prenatal exposure to PFOA and to determine the corresponding internal dosimetry associated with these effects.

MATERIALS AND METHODS

Animals

Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). Pregnant dams were housed individually in polypropylene cages and received chow and tap water ad

libitum; both were known to contain PFOA below the levels of detection. Animal facilities were maintained on a 12:12 h light-dark cycle at 20°C–23°C and 40–50% relative humidity.

Chemicals

PFOA (PFOA as its ammonium salt APFO, > 98% pure) was purchased from Fluka Chemical (Steinhiem, Switzerland), and the PFOA lot number was identical to previous studies (White et al., 2007, 2009). PFOA dosing solutions were prepared fresh daily in deionized water, agitated immediately prior to administration, and were given at a volume of 10-µl solution/g BW.

Experimental Design

Full-gestation exposure study. Fifty-two dams arrived at the animal facility on GD 0 and were divided into four treatment groups (n = 13 per treatment). Each group was gavage dosed with deionized water as vehicle or 0.3, 1.0, or 3.0 mg PFOA/kg BW once daily from GD 1 to 17 (shown as mg/kg). Upon parturition, the litters were equalized to 10 pups of equal male and female representation (5:5) when possible. Approximately 15% of the dams were not pregnant, as expected. The desired number of female offspring was not always achieved due to inequities in litter sex ratios.

Late-gestation exposure and early development study. The second study was performed in two blocks (block 1 = 20 dams, block 2 = 32 dams). Mice arrived at the animal facility on GD 9 and were divided into four treatment groups of equal size (block 1 = 5 dams per treatment, block 2 = 8 dams per treatment). Each group was gavage dosed with deionized water as vehicle or 0.01, 0.1, or 1.0 mg PFOA/kg BW once daily (shown as mg/kg) from GD 10 to 17. Approximately 15% of the dams were not pregnant, again unrelated to PFOA exposure. To be consistent with previous work (*36*), on postnatal day (PND) 1, pups within a treatment group were pooled and randomly distributed among the dams of their respective treatment group, resulting in a final litter size of 7–9 pups per dam, with an unequal sex ratio (n = 4–7 females per litter).

Due to the short acclimation periods for both studies (1 day), the dams were observed by the veterinary staff and study personnel for signs of maternal stress, such as lack of weight gain or aggressiveness. None were observed.

Necropsy

For the full-gestation study, 6 pups per treatment group (1–2 per dam) were weighed and then necropsied at PNDs 7, 14, 21, 28, 42, 63, and 84 following decapitation. The trunk blood was collected and centrifuged, and the serum was collected and stored at −80°C. Liver, brain, and one set of fourth and fifth mammary glands were collected, weighed, and stored at −80°C for future RNA analyses. The contralateral fourth and fifth inguinal mammary glands were removed from female pups for preparation as a whole mount. Dams were sacrificed on PND 24, and afterward male and female pups were housed separately. Due to low female pup numbers in the control group, on PND 63, control females were omitted from the necropsy schedule.

In the late-gestational study, female pups from at least three litters per treatment group were weighed, then necropsied on PNDs 1, 4, 7, 14, and 21 following decapitation and the trunk blood was collected, and serum stored as indicated above. On PND 1 only, serum samples were pooled from several pups, both male and female, within a litter to achieve sufficient volume for PFOA analysis. In all further instances, only female endpoints were measured. Liver and a single set of fourth and fifth inguinal mammary glands were collected, weighed, and stored at −80°C for future RNA and protein analyses. The contralateral fourth and fifth inguinal mammary gland tissues were removed to be prepared as whole mounts. Due to lower than expected female pup numbers, PND 4 measurements were not collected for the 0.1 mg/kg treatment group.

Mammary Gland Preparations

For whole-mount preparations, mammary tissues were flattened onto glass slides, fixed in Carnoy's solution, stained with Carmine alum, and cleared in xylene as previously described in Fenton et

al. (2002). Mammary glands were evaluated by light microscopy (Leica Z16 APO and Leica DFC295 [Camera], Leica Microsystems, Frankfurt, Germany) and assessed an overall developmental score based on a 1–4 scale (1 = poor development, 4 = best development) at each age, similar to methods previously reported (*39-41*). Scores were based on qualitative and quantitative histological characteristics of each developmental time point, including, but not limited to, lateral and longitudinal epithelial growth, change in epithelial growth, appearance of budding from the ductal tree, branching density, and number of differentiating duct ends (*39*). Where applicable, at a given time point, mammary glands from both studies were compared on the microscope to ensure consistency in the scoring scale between studies.

To better understand the morphological differences between the developmental scores assessed, in the late-gestational study, several endpoints were quantitatively measured. Longitudinal growth was defined as the distance from the nipple end of the primary duct to the most distal terminal duct. Lateral growth was defined as the distance between the most distal terminal ducts with the collecting duct as an axis. Terminal end buds (TEBs) were defined as densely staining, bulbous structures located at the end of mammary ducts that were at least twice the width of the duct. These endpoints were measured using the Leica Application Suite (Version 3.5.0, Leica Microsystems). Quantitative measurements were recorded by one staff member; scoring was performed by two staff without knowledge of treatment, and the two scores were averaged. Mean scores for treatment groups were calculated for each time point and analyzed for statistical significance. Quantitative criteria were analyzed for statistical significance as well.

Dosimetry

Chromatographic and mass spectrometer conditions. The quantitative analysis of PFOA was performed using an Agilent 1100 High Performance Liquid Chromatograph (HPLC; Agilent Technologies, Palo Alto, CA) interfaced with a Sciex 3000 triple-quadrupole mass spectrometer (HPLC-MS/MS, Applied Biosystems/MDS Sciex, Foster City, CA).

Blank matrices and quality control. Swiss-Webster mouse serum (with trace hemolysis) was purchased from Pel-Freeze Biologicals (Rogers, AR) to prepare blank and spiked quality control (QC) samples and for calibration curve construction. Liver and brain tissues used to prepare blank and spike QC samples were obtained from non-pregnant control CD-1 animals that were sacrificed upon arrival.

A standard curve was prepared by spiking six solutions with serial dilutions of a 10,000 ng PFOA/µl standard in methanol to obtain final concentrations across the ranges of PFOA expected in the respective matrices to be analyzed. For example, for the late-gestation study, serum samples were analyzed with four separate calibration curves covering 5–50,000 ng PFOA/ml (5–100 ng/ml for control animals; 5–1000 ng/ml for 0.01 mg/kg animals; 100–5000 ng/ml for 0.1 mg/kg animals; 1000–50,000 ng/ml for 1.0 mg/kg animals). For the full-gestation study, serum samples were analyzed with one calibration curve covering 10–40,000 ng PFOA/ml. QC samples were prepared in 25 µl of Pel-Freeze mouse serum by enrichment with PFOA to obtain multiple concentrations. For every treatment group, two QC samples were used, one spiked with a low concentration of PFOA and another spiked with a high concentration of PFOA relative to the range of the calibration curve.

Calibration curves were generated by plotting the ratio of PFOA peak area to 13C2-PFOA peak area versus concentration and were fitted to a linear regression equation with 1/x weighting. Batch-specific calibration curves were prepared to obtain linear curves in the ranges utilized for each treatment group. A minimum of six standards were used to generate each calibration curve and the coefficients of determination (r2) values were 0.99 or greater for each analysis. Matrix-matched standard curves and QC pools were used to minimize potential errors associated with matrix enhancement or suppression of analyte signal due to coeluting matrix interferents.

Method accuracy and precision were determined by analyzing the QC sample repeatedly, using the complete analytical method. Accuracy was calculated as the percentage of the concentration

measured compared with the theoretical concentration. The precision of the methods was determined by calculating the average relative SD of the replicate analysis of the QC materials.

Preparation of serum samples. Serum samples were prepared as previously described in Reiner et al. (2009). Briefly, 25 µl of mouse serum from the collected samples was transferred into 15 ml polypropylene tubes. An appropriate amount of internal standard (13C2-PFOA) was added to achieve the approximate midpoint of the calibration curve of the anticipated sample range. For example, for serum samples expected to be in the 5–100 ng/ml range, the standardized amount of labeled PFOA was added to the sample with 1 ml acetonitrile so that it would fall at the midpoint of the calibration curve (50 ng/ml). Formic acid (0.1M) was added to the serum to denature the proteins at a volume at least four times the serum volume. The samples were vortexed, and the cold (−20°C) acetonitrile internal standard mixture was added to precipitate proteins in a volume of at least 10 times the formic acid volume used. The sample was then vortexed and centrifuged at 2000 × g for 3 min. A 200-µl aliquot of the acetonitrile supernatant was placed in an HPLC vial with 2mM ammonium acetate buffer (pH 6.5; 1:1 vol/vol), and the PFOA concentration was determined using HPLC-MS/MS analysis.

Preparation of liver and brain samples. One gram of thawed tissue was placed in 6 ml of distilled water and ground into a homogenized mixture. Briefly, 50 µl of the liver or brain homogenate was combined with 100 µl of 0.1M formic acid and 1 ml of acetonitrile spiked with 13C2-PFOA. The samples were vortexed and centrifuged at 3500 × g for 3 min. A 200-µl aliquot of the acetonitrile supernatant was placed in an HPLC vial with 2mM ammonium acetate buffer (pH 6.5) (1:1), and the PFOA concentration was determined using HPLC-MS/MS analysis.

Sample analysis. Each sample batch of tissue or fluids contained a maximum of 30 unknowns along with at least 10% QC samples. In addition, each sample batch included a minimum of six calibration standards, a matrix blank (containing internal standard and formic acid), and a method blank (containing internal standard, formic acid, and blank mouse serum, liver, or brain). Blanks, calibration

curve standards, and QCs were all subjected to the same preparation procedures as the unknown samples. QC samples were intermixed with unknown samples. If a QC sample exceeded the range of acceptable variance from that of the theoretical value, the entire batch was rejected and new aliquots of the samples would be put through the sample preparation procedure again and rerun. Analytical batches were considered to be acceptable if the standard curve and QC samples were ± 20% of the theoretical value. Quantification of some serum samples from the 0.1 mg/kg group from the late-gestation cohort were taken from a batch run in which the high QC value was just out of the range of the 20% acceptance criteria compared with the standard curve. However, the unknown sample values were within the range of the standard curve that was verified by the low QC value, and thus, these values were deemed acceptable. The limit of quantitation (LOQ) is defined as the lowest point on the stand curve with back prediction within ± 30% of the theoretical value. For the serum samples from the full-gestation study, the LOQ ranged from 10 to 20 ng/ml, whereas the LOQ for the liver, brain, and late-gestation serum samples were 35 ng/g, 35 ng/g, and 5 ng/ml, respectively. Analytical values below the LOQ were reported as the calculated value of LOQ/√2 for purposes of statistical comparisons.

Statistical Analysis

Dams, or litter, were utilized as the unit of measure for statistical analysis throughout both studies. Thus, when more than one pup was necropsied from one dam, the values were averaged and the mean was used for statistical analysis. Data were evaluated by age and treatment using general linear model ANOVA in SAS 9.1 (SAS Institute, Inc., Cary, NC). In the late-gestation study, data were evaluated for block and treatment effects by ANOVA mixed model analysis. Due to slight variance in litter sizes, litter and litter × treatment effects were evaluated and data were assessed for normal distribution. In cases where a litter × treatment effect existed, data within a treatment group were evaluated for significant outliers calculated by Grubbs' test utilizing GraphPad's QuickCalc outlier online calculator (2005) and were removed from analysis if detected. For all measurements, data are reported

as the mean ± SEM. Differences between PFOA-treated groups and controls were determined using Dunnett's t-tests. Differences within PFOA-treated groups over time were determined utilizing Tukey's t-tests. Differences between the 1.0 mg/kg full-gestation and late-gestation groups were determined using Student's t-tests.

In the full-gestation study, due to the lack of female controls at PND 63, no statistical differences were determined for that time point. Due to low male numbers throughout the study, the male data were considered incomplete and were statistically analyzed with low power. They are briefly discussed herein, and the data are provided in Tables 2-2 and 2-5.

Net pup weights were calculated to determine if PFOA-induced increases in liver weight disguised a BW effect. They were calculated by subtracting liver weight from BW for an individual. Means were then calculated for each dose group. Relative tissue weights were calculated by dividing the absolute tissue weight by the individual BW. For relative tissue weights and dosimetry data, the values were log transformed prior to statistical analysis. For mammary gland endpoints in the late-gestation study, change in epithelial growth was calculated as the difference between the measured length at a given time point from the respective treatment mean values at PND 1. A value of $p \leq 0.05$ was used as the limit to determine statistical significance.

RESULTS

Full-Gestation PFOA Exposure Study

Body weights.

A comparison of female and male offspring BWs over time are shown in Tables 2-1 and 2-2. Also shown are net pup weights, following subtraction of liver weights. PFOA (0.1–3.0 mg/kg) did not affect the absolute or net weights of either female or male offspring (Tables 2-1 and 2-2).

Liver weights.

Gestational exposure to low doses of PFOA elevated the absolute and relative liver weights in both females and males compared with respective controls (Tables 2-1 and 2-2). Importantly, on PND 7, prenatal PFOA exposure elevated relative liver weights in the 0.3 mg/kg treatment group in both females and males, a dose that is lower than the previously reported LOAEL in CD-1 mice (*29, 42*). This effect had dissipated after PND 7. In the 1.0 mg/kg group, the relative liver weights remained elevated in females on PNDs 7 and 14 ($p < 0.05$) and were also elevated on PNDs 7 and 42 in the males ($p = 0.02$). In the 3.0 mg/kg group, the relative liver weights remained elevated from PNDs 7 to 28 in the females ($p \leq 0.05$) and similarly elevated at PNDs 7, 14, 21, and 42 in the males ($p < 0.001$). These elevated liver weights are indicative of hepatomegaly, a common effect of PFOA exposure. The extent of liver weight elevation illustrates the dose-dependent effect of PFOA for this endpoint.

Brain weights.

Gestational PFOA exposure decreased absolute brain weights only at PND 63 in the males of the two highest treatment groups (1.0 and 3.0 mg/kg; $p < 0.05$, Table 2-2); yet, there were no statistical differences in the PFOA-treated females at any time point (Table 2-1). Also, there were no differences in relative brain weights of either gender. These data suggest that, across dose and time, PFOA exposure has little (male) or no effect (female) on brain weight.

Mammary glands.

Mammary glands from PFOA-treated groups exhibited histological characteristics of delayed epithelial growth, similar to those documented in rats neonatally exposed to endocrine-disrupting compounds (*35, 43, 44*) and had lower developmental scores compared with controls (Table 2-3). Even the lowest treatment group (0.3 mg/kg) had developmental scores that were statistically lower compared with controls at numerous time points (PNDs 14, 21, 42, and 84). Representative mammary gland whole mounts from the control, 0.3 mg/kg, and 1.0 mg/kg treatment groups at PNDs 21 and 84

are shown in Figure 2-1. In normal mouse mammary gland development, from PNDs 1 to 14, the primary duct extends from the nipple area toward the lymph node, branching to fill the mammary fat pad. After PND 14, the mouse mammary gland forms TEBs, which are the precursors of future branching (*45*). As the mammary gland grows, it continues to branch and form additional TEBs until the entire fat pad has been filled. The branches extend toward the ends of the fat pad, and the TEBs disappear as they differentiate into terminal structures around PND 63 (*45*), so that in the adult mouse at PND 84, there are no TEBs. The presence of numerous TEBs at PNDs 63 and 84 in the offspring of the PFOA-treated animals in this study are indicative of substantial mammary gland developmental delays. In the absence of control glands at PND 63, we compared our PFOA-treated glands to historical controls from previous studies (*37*); thus, these data may necessitate repeating. Nevertheless, epithelial branching in treated glands did not fill the mammary fat pad as completely as the controls, which was highly evident at PND 84 (Figure 2-1).

Serum PFOA concentrations.

Serum PFOA concentration data from female offspring are summarized in Figure 2-2 and detailed in the Table 2-4, and male offspring data are detailed in Table 2-5. As would be expected, the highest concentrations of PFOA were found in the serum of pups in the 3.0 mg/kg group. In the females of the 1.0 and 3.0 mg/kg treatment groups, the mean serum PFOA concentrations peaked at PND 14 and then gradually declined through the remainder of the study. However, in the 0.3 mg/kg group, the highest serum concentrations occurred at PND 7 (Figure 2-2, Table 2-4). The serum concentrations of all PFOA-treated groups were statistically elevated from PNDs 7 until 42 and in the two highest exposure groups (1.0 and 3.0 mg/kg) until PND 84 in females.

Unlike the adult, in the developing animal, the BW and blood volumes increase rapidly from birth to weaning. The increasing blood volumes and BWs of the offspring were taken into account to estimate the total blood burdens of PFOA in the pups from this study to understand when the body

burden peaks. We developed a formula to calculate the estimated total amount of PFOA in the blood per pup based on weight and serum PFOA concentrations. This calculation is based on the assumption that there is approximately 58.5 ml of blood/kg BW in the mouse and that 55% of the whole blood is serum (Hoff, 2000) resulting in the equation: blood burden = [BW (58.5 ml/kg/1000) × serum concentration × 55%]. The total blood burdens were calculated using the analytical values from the HPLC-MS/MS, regardless if values were below the LOQ for each individual sample. Developmental effects are heavily influenced by timing of exposure, in relation to organogenesis, as well as dose. Thus, comparisons of total blood burdens over time may provide insight into the timing or extent of adverse effects of toxicant exposure, particularly in the developing animal. In addition, blood burdens may be more useful for interspecies comparisons "(o)wing to the gender and species difference in elimination" of PFOA and other perfluoroalkyl acids (*34*). Evaluation of total blood content of PFOA over time resulted in an inverted U-shaped curve in females (Figure 2-3; Table 2-4), with a peak at PND 14 for all doses, which happen to occur after lactation peaks and just before pups begin to rely on solid food and water for their sustenance. As with the serum concentrations, all treatment groups had calculated total blood burdens of PFOA that were statistically greater than controls from PNDs 7 to 42.

Liver PFOA Concentrations.

It should be noted that the livers were not perfused before analysis; therefore, residual amounts of blood were potentially analyzed with these tissues. The concentration of PFOA was greater in liver samples of females in all treated groups compared with controls from PNDs 7 to 42 (Table 2-4). The highest liver concentrations of PFOA were found at PND 7, and unlike serum concentrations, the liver concentrations decreased or remained constant from PND 7 to PND 14 (Figure 2-2; Table 2-4). At the two latest time points evaluated, the mean PFOA concentrations in the liver were higher compared with concentrations in the serum (with exception of 1.0 mg/kg at 84 days). This trend has been observed in

other studies (*46*) and suggests that the liver may be a more sensitive biomarker than serum for long-term PFOA exposure estimates.

Brain PFOA concentrations

As with the livers, the brain samples were not perfused before analysis of PFOA content. PFOA was present in the brain on PND 7 in all treatment groups but at substantially (10- to 30-fold) lower levels than in serum or liver. Judging from these diminishing trends, our data demonstrated that, relative to serum and liver, PFOA is more readily eliminated from the brain. Brain PFOA concentrations in the females remained statistically elevated compared with controls on PND 7 and PND 14 in the 0.3 and 1.0 mg/kg treatment group and from PND 7 until PND 28 in the 3.0 mg/kg group (Table 2-4).

Late-Gestation PFOA Exposure Study

Body weights.

No significant differences in BW or net BWs were observed at any time point in the PFOA-exposed groups when compared with controls (Table 2-6). No differences in BW were expected at these exposure levels based on previous data from Lau et al. (2006).

Liver weights.

Absolute liver weights in the highest treatment group (1.0 mg/kg) were significantly increased compared with controls from PND 4 through PND 7 ($p < 0.05$, Table 2-6). The relative liver weights were also significantly increased in this treatment group from PND 4 through 14 ($p < 0.05$, Table 2-6). There was no effect at the 0.1 mg/kg dose or lower. The effects in the 1.0 mg/kg group were independent of BW and demonstrate that liver weight effects are dose and exposure length dependent; the LOAEL for hepatomegaly from late-gestation exposure was 1.0 mg PFOA/kg, whereas 0.3 mg PFOA/kg was adequate to induce significant hepatomegaly in the full-gestation exposure.

Mammary glands.

Upon visual observation, mammary glands of PFOA-exposed mice displayed aberrant morphology and thus were assessed lower developmental scores compared with controls (Figure 2-4). It should be noted that statistical differences found in a single quantitative endpoint did not necessarily determine aberrant development; rather, all quantitative and qualitative measurements were collectively utilized to determine overall developmental mammary gland scores. At PND 14, the longitudinal epithelial growth of the mammary glands from the 0.1 and 1.0 mg/kg groups was reduced compared with controls by 14.4% ($p = 0.04$) and 37.3% ($p = 0.01$), respectively, and the change in longitudinal growth from PNDs 1 to 14 was reduced by 27.4% ($p = 0.005$) and 56.5% ($p = 0.002$), respectively. Developmental delays were most evident at PND 21, and all treated groups exhibited statistically lower developmental scores compared with controls ($p < 0.02$; Table 2-7). Developmental scores and quantitative endpoints at PND 21 are presented in Table 2-7; mammary gland whole mounts from PND 21 are presented in Figure 2-4. At PND 21, the pups in the highest dose group (1.0 mg/kg) displayed the most impaired mammary gland growth, with low mammary gland scores, poor longitudinal epithelial growth, and fewer TEBs ($p < 0.05$; Table 2-7). Taking all developmental criteria into consideration, the lowest dose at which mammary gland developmental abnormalities were visible was 0.01 mg PFOA/kg.

Serum PFOA concentrations.

Serum PFOA concentrations in all exposed litters were statistically higher than controls at all time-points and in a dose-dependent manner (Figure 2-5). The highest serum concentrations were found at PND 1 and gradually declined until the end of the study. Even at the lowest dose (0.01 mg/kg), the mean serum PFOA concentration was not reduced to the levels of controls before the conclusion of the study at PND 21.

As with the full-gestational cohort, we also assessed the total PFOA blood content per pup as an indicator of body burden. In all treatment groups, the total blood PFOA content did not change from

PND 7 to PND 14 and then declined substantially thereafter (Figure 2-6; Table 2-8). PFOA blood burdens among the treated groups were statistically elevated at all time-points measured ($p < 0.05$).

To better understand the developmental toxicity of PFOA, we compared the dosimetry data from the 1.0 mg/kg females of the full-gestation study to those of the late-gestation study. These comparisons are highlighted in Figure 2-7 and also in Tables 2-4 and 2-8 and revealed unexpected similarities in PFOA serum dosimetry. However, it should be noted that slight differences in timing of necropsies or litter size may have some influence on the serum concentrations of offspring, and thus, these comparisons have been cautiously interpreted. There were no differences detected in the serum or calculated blood burdens at PND 7 of the two study groups, although the pups in the full-gestation study received 10 additional days of exposure. Large differences were expected at this point. The full-gestation 1.0 mg/kg females had mean serum and calculated blood burdens that were nearly twofold greater than those of the late-gestation study at PND 14 and PND 21, as expected.

DISCUSSION

The present studies demonstrate that exposure to PFOA throughout gestation (GD 1–17) or during the latter half of gestation (GD 10–17) at doses 10- to 30-fold lower than previously investigated are sufficient to produce abnormal mammary gland development in CD-1 mice. In both studies, low doses of PFOA elevated relative liver weights and stunted mammary gland development. Full-gestational exposure to ≥ 0.3 mg/kg PFOA resulted in reduced mammary gland developmental scores compared with controls at perinatal (PNDs 14 and 21), peripubertal (PND 42), and adult (PND 84) time points. Moreover, exposure to 30-fold lower doses of PFOA (0.01 mg/kg) during GD 10–17 suppressed mammary gland development as well. These data suggest that prenatal exposure to PFOA may alter mammary gland development in CD-1 mice at doses lower than investigated here. Additionally, effects on mammary tissue were observed at doses of PFOA lower than those required to exert an effect on the

liver and the mammary effects persisted longer. These findings implied that in CD-1 mice, the mammary gland was more sensitive to prenatal PFOA exposure than was the liver.

In a previous study by Yang et al. (2009), peripubertal exposures to PFOA in Balb/C and C57Bl/6 mice resulted in mammary gland growth effects at doses ≥ 5 mg/kg but not at ≤ 1 mg/kg, even though elevated liver weights were observed at 1 mg/kg. Thus, the mammary gland effects presented from our studies appear to be the result of an increased sensitivity in the CD-1 mouse strain. We postulate that intra-species differences in effects are more likely due to the timing of exposure, as there are strain differences in timing of puberty (*47*); yet, mammary gland morphology remains fairly consistent during stages of development (i.e., puberty and pregnancy). Further research is needed to determine if the sensitivity is attributed to timing of exposure or the mouse strain utilized or if there are other novel mechanisms underlying this apparent sensitivity in CD-1 mice.

The developmental scoring method utilized in these studies, which incorporated both qualitative and quantitative endpoints, accurately predicted the long-term mammary gland developmental delays seen in PND 84 full-gestation–treated animals. Following full-gestational PFOA exposure, several TEBs remained in PFOA-treated mammary glands at PND 84. Other studies have shown that the extended presence of TEBs, in general, can lead to long-term adverse effects on the gland, including a higher risk for mammary tumor formation following exposure to carcinogens (*48*) and altered lactation (*49*). Although it is unclear whether there are lasting adult effects on the mammary gland due to late-gestation exposure, evaluation of mammary tissues indicated that prenatal exposure to doses as low as 0.01 mg PFOA/kg can also lead to developmental delays. Thus, we did not identify an NOAEL for PFOA-induced mammary gland developmental effects in CD-1 mice.

In the full-gestational study, relative liver weights were elevated in the 0.3 mg/kg group, which is lower than the previously reported liver LOAEL of 1.0 mg/kg in this mouse strain (1.0 mg/kg was the lowest dose utilized; (*29, 42*). Another recent study found that prenatal exposure to 0.3 mg/kg of PFOA

elevated absolute liver weights independent of BW differences (*32*). Importantly, in the late-gestation study, we used exposure periods that were half the length previously used; yet, this study produced comparable effects on liver weights at the same doses reported previously (*29*). This warrants strength to the supposition that the LOAEL for PFOA-induced liver hepatomegaly in CD-1 mice is lower than 1.0 mg/kg and that the NOAEL is approximately 0.1 mg/kg.

PFOA did not appear to affect brain weights in our studies. However, the presence of PFOA in the tissue warrants further investigation, as it may impart other effects to the brain. A recent study (*33*) showed that low-level developmental exposure to PFOA produced behavioral effects in mice that extended into adulthood. Onishchenko *et al*. (2011) reported that prenatal exposure to 0.3 mg/kg affected activity levels in mice independent of brain weight changes. The presence of PFOA in the neonatal brain, coupled with its absence after four weeks of age, suggests that PFOA passes through the fetal mouse blood-brain barrier but is not able to pass through the fully functional barrier that is normally formed by the time of birth (*50*).

Theoretically, animals dosed for longer periods are expected to exhibit higher serum concentrations than those exposed for shorter periods. However, as seen in Figure 2-7, there are similarities in the serum PFOA concentration of 1.0 mg/kg females from the full-gestation study compared with those of the late-gestation study at PND 7. Other studies have found that there are comparable serum concentrations in adult CD-1 mice following differing lengths of exposure (*29*). It is postulated that these similarities in PFOA serum concentrations are attributable to differences in clearance rates relative to exposure length. In a study by Lou et al. (2009), with repeated low doses of PFOA, net urinary elimination rates of PFOA were found to be high, potentially due to saturation of renal resorption. However, in the same study, urinary elimination rates were found to be low when resorption saturation was not reached. Thus, it is speculated that the longer exposure parameters for the full-gestation study offspring resulted in higher urinary elimination rates and consequently higher blood

clearance rates, which may have reduced the serum PFOA concentration to comparable levels of the offspring exposed during late gestation. Additionally, it is important to consider the functional capacity of the developing urinary system in pups at this age relative to their ability to eliminate toxicants.

A temporal comparison revealed that the mean serum PFOA concentration and calculated blood burden of the 1.0 mg/kg group female offspring were greatest at PND 14 in the full-gestation study, whereas in the 1.0 mg/kg female offspring of the late-gestation study, the mean serum concentration was greatest at PND 1 and the calculated PFOA blood burden was greatest from PNDs 7 to 14 (Figure 2-7, Tables 2-4 and 2-8). A similar trend to that observed in the late-gestation females was also seen in mice given a single prenatal dose of PFOA with analytically measured, not calculated, body burdens (*9*).

After parturition, offspring of PFOA-treated lactating dams experience extended exposure via milk consumption (*9*). In addition to the PFOA that is transported into milk from the blood supply, the grooming habits of rodents further contribute to milk-borne exposure; PFOA present in urine of PNDs 1–10 offspring is consumed by dams and subsequently recirculated back into the maternal system (*52*). Due to the relationship between maternal grooming habits and pup exposure, pup urinary excretion rates can also influence the availability of PFOA for recycling by the dam. Therefore, higher urinary excretion rates in pups may indeed account for greater serum PFOA concentrations and blood burdens at the second week of age in those exposed throughout gestation compared with those exposed from GD 10 to 17.

Importantly, serum PFOA concentrations found in the 0.01 mg/kg group are lower than those measured in young children living in areas highly contaminated with PFOA, such as the Ohio River Valley in West Virginia (*15*). Emmett et al. (2006) reported that the 2004 mean serum PFOA concentrations were near 600 ng/ml in children aged 2–5 years from the Ohio River Valley area. Approximately 2 years later, and after some exposure intervention, Frisbee et al. (2009) reported mean serum concentration of 77.6 and 59.9 ng/ml in children from the C8 Health Project of age < 12 and 12–19 years, respectively.

The late-gestation study conducted here observed mice only until weaning, which would be equivalent to 2–3 years of age in humans. Due to the elimination rates of PFOA in female mice (t1/2 ~16 days, (*51*)), it can be assumed that serum PFOA levels of pups in the 0.1 mg/kg treatment group would have decreased after PND 21 to levels approaching, if not lower, than those reported in children by Frisbee et al. (2009). Importantly, from the full-gestation study mammary glands, we observed that the developmental effects were not transient and in fact were apparent at adulthood, although PFOA exposure had ceased 12 weeks earlier. These findings are of great concern considering that children are likely to be exposed to PFOA prenatally, as well as throughout life. Therefore, it is important to determine the human relevance of the observed endpoints in relation to internal dosimetry to establish a benchmark dose for the PFOA mammary gland effects.

It is also critical to establish a mode of action (MOA) for the developmental mammary gland growth effects following PFOA exposure to determine whether this MOA is biologically relevant to humans. The liver toxicity and general developmental effects of PFOA are believed to be mediated by activation of peroxisome proliferator–activated receptor-alpha (PPARα)(*30, 42, 54*); yet, there are data to suggest that PFOA-induced mammary gland effects are mediated by other pathways (*55*). Previous studies using PPAR-α knockout (KO) mice reported normal lactation after exposure to PFOA (*55*). Changes in serum progesterone reported in the study of Zhao et al. (2010) suggest that stimulatory, and potentially inhibitory, mammary gland effects may be mediated through endocrine disruption. PFOA may indirectly affect branching morphogenesis through modulation of progesterone synthesis (*55*), and other endocrine-disrupting effects of PFOA have been reported (*23*). In the future, we plan to further compare mammary gland developmental effects in PPARα wild-type and KO mice after prenatal PFOA exposure to determine whether PPARα activation is involved in this outcome.

In summary, an NOAEL was not achieved in either study for PFOA-induced mammary gland effects in CD-1 mice, as altered mammary gland development was observed in offspring of dams treated with the lowest PFOA dose utilized in each study. As these are the lowest doses of PFOA tested in CD-1 mice thus far, additional studies are necessary to determine an NOAEL, as well as to establish the human relevance of PFOA-induced mammary gland effects.

Table 2-1. Weight indices of female offspring from the full gestation exposure study.

	PND 7 (n)	PND 14 (n)	PND 21 (n)	PND 28 (n)	PND 42 (n)	PND 63 (n)	PND 84 (n)
Pup Weight (g)							
Control	4.12 ± 0.23 (5)	8.16 ± 0.06 (4)	13.73 ± 0.63 (6)	20.40 ± 1.05 (4)	28.21 ± 1.74 (4)	-	34.65 ± 0.05 (2)
0.3 mg/kg	4.76 ± 0.09 (4)	8.15 ± 0.39 (6)	12.02 ± 1.23 (5)	20.77 ± 0.92 (6)	25.13 ± 3.91 (6)	28.90 ± 1.88 (4)	31.71 ± 2.20 (5)
1.0 mg/kg	4.30 ± 0.32 (5)	7.97 ± 0.73 (6)	12.29 ± 1.10 (5)	19.39 ± 1.74 (4)	25.89 ± 1.00 (6)	28.83 ± 0.84 (5)	35.58 ± 3.03 (4)
3.0 mg/kg	3.78 ± 0.22 (4)	7.65 ± 0.63 (6)	14.78 ± 0.96 (3)	19.77 ± 0.82 (6)	25.43 ± 1.70 (4)	29.60 ± 0.90 (2)	37.30 ± 4.60 (2)
Net Pup Weight (g)							
Control	3.97 ± 0.22 (5)	7.85 ± 0.06 (4)	12.90 ± 0.59 (6)	19.10 ± 0.98 (4)	26.51 ± 1.61 (4)	-	32.84 ± 0.11 (2)
0.3 mg/kg	4.56 ± 0.09 (4)	7.81 ± 0.38 (6)	11.34 ± 1.15 (5)	19.49 ± 0.88 (6)	23.44 ± 3.87 (6)	27.15 ± 1.73 (4)	30.08 ± 2.11 (5)
1.0 mg/kg	4.08 ± 0.31 (5)	7.58 ± 0.68 (6)	11.49 ± 1.02 (5)	18.13 ± 1.65 (4)	24.30 ± 0.93 (6)	27.19 ± 0.79 (5)	33.89 ± 2.93 (4)
3.0 mg/kg	3.53 ± 0.21 (4)	7.19 ± 0.59 (4)	13.69 ± 0.88 (3)	18.39 ± 0.75 (6)	23.78 ± 1.58 (4)	28.08 ± 0.90 (2)	35.24 ± 4.52 (2)
Absolute Liver Weight (g)							
Control	0.15 ± 0.01 (5)	0.31 ± 0.01 (4)	0.83 ± 0.04 (6)	1.31 ± 0.08 (4)	1.70 ± 0.13 (4)	-	1.81 ± 0.06 (2)
0.3 mg/kg	0.21 ± 0.01* (4)	0.34 ± 0.02 (6)	0.68 ± 0.09 (5)	1.28 ± 0.04 (6)	1.70 ± 0.14 (6)	1.75 ± 0.19 (4)	1.63 ± 0.10 (5)
1.0 mg/kg	0.22 ± 0.01** (5)	0.39 ± 0.05 (6)	0.80 ± 0.08 (5)	1.26 ± 0.09 (4)	1.59 ± 0.08 (6)	1.64 ± 0.05 (5)	1.68 ± 0.13 (4)
3.0 mg/kg	0.24 ± 0.01*** (4)	0.46 ± 0.04 (4)	1.09 ± 0.09 (3)	1.38 ± 0.08 (6)	1.65 ± 0.12 (4)	1.53 ± 0.01 (2)	2.06 ± 0.08 (2)

Relative Liver							
Control	0.037 ± 0.002 (5)	0.038 ± 0.001 (4)	0.061 ± 0.001 (6)	0.064 ± 0.001 (4)	0.060 ± 0.001 (4)	-	0.052 ± 0.002 (2)
0.3 mg/kg	0.044 ± 0.002* (4)	0.042 ± 0.003 (6)	0.056 ± 0.002 (5)	0.062 ± 0.001 (6)	0.091 ± 0.033 (6)	0.060 ± 0.004 (4)	0.051 ± 0.002 (5)
1.0 mg/kg	0.051 ± 0.001*** (5)	0.048 ± 0.002** (6)	0.065 ± 0.001 (5)	0.065 ± 0.002 (4)	0.061 ± 0.002 (6)	0.057 ± 0.000 (5)	0.048 ± 0.002 (4)
3.0 mg/kg	0.065 ± 0.002*** (4)	0.060 ± 0.001*** (4)	0.074 ± 0.002*** (3)	0.070 ± 0.002* (6)	0.065 ± 0.001 (4)	0.052 ± 0.002 (2)	0.056 ± 0.005 (2)
Absolute Brain Weight (g)							
Control	0.26 ± 0.01 (5)	0.39 ± 0.01 (4)	0.43 ± 0.01 (6)	0.45 ± 0.02 (4)	0.50 ± 0.01 (4)	-	0.55 ± 0.04 (2)
0.3 mg/kg	0.28 ± 0.00 (4)	0.38 ± 0.00 (5)	0.41 ± 0.01 (5)	0.45 ± 0.01 (6)	0.47 ± 0.01 (6)	0.53 ± 0.03 (4)	0.53 ± 0.01 (5)
1.0 mg/kg	0.25 ± 0.01 (5)	0.38 ± 0.02 (6)	0.41 ± 0.01 (5)	0.46 ± 0.02 (4)	0.46 ± 0.01 (6)	0.47 ± 0.01 (5)	0.51 ± 0.02 (4)
3.0 mg/kg	0.26 ± 0.02 (4)	0.40 ± 0.02 (4)	0.48 ± 0.00 (3)	0.45 ± 0.00 (6)	0.45 ± 0.02 (4)	0.48 ± 0.01 (2)	0.53 ± 0.01 (2)
Relative Brain							
Control	0.063 ± 0.002 (5)	0.048 ± 0.001 (4)	0.031 ± 0.001 (6)	0.022 ± 0.001 (4)	0.018 ± 0.001 (4)	-	0.016 ± 0.001 (2)
0.3 mg/kg	0.058 ± 0.001 (4)	0.040 ± 0.008 (5)	0.035 ± 0.003 (5)	0.022 ± 0.001 (6)	0.026 ± 0.010 (6)	0.019 ± 0.002 (4)	0.017 ± 0.001 (4)
1.0 mg/kg	0.059 ± 0.003 (5)	0.048 ± 0.003 (4)	0.034 ± 0.002 (5)	0.024 ± 0.001 (4)	0.018 ± 0.000 (6)	0.016 ± 0.001 (5)	0.015 ± 0.001 (5)
3.0 mg/kg	0.070 ± 0.008 (4)	0.053 ± 0.002 (4)	0.032 ± 0.002 (3)	0.023 ± 0.001 (6)	0.018 ± 0.002 (4)	0.016 ± 0.000 (2)	0.015 ± 0.002 (2)

Note. Weight indices of female offspring from the full gestation exposure study. Data presented are mean ± SE. Dashes (-) signify time points where no measure was taken for a treatment group. Net pup weights were calculated as the individual liver weights subtracted from the individual pup weights and are presented as the mean values for each treatment group. Relative weights were calculated as the individual absolute tissue weight divided by the individual body weight and are presented as the mean values for each treatment group. Not able to determine significance at PND 63 due to absence of controls.

Significant treatment effect compared to controls; * p≤0.05 ** p≤0.01, *** p≤0.001.

Table 2-2. Weight indices of male offspring from the full gestation exposure study

	PND 7 (n)	PND 14 (n)	PND 21 (n)	PND 28 (n)	PND 42 (n)	PND 63 (n)	PND 84 (n)
Pup Weight (g)							
Control	4.63 ± 0.20 (6)	8.24 ± 0.45 (4)	13.95 ± 0.32 (4)	24.71 ± 0.82 (4)	33.21 ± 1.67 (5)	43.93 ± 1.98 (4)	40.40 ± 4.61 (3)
0.3 mg/kg	4.13 ± 0.53 (5)	7.92 ± 0.40 (5)	13.43 ± 1.09 (4)	21.75 ± 1.05 (4)	32.95 ± 0.68 (6)	38.17 ± 1.06 (3)	40.13 ± 2.39 (3)
1.0 mg/kg	4.70 ± 0.21 (3)	8.10 ± 0.98 (4)	12.71 ± 1.00 (4)	25.69 ± 0.83 (4)	31.30 ± 3.40 (3)	38.70 ± 2.51 (3)	44.94 ± 2.00 (5)
3.0 mg/kg	4.15 ± 0.34 (4)	7.32 ± 0.54 (6)	13.11 ± 1.28 (4)	22.30 ± 1.33 (4)	31.86 ± 1.00 (6)	37.38 ± 0.58 (2)	38.58 ± 2.48 (3)
Net Pup Weight (g)							
Control	4.47 ± 0.20 (6)	7.90 ± 0.43 (4)	13.13 ± 0.31 (4)	23.02 ± 0.83 (4)	31.22 ± 1.58 (5)	41.44 ± 1.82 (4)	38.24 ± 4.37 (3)
0.3 mg/kg	3.95 ± 0.51 (5)	7.58 ± 0.40 (5)	12.59 ± 1.00 (4)	20.26 ± 0.95 (4)	30.94 ± 0.62 (6)	36.07 ± 0.99 (3)	38.11 ± 2.29 (3)
1.0 mg/kg	4.45 ± 0.20 (3)	7.70 ± 0.91 (4)	11.90 ± 0.92 (4)	24.00 ± 0.75 (4)	29.22 ± 3.17 (3)	36.40 ± 2.31 (3)	42.57 ± 1.84 (5)
3.0 mg/kg	3.87 ± 0.31 (4)	6.89 ± 0.50 (6)	12.08 ± 1.18 (4)	20.65 ± 1.20 (4)	29.64 ± 0.93 (6)	35.07 ± 0.44 (2)	36.48 ± 2.23 (3)
Absolute Liver Weight (g)							
Control	0.16 ± 0.01 (6)	0.34 ± 0.03 (4)	0.82 ± 0.03 (4)	1.69 ± 0.03 (4)	1.99 ± 0.10 (5)	2.48 ± 0.18 (4)	2.16 ± 0.24 (3)
0.3 mg/kg	0.18 ± 0.03 (5)	0.34 ± 0.02 (5)	0.83 ± 0.10 (4)	1.49 ± 0.11 (4)	2.01 ± 0.07 (6)	2.10 ± 0.08 (3)	2.03 ± 0.15 (3)
1.0 mg/kg	0.25 ± 0.02* (3)	0.40 ± 0.07 (4)	0.82 ± 0.09 (4)	1.69 ± 0.09 (4)	2.08 ± 0.23 (3)	2.31 ± 0.21 (3)	2.37 ± 0.17 (5)
3.0 mg/kg	0.28 ± 0.03** (4)	0.43 ± 0.04 (6)	1.04 ± 0.10 (4)	1.65 ± 0.13 (4)	2.22 ± 0.09 (6)	2.31 ± 0.14 (2)	2.11 ± 0.25 (5)

Relative Liver							
Control	0.034 ± 0.002 (6)	0.041 ± 0.003 (4)	0.059 ± 0.002 (4)	0.061 ± 0.008 (4)	0.060 ± 0.001 (5)	0.056 ± 0.002 (4)	0.053 ± 0.001 (3)
0.3 mg/kg	0.043 ± 0.002** (5)	0.043 ± 0.003 (5)	0.061 ± 0.003 (4)	0.068 ± 0.002 (4)	0.061 ± 0.001 (6)	0.055 ± 0.001 (3)	0.051 ± 0.003 (3)
1.0 mg/kg	0.054 ± 0.003*** (3)	0.048 ± 0.003 (4)	0.064 ± 0.002 (4)	0.066 ± 0.001 (4)	0.066 ± 0.000* (3)	0.059 ± 0.002 (3)	0.052 ± 0.002 (5)
3.0 mg/kg	0.067 ± 0.002*** (4)	0.058 ± 0.001*** (6)	0.079 ± 0.001*** (4)	0.074 ± 0.002 (4)	0.070 ± 0.001*** (6)	0.062 ± 0.003 (2)	0.054 ± 0.003 (3)
Absolute Brain Weight (g)							
Control	0.27 ± 0.01 (6)	0.40 ± 0.01 (4)	0.43 ± 0.01 (4)	0.49 ± 0.01 (4)	0.51 ± 0.01 (5)	0.60 ± 0.04 (4)	0.51 ± 0.02 (3)
0.3 mg/kg	0.25 ± 0.02 (5)	0.38 ± 0.01 (4)	0.42 ± 0.01 (4)	0.46 ± 0.01 (4)	0.48 ± 0.01 (6)	0.50 ± 0.00 (3)	0.51 ± 0.00 (3)
1.0 mg/kg	0.26 ± 0.00 (3)	0.40 ± 0.01 (4)	0.42 ± 0.01 (4)	0.47 ± 0.01 (4)	0.48 ± 0.03 (3)	0.46 ± 0.02* (3)	0.50 ± 0.01 (5)
3.0 mg/kg	0.25 ± 0.01 (4)	0.39 ± 0.01 (6)	0.46 ± 0.02 (4)	0.45 ± 0.01(4)	0.48 ± 0.01 (6)	0.47 ± 0.03* (2)	0.51 ± 0.01 (3)
Relative Brain							
Control	0.059 ± 0.002 (6)	0.049 ± 0.002 (4)	0.031 ± 0.000 (4)	0.020 ± 0.001 (4)	0.016 ± 0.001 (5)	0.014 ± 0.001 (4)	0.013 ± 0.001 (3)
0.3 mg/kg	0.063 ± 0.003 (5)	0.039 ± 0.010 (5)	0.032 ± 0.002 (4)	0.021 ± 0.001 (4)	0.015 ± 0.001 (6)	0.013 ± 0.001 (3)	0.013 ± 0.001 (3)
1.0 mg/kg	0.055 ± 0.002 (3)	0.051 ± 0.006 (4)	0.033 ± 0.002 (4)	0.018 ± 0.001 (4)	0.015 ± 0.001 (3)	0.011 ± 0.001 (3)	0.011 ± 0.001 (5)
3.0 mg/kg	0.061 ± 0.002 (4)	0.055 ± 0.003 (6)	0.035 ± 0.003 (4)	0.020 ± 0.001 (4)	0.015 ± 0.000 (6)	0.013 ± 0.001 (2)	0.013 ± 0.001 (3)

Note. Weight indices of male offspring from the full gestational study. Data presented are mean ± SE. Net pup weights were calculated as the individual liver weights subtracted from the individual pup weights and are presented as the mean values for each treatment group. Relative weights were calculated as the individual absolute tissue weight divided by the individual body weight and are presented as the mean values for each treatment group.

Significant treatment effect compared to controls; * p≤0.05 ** p≤0.01, *** p≤0.001.

Table 2-3. Mammary gland developmental scores from the full gestation exposure study.

	PND 7 (n)	PND 14 (n)	PND 21 (n)	PND 28 (n)	PND 42 (n)	PND 63 (n)	PND 84 (n)
Control	3.3 ± 0.2 (5)	3.2 ± 0.3 (4)	3.4 ± 0.3 (3)	3.4 ± 0.3 (4)	3.8 ± 0.1 (4)	-	4.0 ± 0.0 (2)
0.3 mg/kg	2.4 ± 0.4 (4)	1.5 ± 0.2*** (6)	1.9 ± 0.2** (5)	2.8 ± 0.2 (6)	2.8 ± 0.3* (5)	2.4 ± 0.6 (3)	2.4 ± 0.3* (3)
1.0 mg/kg	2.2 ± 0.3* (5)	1.5 ± 0.1*** (6)	1.3 ± 0.1*** (5)	2.3 ± 0.3**(4)	2.0 ± 0.3*** (6)	2.9 ± 0.4 (5)	2.2 ± 0.2** (4)
3.0 mg/kg	1.6 ± 0.2** (4)	1.7 ± 0.3** (4)	1.6 ± 0.4** (3)	1.8± 0.2*** (6)	2.8 ± 0.2 (4)	1.8 ± 0.5 (2)	2.9 ± 0.4 (2)

Note. Mammary gland developmental scores from the full gestation exposure study. Data presented are mean ± SE. Dashes (-) signify time points where no measure was taken for a treatment group. Not able to determine significance at PND 63 due to absence of controls.

Significant treatment effect compared to controls; * p≤0.05 ** p≤0.01, *** p≤0.001.

Table 2-4. PFOA dosimetry of female offspring from the full gestation exposure study.

	PND 7[d,e] (n)	PND 14[a,b,c] (n)	PND 21 (n)	PND 28 (n)	PND 42 (n)	PND 63 (n)	PND 84 (n)
Serum (ng/ml)							
Control	< 20, LOQ (5)	12 ± 2 (4)	< 20, LOQ (6)	< 20, LOQ (3)	<10, LOQ (4)	-	<10, LOQ (2)
0.3 mg/kg	4980 ± 218*** (4)	4535 ± 920*** (6)	1194 ± 394*** (5)	630 ± 162*** (6)	377 ± 81*** (6)	55 ± 17 (3)	16 ± 5 (5)
1.0 mg/kg	11026 ± 915*** (5)	16950 ± 3606*** (6)	3770 ± 607*** (5)	1247 ± 208*** (4)	663 ± 185*** (6)	176 ± 85 (2)	71 ± 8* (2)
3.0 mg/kg	20700 ± 3900*** (5)	26525 ± 2446*** (4)	8343 ± 1078*** (3)	4883 ± 1378*** (6)	2058 ± 348*** (4)	-	125* (1)
Calculated Blood Burden (ng)							
Control	0.3 ± 0.2 (5)	0.5 ± 0.5 (4)	1.0 ± 0.7 (6)	1.7 ± 1.7 (3)	1.5 ± 1.5 (4)	-	0.0 ± 0.0 (2)
0.3 mg/kg	762.4 ± 32.2*** (4)	1166.8 ± 206.3*** (6)	412.3 ± 72.4*** (5)	409.7 ± 103.1*** (6)	306.7 ± 95.8* (6)	46.8 ± 14.1 (3)	15.5 ± 3.9 (5)
1.0 mg/kg	1560.5 ± 241.7*** (5)	3728..8 ± 989.4*** (6)	1408.7 ± 172.6*** (5)	727.2 ± 60.5*** (4)	547.1 ± 127.8** (6)	165.6 ± 77.9 (2)	82.1 ± 3.1 (2)
3.0 mg/kg	2514.5 ± 730.7*** (5)	6422 ± 457.5*** (4)	4038.3 ± 628.4*** (3)	3245.5 ±1080.3*** (6)	1640.0 ± 211.8** (4)	-	131.5 (1)
Liver (ng/g)							
Control	<35, LOQ (5)	<LOQ (4)	<LOQ (6)	<LOQ (4)	<LOQ (4)	-	<LOQ (2)
0.3 mg/kg	2078 ± 90*** (4)	972 ± 124*** (6)	1188 ± 182*** (5)	678 ± 130*** (6)	342 ± 87** (6)	118 ± 22 (3)	43 ±12 (5)
1.0 mg/kg	8134 ± 740*** (5)	4152 ± 483*** (6)	1939 ± 637*** (5)	2007 ± 560*** (4)	617 ± 145*** (6)	320 ± 113 (5)	55 ± 12 (4)
3.0 mg/kg	16700 ± 749*** (4)	10290 ± 1028*** (4)	2339 ± 1241*** (3)	7124 ± 1081*** (6)	1145 ± 274*** (4)	417 ± 160 (2)	235 ± 79** (2)

Brain (ng/g)							
Control	<35, LOQ (5)	<LOQ (4)	<LOQ (6)	<LOQ (4)	<LOQ (4)	-	<LOQ (2)
0.3 mg/kg	150 ± 26*** (4)	65 ± 12** (6)	<LOQ (5)	<LOQ (6)	<LOQ (6)	<LOQ (3)	<LOQ (5)
1.0 mg/kg	479 ± 41*** (5)	241 ± 20*** (6)	31 ± 5 (5)	<LOQ (4)	<LOQ (6)	<LOQ (5)	<LOQ (4)
3.0 mg/kg	1594 ± 162*** (5)	650 ± 44*** (4)	133 ± 23*** (3)	62 ± 93*** (6)	<LOQ (4)	<LOQ (2)	<LOQ (2)

Note. PFOA dosimetry data for female pups from the full gestation study. Data presented are mean ± SE. Dashes (-) signify time points where no measure was taken for a treatment group. Calculated blood burdens were determined by the equation (BW x (58.5/1000) x serum x 0.55). Not able to determine significance at PND 63 due to absence of controls. LOQ=Limit of Quantitation.

Significant treatment effect compared to controls; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[a] Mean serum concentration of each PFOA-treated group within PND: 14> 21, 21, 42, 63, and 84, $p<0.001$.

[b] Mean calculated blood burden of 0.3 and 1.0 mg/kg group within PND: 14> 21, 28, 42, 63, and 84, $p<0.05$.

[c] Mean calculated blood burden of 3.0 mg/kg group within PND: 14> 42 and 84, $p\leq0.05$.

[d] Mean liver concentration of each PFOA-treated group within PND: 7> 14, 21, 28, 42, 63, 84, $p<0.01$.

[e] Mean brain concentration of each PFOA-treated group within PND: 7 > 14, 21, 28, 42, 63, 84, $p<0.001$.

Table 2-5. PFOA dosimetry of male offspring from the full gestation exposure study.

	PND 7 (n)	PND 14 (n)	PND 21 (n)	PND 28 (n)	PND 42 (n)	PND 63 (n)	PND 84 (n)
Serum (ng/ml)							
Control	< 20, LOQ (1)	2292 ± 2278 (2)	-	< 20, LOQ (2)	< 10, LOQ (2)	< 10, LOQ (4)	< 10, LOQ (3)
0.3 mg/kg	5940* (1)	-	597 (1)	-	-	74 ± 24 ***(3)	39*** (1)
1.0 mg/kg	11600* (1)	-	2840 (1)	1833 ± 1217* (2)	-	130*** (1)	29*** (1)
3.0 mg/kg	27050 ± 1550* (2)	23650 ± 2850 (2)	11440 ± 1060 (3)	-	3245 ± 255*** (2)	118** (1)	-
Calculated Blood Burden (ng)							
Control	1.1 (1)	600.4 ± 598.0 (2)	-	3.0 ± 3.0 (2)	0.0 ± 0.0 (2)	0.0 ± 0.0 (4)	2.5 ± 2.5 (3)
0.3 mg/kg	716.7 (1)	-	263.2 (1)	-	-	93.7 ± 29.9 (3)	58.4 (1)
1.0 mg/kg	1642.2* (1)	-	1261 .0 (1)	1625.0 ± 1103.0 (2)	-	169.0 (1)	39.2 (1)
3.0 mg/kg	3900.4 ± 700.6* (2)	5128.5 ± 243.1 (2)	4243.2 ± 505.1 (3)	-	3384.8 ± 489.1 (2)	138.2 (1)	-
Liver (ng/g)							
Control	<35, LOQ (1)	< LOQ (2)	-	< LOQ (2)	< LOQ (2)	< LOQ (4)	< LOQ (3)
0.3 mg/kg	2600 ± 490** (2)	-	1015 (1)	-	-	220 ± 67*** (3)	83 (1)
1.0 mg/kg	6490** (1)	-	654 (1)	3132 ± 2412* (2)	-	406*** (1)	172 ± 97** (2)
3.0 mg/kg	17450 ± 450** (2)	11030 ± 1170*** (2)	3383 ± 562 (3)	-	5758 ± 2713** (2)	2384 ± 921*** (2)	421 ± 28*** (3)

Brain (ng/g)							
Control	<35, LOQ (1)	< LOQ (2)	-	< LOQ (2)	< LOQ (2)	< LOQ (4)	< LOQ (3)
0.3 mg/kg	188 ± 48 (2)	-	< LOQ (1)	-	-	< LOQ (3)	< LOQ (1)
1.0 mg/kg	412 (1)	-	< LOQ (1)	< LOQ (2)	-	< LOQ (1)	< LOQ (2)
3.0 mg/kg	1256 ± 305* (2)	751 ± 61*** (2)	181 ± 20 (3)	-	32 ± 7 (2)	< LOQ (2)	< LOQ (3)

Note. PFOA dosimetry data for male offspring from the full gestation study. Data presented are mean ± SE. Dashes (-) signify time points where no measure was taken for a treatment group. Calculated blood burdens were determined by the equation (BW x (58.5/1000) x serum x 0.55). As calculated blood burdens were log transformed to determine significance and the control value was 0.0, no significance could be determined at PND 42 or 63. Not able to determine significance at PND 21 due to absence of controls. LOQ=Limit of Quantitation.

Significant treatment effect compared to controls; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2-6. Weight indices of female offspring from the late gestation exposure study.

	PND 1 (n)	PND 4 (n)	PND 7 (n)	PND 14 (n)	PND 21 (n)
Pup Weight (g)					
Control	1.78 ± 0.05 (8)	3.43 ± 0.03 (3)	5.53 ± 0.22 (11)	10.54 ± 0.41 (11)	16.02 ± 0.57 (10)
0.01 mg/kg	1.75 ± 0.07 (8)	3.39 ± 0.12 (4)	5.48 ± 0.14 (11)	9.90 ± 0.38 (11)	14.52 ± 0.56 (11)
0.1 mg/kg	1.76 ± 0.09 (7)	-	5.48 ± 0.18 (9)	10.31 ± 0.31 (8)	15.51 ± 0.43 (7)
1.0 mg/kg	1.74 ± 0.05 (9)	3.52 ± 0.19 (5)	5.10 ± 0.19 (11)	9.97 ± 0.53 (11)	14.99 ± 0.52 (11)
Net Weight (g)					
Control	1.74 ± 0.10 (4)	3.31 ± 0.03 (3)	5.85 ± 0.23 (5)	10.92 ± 0.51 (5)	15.75 ± 0.68 (5)
0.01 mg/kg	1.63 ± 0.05 (4)	3.27 ± 0.12 (4)	5.64 ± 0.19 (4)	10.23 ± 0.56 (4)	14.82 ± 0.42 (4)
0.1 mg/kg	1.69 ± 0.21 (3)	-	5.66 ± 0.35 (3)	10.16 ± 0.42 (2)	13.78 ± 0.27 (2)
1.0 mg/kg	1.63 ± 0.05 (3)	3.34 ± 0.18 (5)	5.33 ± 0.22 (5)	10.91 ± 0.49 (5)	15.09 ± 0.48 (5)
Absolute Liver Weights (g)					
Control	0.08 ± 0.01 (4)	0.13 ± 0.00 (3)	0.22 ± 0.01 (5)	0.45 ± 0.04 (5)	0.89 ± 0.04 (5)
0.01 mg/kg	0.06 ± 0.00 (4)	0.12 ± 0.01 (4)	0.22 ± 0.01 (4)	0.40 ± 0.04 (4)	0.81 ± 0.03 (4)
0.1 mg/kg	0.08 ± 0.02 (3)	-	0.21 ± 0.02 (3)	0.44 ± 0.03 (2)	0.77 ± 0.02 (2)
1.0 mg/kg	0.09 ± 0.01 (5)	0.18 ± 0.02* (5)	0.29 ± 0.01** (5)	0.55 ± 0.02 (5)	0.90 ± 0.02 (5)
Relative Liver					
Control	0.043 ± 0.001 (4)	0.037 ± 0.001 (3)	0.036 ± 0.001 (5)	0.039 ± 0.001 (5)	0.053 ± 0.001 (5)
0.01 mg/kg	0.035 ± 0.002 (4)	0.036 ± 0.002 (4)	0.037 ± 0.001 (4)	0.038 ± 0.002 (4)	0.052 ± 0.002 (4)
0.1 mg/kg	0.044 ± 0.005 (3)	-	0.036 ± 0.002 (3)	0.041 ± 0.001 (2)	0.053 ± 0.002 (2)
1.0 mg/kg	0.055 ± 0.004 (5)	0.052 ± 0.004* (5)	0.051 ± 0.001*** (5)	0.048 ± 0.001** (5)	0.057 ± 0.001 (5)

Note. Weight indices of female offspring from the late gestation study. Data presented are mean ± SE. Dashes (-) signify time points where no measure was taken for a treatment group. Net weights were calculated as the individual liver weights subtracted from the individual pup weights and are presented as the mean values for each treatment group. Relative liver weights were calculated as the individual absolute liver weight divided by the individual body weight and are presented as the mean values for each treatment group.

Significant treatment effect compared to controls; * $p<0.05$ ** $p<0.01$, *** $p<0.001$.

Table 2-7. Late gestation female mammary gland measurements at PND 21

	Developmental score	Longitudinal growth (μm)	Lateral growth (μm)	Δ Longitudinal growth (μm)	Δ Lateral growth (μm)	Terminal End Buds (TEBs)	Terminal ends (TEs)
Control (n)	3.3 ± 0.3 (5)	4321 ± 306 (5)	5941 ± 280 (5)	3394 ± 306 (5)	4358 ± 280 (5)	40 ± 4 (5)	81 ± 12 (5)
0.01 mg/kg (n)	2.2 ± 0.2* (4)	3803 ± 386 (4)	5420 ± 326 (4)	3087 ± 386 (4)	3899 ± 326 (4)	33 ± 4 (4)	61 ± 8 (4)
0.1 mg/kg (n)	1.8 ± 0.3** (3)	3615 ± 320 (3)	4822 ± 672 (3)	2370 ± 320 (3)	3035 ± 672 (3)	24 ± 4* (3)	58 ± 4 (3)
1.0 mg/kg (n)	1.6 ± 0.1*** (5)	2775 ± 285** (5)	4822 ± 313 (5)	1553 ± 301** (5)	3380 ± 313 (5)	15 ± 2***(5)	47 ± 11 (5)

Note. Mammary gland measurements from late gestation females at PND 21**.** Data presented as the mean ± SEM. n=4-7.

Significant effects compared to controls, * p<0.05, **p<0.01, ***p<0.001.

Table 2-8. PFOA dosimetry of female offspring from the late gestation exposure study.

	PND 1[a] (n)	PND 4 (n)	PND 7 (n)	PND 14 (n)	PND 21[b] (n)
Serum (ng/ml)					
Control	22.6 ± 5.5 (4)	8.6 ± 0.5 (2)	7.8 ± 2.1 (5)	7.8 ± 1.5 (8)	4.1 ± 0.6 (7)
0.01 mg/kg	284.5 ± 21.0* (3)	184.1 ± 12.1* (2)	150.7 ± 20.9* (7)	80.2 ± 13.9* (8)	16.5 ± 2.1* (10)
0.1 mg/kg	2303.5 ± 114.1* (2)	-	1277.8 ± 122.6* (8)	645.4 ± 114.2* (7)	131.7 ± 24.5* (7)
1.0 mg/kg	16305.5 ± 873.5* (7)	-	11880.3 ± 1447.6* (11)	6083.7 ± 662.6* (11)	2025.1 ± 281.9* (11)
Calculated Blood Burden (ng)					
Control	1.3 ± 0.38 (4)	0.9 ± 0.1 (2)	1.5 ± 0.4 (5)	2.7 ± 0.7 (8)	1.2 ± 0.6 (7)
0.01 mg/kg	15.2 ± 1.7* (3)	20.6 ± 0.1* (2)	27.3 ± 3.8* (7)	27.0 ± 4.6* (8)	7.9 ± 1.0* (10)
0.1 mg/kg	114.3 ± 5.4* (2)	-	221.7 ± 24.9* (8)	218.5 ± 39.8* (7)	66.4 ± 12.8* (7)
1.0 mg/kg	926.0 ± 47.6* (7)	-	1965.9 ± 256.7* (11)	2033.6 ± 293.5* (11)	984.9 ± 142.8* (11)

Note. PFOA Dosimetry data for female offspring from late gestation study. Data presented are mean ± SE. Dashes (-) signify time points where no measure was taken for a treatment group. Calculated blood burdens were determined by the equation (BW x (58.5/1000) x serum x 0 .55).

Significant treatment effect compared to controls; * $p<0.001$.

[a] Mean serum concentration of each PFOA-treated group within PND: 1> 4, 7, 14, and 21, $p<0.05$.

[b] Mean calculated blood burdens of each PFOA-treated group within PND: 21< 7 and 14, $p<0.02$.

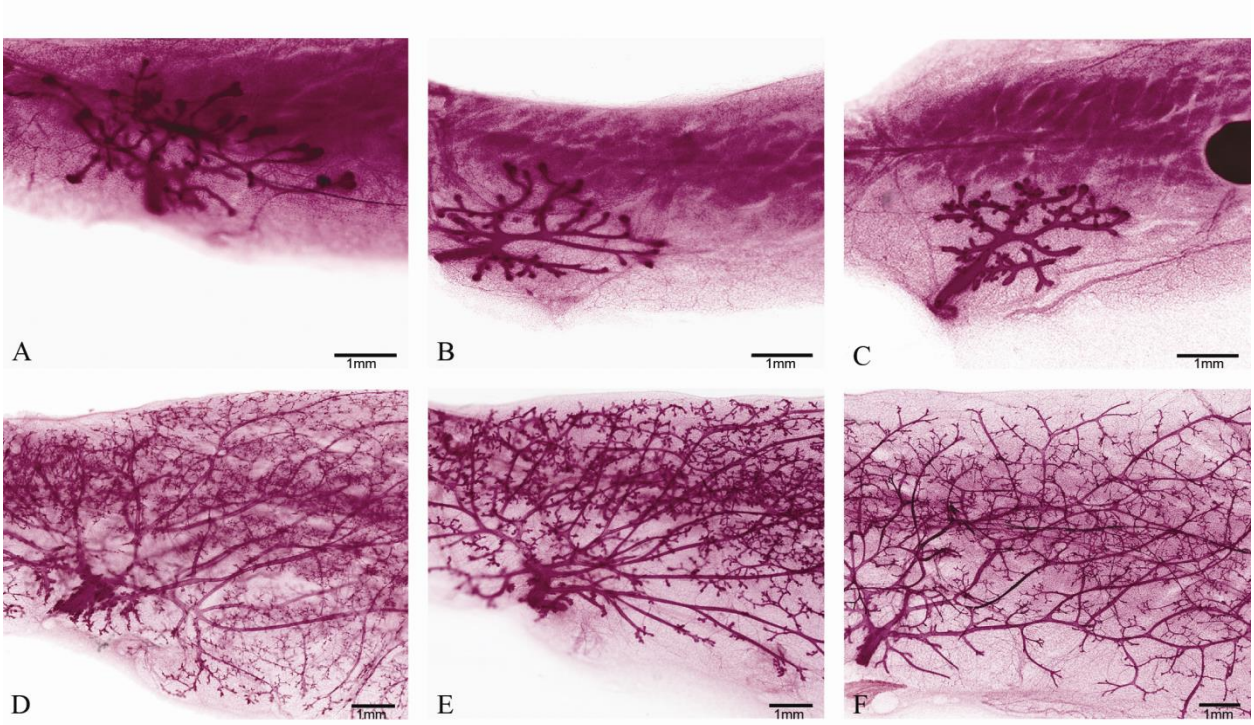

Figure 2-1. Offspring mammary gland whole mounts in the full gestation studyControl glands from (A) PND 21 and (D) 84; 0.3 mg/kg glands from (B) PND 21 and (E) 84; 1.0 mg/kg glands from (C) PND 21 and (F) 84. Glands pictured are representative of mean score for each treatment group; n=3-5 (PND 21), 2-8, (PND 84). At PND 21 (top panel), the two treated glands (B, C) are smaller in overall size, with poor branching patterns and fewer visible terminal end buds (TEBs) when compared to the control gland(A). At PND 84 (bottom panel), the two treated glands (E, F) have poor branching patterns, poor differention and several TEBs. All PFOA-treated mammary glands received significantly lower developmental scores compared to controls at both time points (p<0.02).

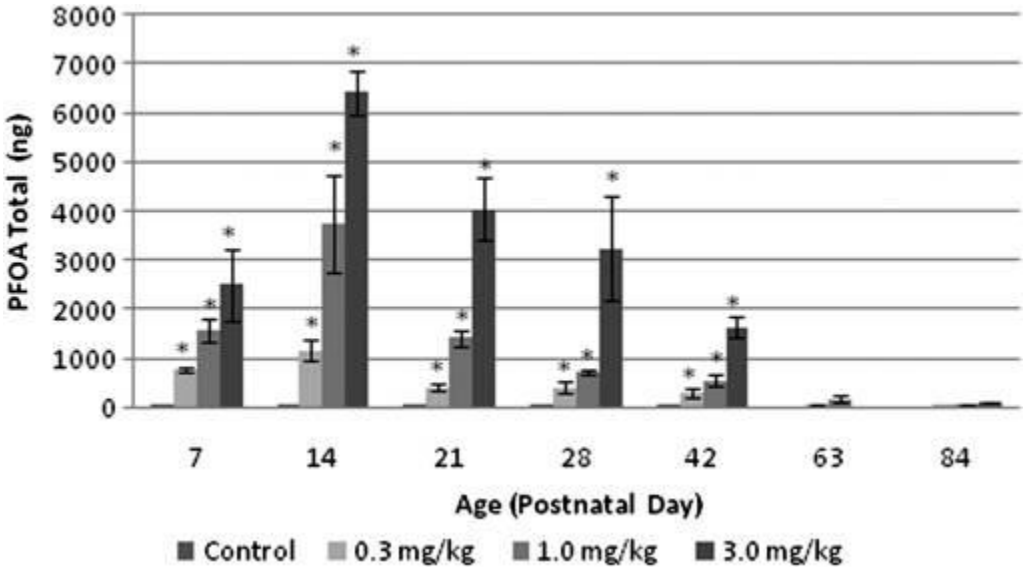

Figure 2-2. Serum PFOA concentration in female offspring following the full gestation exposure. Data presented as the mean ± SEM. Litter n=2-5 (PND7), 4-6(PND 14); individual n=3-6 (PND 21), 4-6 (PND 28), 4-6 (PND 42), 2-5 (PND 63), 1-3 (PND 84). In all PFOA treatment groups mean serum PFOA concentrations were greater at PND 14 than respective treatments means at PND 21, 28, 42, 63, 84, $p<0.001$. *Significant treatment effect compared to controls ($p \leq 0.01$).

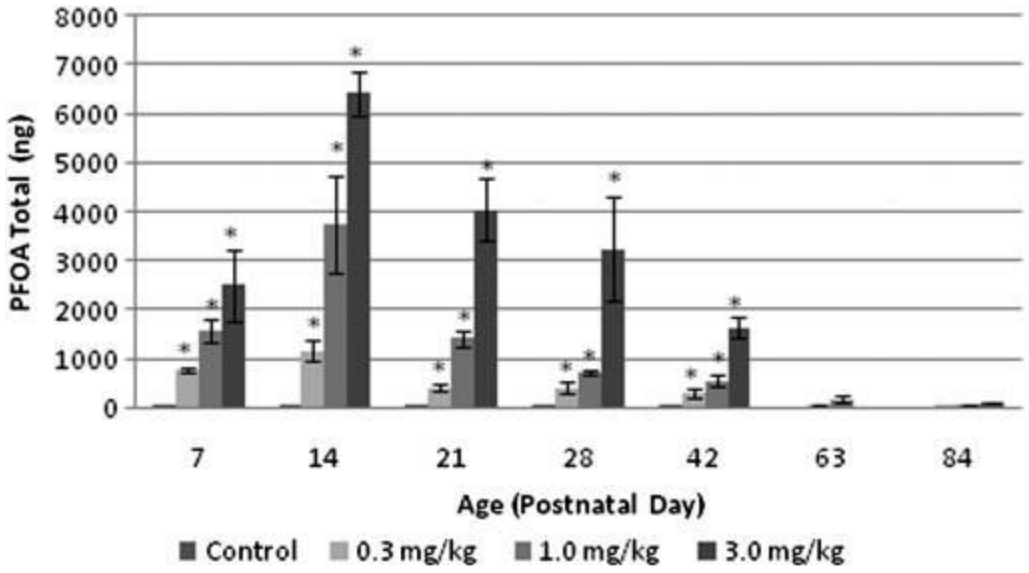

Figure 2-3. Calculated PFOA blood burdens in female offspring following full gestation exposure. Data presented as the mean ± SEM. Calculated blood burden is determined by the equation [BW x (58.5 ml/kg/1000) x Serum x 55%] for litters/individual pups and mean values are presented for each treatment group. Litter n=2-5 (PND7), 4-6(PND 14); individual n=3-6 (PND 21), 4-6 (PND 28), 4-6 (PND 42), 2-5 (PND 63), 1-3 (PND 84). In the 0.3 and 1.0 mg/kg treatment group mean calculated PFOA blood burdens were greater at PND 14 compared to respective treatment mean burdens at PND 21, 28, 42, 63, and 84, $p<0.05$. In the 3.0 mg/kg group mean serum concentration at PND 14 was greater than means at PND 42 and 84, $p\leq0.05$. *Significant treatment effect compared to controls ($p\leq0.01$).

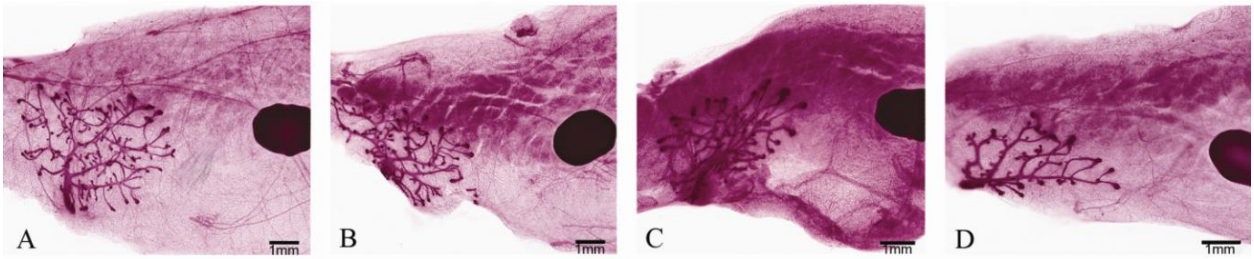

Figure 2-4. Offspring mammary gland whole mounts at PND 21 in the late gestation study from (A) controls, (B) 0.01 mg/kg, (C) 0.1 mg/kg and (D) 1.0 mg/kg. Glands pictured are representative of the mean score for each treatment group; n=4-7. The 0.01 mg/kg representative gland has poor branching patterns; the 0.1 mg/kg representative gland is smaller in size and fewer visible terminal end buds (TEBs) relative to controls; 1.0 mg/kg representative gland has poor branching patterns, few TEBs and small size. All PFOA-treated mammary glands received significantly lower developmental scores compared to controls ($p \leq 0.0004$).

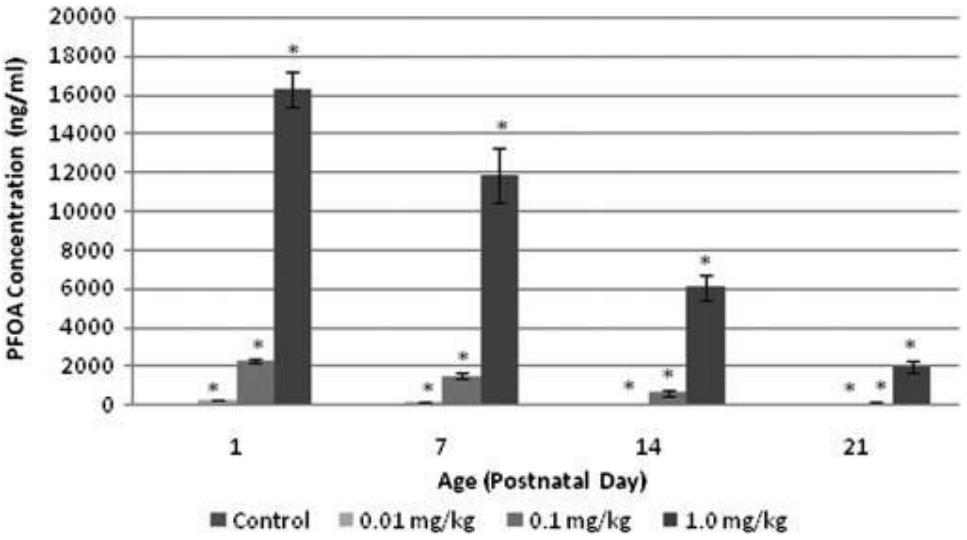

Figure 2-5. Serum PFOA concentration of female offspring from the late gestation study. Data presented as the mean ± SEM. Litter n= 2-7 (PND 1), 2 (PND 4), 5-11 (PND 7), 7-11 (PND 14). Individual pup n=7-11. Mean serum concentration of each PFOA-treated group at PND 1 was greater than respective treatment mean concentration at PND 4, 7, 14, and 21, $p < 0.05$. *Significant treatment effect compared to controls ($p \leq 0.0004$).

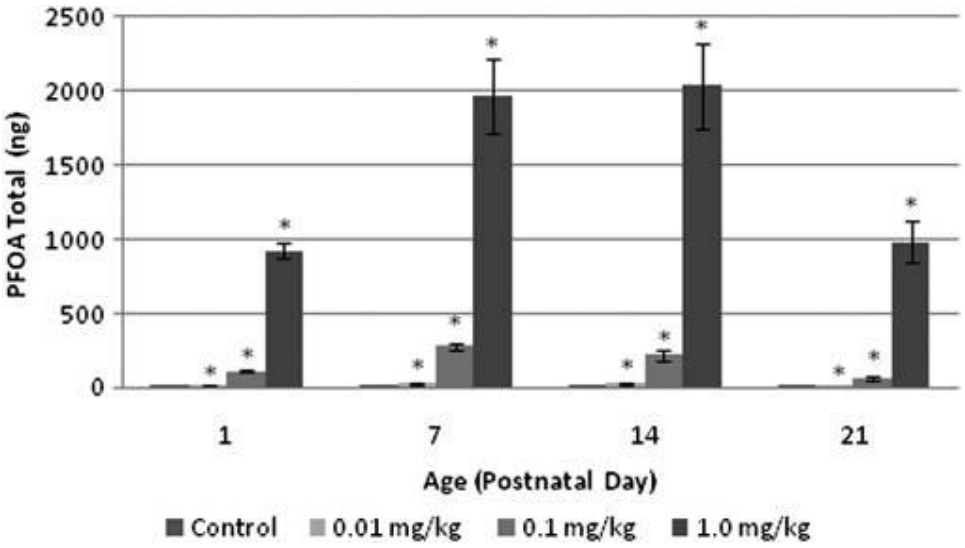

Figure 2-6. Calculated PFOA blood burdens of offspring from the late gestation study. Data presented as the mean ± SEM. Calculated blood burden is determined by the equation [BW x (58.5 ml/kg/1000) x Serum x 55%] for litters/individual pups and mean values are presented for each treatment group. Litter n= 2-7 (PND 1), 2 (PND 4), 5-11 (PND 7), 7-11 (PND 14). Individual pup n=7-11 (PND 21). Mean calculated blood burdens of each PFOA-treated group at PND 21 was lower than respective treatment mean burden at PND 7 and 14, $p<0.02$. *Significant treatment effect compared to controls ($p \leq 0.0008$).

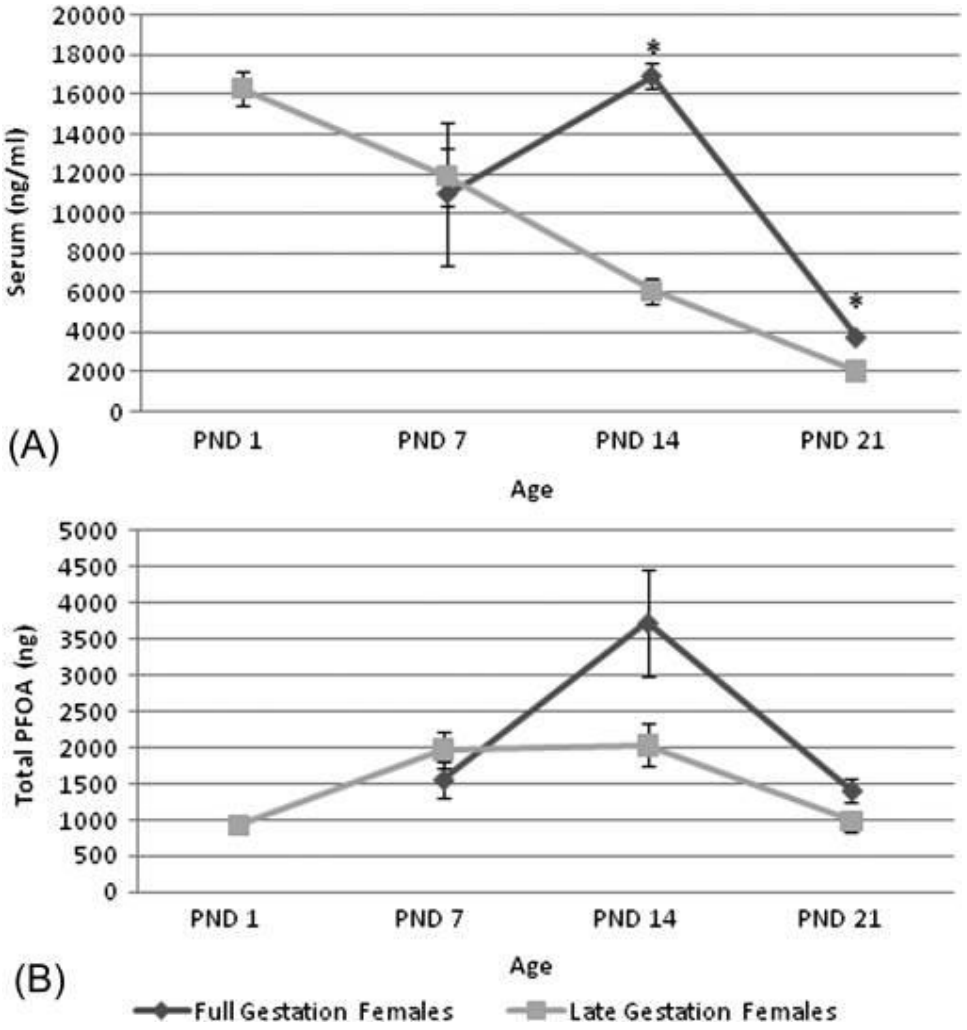

Figure 2-7. Comparison of 1.0 mg/kg treatment group dosimetry in full and late gestation study (A) Serum PFOA concentrations and (B) calculated PFOA blood burdens of female offspring from the 1.0 mg/kg treatment group from the full and late gestation study preweaning. Data presented as the mean ± SEM. Calculated blood burden is determined by the equation [BW x (58.5 ml/kg/1000) x Serum x 55%] for litters/individual pups and mean values are presented for each treatment group. Litter n= 7 (PND 1), 5-11 (PND 7), 6-11 (PND 14). Individual pup n=5-11 (PND 21). *Significant differences by ANOVA ($p \leq$ 0.0008).

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CHAPTER 3

Prenatal PFOA exposure Alters Gene Expression Pathways in Neonate Murine Mammary Glands[3]

OVERVIEW

Perfluorooctanoic acid (PFOA) is a synthetic surfactant that was previously shown to delay mammary gland development in CD-1 mice. The objective of this study was to elucidate the targeted signaling pathways involved in this effect. Timed-pregnant CD-1 mice were treated with 0, 0.01, 0.1, or 1.0 mg/kg/day of PFOA from gestational days (GD) 10-17 via oral gavage. Mammary glands from postnatal days (PND) 7 and 14 from control and 1.0 mg/kg PFOA treated mice were evaluated for gene expression changes via genome-wide microarray analysis to identify candidate gene pathways. Selected genes from candidate pathways were evaluated by real-time PCR (RT-PCR) and Western blot analysis for RNA and protein expression changes, respectively, using samples from all treatment groups from PND 7-21. Microarray analysis revealed that PFOA altered expression of genes involved in RNA post-transcriptional modification, lipid metabolism, and cholesterol biosynthesis. These transcriptional changes were predicted to be regulated by peroxisome proliferator-activated receptor (Ppar) and endocrine related genes. Selected genes from candidate gene pathways Ppar, estrogen receptor alpha (Erα/Esr1), and Wnt were evaluated with RT-PCR. Results from the RT-PCR analysis confirmed that genes in all 3 pathways were altered at varying levels in PFOA-exposed mammary glands. At PND 7, expression of PPARγ and ERα protein were increased in a dose-dependent manner. At PND 21 ERα protein

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expression was down-regulated. PPARα protein levels were not affected. These results suggest that PFOA-induced mammary gland delays are modulated by changes in PPAR and endocrine related genes.

INTRODUCTION

Perfluorooctanoic acid (PFOA) is a synthetic surfactant that is used in a variety of consumables as it ammonium salt, ammonium perfluorooctanoate (APFO). PFOA belongs to a class of chemicals called perfluoroalkyl acids (PFAAs) or perfluorinated compounds (PFCs). It is a fully fluorinated 8-carbon compound that is used to make fluoropolymers which are found in common consumer products including non-stick and stain resistant products, water proof materials, paint and paint thinners, and flame retardants. Fluoropolymers can break-down into PFOA due to overheating and other physiochemical stress (*1*). As a result of its widespread commercial use, heat resistance, chemical stability, emissions and pollution, PFOA has become a persistent and ubiquitous environmental contaminant. Although the production and emissions of PFOA has been greatly reduced in the US (USEPA Stewardship program), PFOA can still be found in the human blood in the US and worldwide.

Humans are exposed to PFOA in contaminated drinking water, household dust, food and food products (*2*). It is also found in breast milk and can be transferred through the placenta during development (*3-6*). PFOA is not readily metabolized and as such has a long half-life (2.5-3.8 years (*7*). The average levels of PFOA in serum are approximately 3.1 ng PFOA/ml (Geometric mean) according to US Centers for Disease Control and Prevention latest report on 2009-2010 samples. Children have higher levels of serum PFOA (6.1 ng/ml)(*8*).

Hazard identification of PFOA has been well characterized from animal laboratory, occupational, and epidemiological studies. In rat 2 year cancer studies, adult PFOA exposure leads to hepatocellular carcinoma, Leydig cell tumors, and pancreatic acinar tumors (*9, 10*). PFOA is hepatotoxic, nephrotoxic, neurotoxic, modulates the immune system, causes endocrine disruption, and is a reproductive and

developmental toxicant (*11-15*). Epidemiological studies have found correlations between PFOA exposure and testicular, prostate, kidney, and ovarian cancer, non-Hodgkin lymphomas, ulcerative colitis, gestational hypertension, high cholesterol, and thyroid disease (*16, 17*).

As a developmental toxicant, PFOA also alters mammary gland development and maturation in animals (*11, 15, 18-21*). It has previously been shown in the Fenton laboratory that prenatal exposure to low-doses of PFOA delays mammary gland growth in CD-1 mice (*22*) following full length or abbreviated *in utero* exposure. Although we and others have described the morphological consequences of PFOA exposure, a mode of action remains to be fully characterized. However, some insights have been gained from studies exploring the effects of PFOA following peri-pubertal exposures that indicate disruption of endocrine related genes and their protein expression (*23, 24*). PFOA-induced liver toxicity in adult rodents has been extensively studied; the majority of the effects are thought to be mediated by activation of peroxisome-proliferator activated receptor alpha (Pparα, (*25, 26*). Nevertheless, a growing body of research finds Pparα-independent effects in the liver (*25-28*). Although PFOA is a weak agonist of Pparα, PFOA-induced developmental effects in mice, such as litter resorption and early postnatal mortality, are dependent on this nuclear receptor (*11*). However, prenatal exposure to PPARα agonists does not result in outcomes observed in PFOA-exposed mice (*29*) suggesting that PFOA-induced developmental effects are mediated by other factors in addition to PPARα activation. While others have investigated PFOA effects in the mammary gland of *Pparα* KO mice, the role of PPARα activation in PFOA-mediated mammary gland effects remains unclear (*24, 30*).

In this study, a systems biology approach was used to determine the major signaling pathways involved in PFOA-induced mammary gland delays following prenatal exposure. Mammary tissues from our late gestation exposure study (*22*) were analyzed to characterize the major molecular pathways involved in developmental mammary effects. Mammary tissues from the abbreviated *in utero* exposure

study were used to minimize influence of overt developmental effects, such as body weight, and to focus on the MOA of PFOA-induced mammary toxicity.

MATERIALS AND METHODS

Animals

Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). Pregnant dams were housed individually in polypropylene cages and received chow chow (LabDiet 5001, PMI Nutrition International LLC, Brentwood, MO) and tap water ad libitum; both were known to contain PFOA below the levels of detection. Animal facilities were maintained on a 12:12 h light-dark cycle at 20°C–23°C and 40–50% relative humidity. Animal protocols were approved by USEPA NHEERL Animal Care and Use Committee.

Chemicals

PFOA (PFOA as its ammonium salt APFO, > 98% pure) was purchased from Fluka Chemical (Steinhiem, Switzerland); the PFOA lot number was identical to previous studies (*18, 19*). PFOA dosing solutions were prepared fresh daily in deionized water, agitated immediately prior to administration, and were given at a volume of 10-µl solution/g body weight (BW).

Study Design

Thirty-two time-pregnant CD-1 mice arrived at the animal facility on gestation day (GD) 9 and were divided into four treatment groups of equal size (n=8/treatment). Each dam was orally gavaged with deionized water (vehicle) or 0.01, 0.1, or 1.0 mg PFOA/ kg BW daily. A schematic of the study design is illustrated in Figure 3-1. Approximately 15% of the dams were not pregnant, unrelated to PFOA exposure. To be consistent with previous work (*18*), on postnatal day (PND) 1, pups within a treatment group were pooled and randomly distributed among the dams of their respective treatment group, resulting in a final litter size of 7–9 pups per dam, with an unequal sex ratio (n= 4–7 females per litter).

Due to the short acclimation period (1 day), dams were observed by the veterinary staff and study personnel for signs of maternal stress, such as lack of weight gain or aggressiveness; none were observed. On PND 4, 7, 14 and 21, female pups from at least 5 litters per treatment group were weighed and necropsied. A set of fourth and fifth inguinal mammary glands were removed, weighed, and collected. Mammary glands for microarray analysis were placed in TRI Reagent (Sigma-Aldrich, St. Louis, MO) on dry ice and stored at -80°C; mammary glands for RNA or protein analysis were wrapped in foil and immediately placed on dry ice and stored at -80°C.

Microarray Analysis

Total RNA was extracted from homogenized mammary samples in TRI Reagent with chloroform and isolated using Qiagen RNeasy Mini Kits (Qiagen; Hilen, Germany) according to manufacturer's instructions. Total RNA was quantified using NanoDrop 2000c (ThermoScientific, Wilmington, DE). RNA integrity was determined using the RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Only samples with RNA Integrity Number (RIN) ≥8.0 were used for microarray analysis. A total of 16 mammary samples from control and 1.0 mg/kg treated mice at PND 7 and 14 (4/group; 8/time-point) were used for the microarray analysis. The samples were processed at EPA's Genomic Research Core facilities (RTP, NC) and analyzed on Affymetrix GeneChip Mouse Genome 430.2 Arrays (Affymetrix; Santa Clara, CA). Samples were prepared, normalized, and analyzed as described in Rosen *et al*., 2008 (*25*). Briefly, Affymetrix microarray gene expression data (.cel files) was analyzed and statistically filtered using Rosetta Resolver version 7.0 software (Rosetta Inpharmatics, Kirkland, WA). Statistically significant genes were identified using one-way ANOVA with a false discovery rate (Benjamini–Hochberg test) of 0.05 followed by a post hoc test (Scheffe) for significance. Genes that were statistically altered were then analyzed for gene expression changes using Ingenuity Pathway Analysis (http://www.ingenuity.com/products/ipa).

Real Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from frozen samples by homogenization using MP Lysing Matrix D tubes and the FastPrep® -24 Instrument (MP Biomedicals, Irvine, CA) and trizol/chloroform extraction method. Briefly, samples were transferred to Matrix D tubes, placed in the FastPrep, homogenized for 30 seconds at a speed setting of 6, placed on ice for 5 min; the process was repeated twice. 100-200 µl of chloroform was added to homogenized samples, vortexed for 15 sec, and incubated at RT for 10 min, centrifuged at 14,000g at room temperature (RT) for 15 min. The upper aqueous phase was transferred to a new microcentrifuge tube then incubated at RT for 5 min. An equal volume of 70% ethanol was added and total RNA was extracted using RNeasy Mini kits with the addition of RNase free DNase (Qiagen). Samples were quantified on a NanoDrop 2000c (ThermoScientific) and with the Ribogreen Quantitation Kit (Invitrogen, Carlsbad, CA; R11490) according to the manufacturer's instructions. Taqman (Applied Biosystems, Branchburg, NJ) assays were purchased for Esr1, Errγ, Gpr30, Pgr, and Tbp; all other gene primers were designed in Primer Express 3.0 (Applied Biosystems). Sequence of primers can be found in Table 3-1. Approximately 2 µg of RNA was reverse transcribed to yield cDNA using Superscript III Reverse Transcriptase (Invitrogen) and random hexamers. 25 ng of cDNA of each sample was run in duplicate on a 7900HT Fast Real Time PCR System and measured with SDS Software (Applied Biosystems). (*n*=2-6). Taqman Universal PCR Master Mix (Applied Biosystems) was used for Esr1, Errγ, Gpr30, and Pgr; Power SYBR Green PCR Master Mix (Applied Biosystems) was used for all other genes. The relative amount of each transcript was normalized to the amount of TATA Box binding protein (Tbp) transcript in the same sample and calculated using SDS Relative Quantification Software (version 2.4, Applied Biosystems). Values were included in analysis if the standard error for the duplicates were ≤0.5. The relative amount of each transcript was normalized to the amount of Tbp transcript in the same sample. Fold change was calculated using $2^{-\Delta ct}$; the results are expressed as the ratio of the mean values $2^{-\Delta ct}$ of treated samples over mean values $2^{-\Delta ct}$ of controls as described in Livak and Schmittgen (*31*). Heat

maps of RT-PCR data were created in Cluster 3.0 and visualized with Java Treeview (http://rana.lbl.gov/EisenSoftware.htm) to compare expression changes in several genes over dose and time.

Western Blotting

Frozen tissues were placed in 4 µl of Radio-Immunoprecipitation Assay (RIPA) lysis buffer/mg of tissue containing proteinase inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF). Tissues were homogenized using a BioSpec Tissue Tearor (Bartlesville, OK) and quantified using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Cell lysates containing ~40 µg of protein was loaded onto a Bio-Rad 10% mini-PROTEAN TGX gel (Hercules, CA), transferred to nitrocellulose membranes (Bio-Rad), blocked in milk for 1 hr, probed with appropriate rabbit polyclonal primary antibody overnight at 4°C, incubated with secondary antibody for 1 hr at room temperatrue, developed by SuperSignal West Pico Chemiluminescence Substrates (Thermo Scientific, Rockford, IL), and visualized and analyzed using Carestream software. Images were captured using a Kodak 4000MM image station (Carestream Molecular Imaging), and net band intensities (mean pixel intensity by number of pixels) were determined using MI imaging software (Carestream Molecular Imaging). Net band intensities were normalized to those for the loading controls, and fold changes in protein levels were determined by comparison to the level for the untreated controls. All rabbit polycolonal primary antibodies, positive controls, and the loading control were purchased from Santa Cruz: PPARα H-98 (sc-9000); PPARβ K-20 (sc-1987); PPARγ H-100 (sc-7196); ERα MC-20 (sc-542); Hep G2 cell lysates (sc-2227); Jurkat nuclear extract (sc-2132); U-937 cell lysates (sc-2239); MCF7 cell lysates (sc-2006); vinculin H-300 (sc-5573). Goat anti rabbit HRP secondary antibody was purchased from KLP (074-1506; Gaithersburg, MD,). Dilutions for each antibody can be found in Table 3-2.

Statistical Significance

Microarray data was analyzed for statistical significance as described above. For all measurements, data are reported as the mean ± SEM. For PCR and Western blot analysis, differences between PFOA-treated groups and controls were determined using two-tailed paired, Student's t-tests with a $p \leq 0.05$ used to indicate statistical significance.

RESULTS

Prenatal PFOA exposure leads to transcriptional changes in metabolism, endocrine system, and Ppar

To determine candidate pathways involved in prenatal PFOA-induced mammary gland effects, genome-wide microarray analysis was conducted on mammary glands of control and PFOA treated mice (1.0 mg/kg PFOA) collected on PND 7 and 14 to identify candidate signaling pathways that may mediate the morphological effects. More than 39,000 transcripts and variants of 34,000 genes were analyzed. Initial comparison of controls glands compared to 1.0 mg/kg glands revealed PFOA altered 591 genes at PND 7 and 34 genes at PND 14. A list of genes altered can be found in Supplementary Files. A Venn diagram of PFOA altered genes is illustrated in Figure 3-4A. There were only 6 genes that were altered following PFOA exposure at both PND 7 and 14 and they are listed in Table 3-3. As the majority of gene changes occurred at PND 7, the majority of the results describe the characteristics of the genes altered at that time. A comparison of PND 14 glands to PND 7 glands found 2,975 genes altered over time in controls and 3,222 genes altered in glands of PFOA-treated mice over time (Figure 3-2B). Principal component analysis of the gene expression changes are illustrated in Figure 3-2C.

Ingenuity Pathway Analysis (IPA) analyzed altered genes that were statistically significant and clustered genes based on disease, biological function, and canonical pathways and predicted whether groupings were inhibited or activated based on down- or up-regulation of genes in that cluster. Altered genes were grouped into networks based on p-values associated with those groupings. At PND 7, the Top Network contained 49 genes and was associated with RNA post-transcriptional modification,

cancer, and endocrine system disorders. RNA post-transcriptional modification was also the top Molecular and Cellular Function, followed by lipid metabolism. The Top 5 Molecular and Cellular Functions are listed in Table 3-4 for both PND 7 and 14. Cholesterol biosynthesis was the Top Tox Lists at PND 7 and 14 with the majority of genes up-regulated by PFOA exposure at PND 7 and down-regulated at PND 14 suggesting that there may have been a negative feedback response for this function. Selected Top Tox Lists for PND 7 can be found in Figure 3-3. To focus on clusters related to the mammary gland, lists that were specific for other organs (i.e. cardiac hypertrophy) were removed from analysis. Notably, 4 of the 10 lists altered by PFOA involved mitochondrial damage, a pathology found in PFOA-treated liver tissues in our lab (*32*) and previously by others (*26*).

To focus our efforts, 3 candidate gene pathways were identified from array analysis: Peroxisome proliferator activated receptor (*Ppar*), *Wnt*, and estrogen receptor alpha (*Erα/Esr1*) and related genes. Lipid metabolism and cholesterol biosynthesis are regulated by *Ppars*. *Pparα-Rxrα* Activation was one of the Canonical Pathways altered in PFOA-treated glands at PND 7; evaluation of the pathway suggested that *Pparα* was inhibited (Figure 3-4). However, the PFOA-induced genes altered in this pathway (*Lpl*, *Fatp*) are also known to be altered by all *Ppar* subtypes. Further evaluation of array data indicated that specific Ppar subtypes were altered: aquaporin 7 (*Aqp7*), a *Pparγ* specific target, was down-regulated (-1.46) in controls over time but not in PFOA-exposed glands; hypoxia inducible factor 1 alpha (*Hif1α*), a *Pparβδ* specific target, was up-regulated (1.25) in PFOA-exposed glands compared to controls at PND 7. In addition to the *Ppar* family gene changes, *Wnt* inhibitory factor (*Wif1*) was up-regulated (15.40) suggesting that the *Wnt* pathway was down regulated. *Esr1* was down-regulated (-1.51) and *ErbB*2 interacting protein (*ErbB2ip*) was up-regulated (1.30).

One of the relatively new features of IPA predicts upstream regulators based on changes in gene expression and whether regulators are activated or inhibited based on an activation z-score. Selected predicted regulators are listed in Table 3-5. Based on p-values, *Pparα*, *Pparβδ*, *Pparγ*, *Myc*,

dihydroxytestosterone, and beta-estradiol were predicted to be upstream regulators of PFOA-induced gene expression changes at PND 7. *Pparα* and *Pparγ* were predicted to be inhibited due to negative activation scores (-0.08, -0.78, respectively); *Pparβδ*, *Myc*, dihydroxytestosterone, beta-estradiol were predicted to be activated due to positive activation scores (1.54, 1.98, 0.61, 3.33, respectively). Chemical drugs that act through *Pparα* and *Pparγ* were also predicted to be inhibited (ciprofibrate, -1.80; plioglitazone, -2.91; rosiglitazone, -1.32; troglitazone, -1.74) providing further evidence that genes associated with both *Pparα* and *Pparγ* were inhibited by PFOA exposure in mammary glands. Glucocorticoid receptor (*Nr3c1*), Retinoid X receptor alpha (*Rxrα*), Liver X receptor -alpha and –beta (*Lxr*; *Nr1h3, Nr1h2*), and pregnane X receptor (*Pxr*; *Nr1i2*) were also predicted upstream regulators (-1.86, -0.02, 0.05, 0.94, 1.94, respectively). Glucocorticoid receptors are important in regulation of inflammation and *Ppars* form heterodimers with *Rxr* then translocate to the nucleus and bind to peroxisome proliferator response elements (Ppres). *Rxr* also forms heterodimers with *Lxr* and *Pxr* to increase transcriptional activity. Like *Ppars*, activation of *Lxr* is also associated with lipid metabolism; activation of *Pxr* is associated with metabolism of xenobiotics. Expression of *Lxr* is inversely associated with *Ppar* and thus positive activation scores for *Lxr* also suggest *Ppars* are inhibited in PFOA-treated mammary glands (*33*). Pathways of predicted upstream regulator connections for *Pparα* and *Pparγ* are shown in Figure 3-5 which illustrates the connections of the genes described. Transforming growth factor beta (*Tgfβ*) 1-3, which can act to inhibit both *Ppar* and *Wnt* signaling, were all predicted to be activated. Ccaatt enhancer binding protein beta (*Cebpβ*), a target in the *Wnt*/Beta catenin pathway and a regulator of *Pparγ* (*34*), was the only predicted upstream regulator at PND 14 with an activation score (0.61); *Cebpβ* was also a predicted upstream regulator at PND 7 (activation score 1.75). The predicted regulators correlated with our original candidate gene pathways, with the addition of dihydroxytestosterone.

As mentioned earlier, we also analyzed gene alterations that occurred over time in controls and changes over time in PFOA treated glands. Array analysis of gene alterations in PFOA-exposed glands over time were similar to that of changes in controls, however direction in gene expression differed for Sterol regulatory element binding transcription factor 1 (*Srebf1*, -3.35 versus (vs) 1.36 in PFOA treated vs controls, respectively), Sterol regulatory element binding protein chaperone (*Scap*, -2.72 vs 1.84), *p53* (2.02 vs -0.37), and others (not shown).

Real time RT-PCR of selected genes in Ppar, Wnt and Erα pathways

In an attempt to validate the microarray analysis, selected genes from candidate gene pathways were analyzed using real time reverse transcriptase polymerase chain reaction (RT-PCR) of mammary samples from all treatment groups collected at PND 7-21. Heat maps of the RT-PCR results expressed as fold change relative to controls are illustrated in Figure 3-6 and listed in Table 3-6. Genes from the *Ppar* gene pathway were selected to evaluate this candidate pathway including both gene targets of *Ppar* and genes that modify *Ppar* signaling. Of note, many *Ppar* target genes can also modify transcriptional activity of *Ppars*. In agreement with the microarray analysis, the majority of PFOA-induced gene expression changes occurred at PND 7 then tapered off with increasing age (Figure 3-6). Adiponectin (*Adip*), an adipocyte cytokine, and its receptor (*Adipr*) were significantly increased at PND 7 by RT-PCR; *Adip* was reduced in arrays (-1.20). *Adipr* was the only gene in this pathway that was statistically altered at PND 21. Gene expression of fatty acid binding protein 3 (*Fabp3*), also known as mammary derived growth inhibitor, and peroxisome proliferator activated receptor gamma co-activator 1 (*Pparγcoa1*), a co-activator of all *Ppars*, was significantly reduced at PND 7. At PND 14, *Pparα* was reduced along with uncoupling protein 1 (*Ucp1*), a mitochondrial protein involved in thermogenesis. As the majority of the target genes in the *Ppar* pathway were reduced (Table 3-4), RT-PCR data suggested that *Ppars* were inhibited at the transcriptional level following prenatal PFOA exposure. RT-PCR of *Aqp7*, a downstream

target of *Pparγ*, was also evaluated and found to be significantly reduced at PND 7 which further substantiates that Pparγ transcriptional activity was inhibited by PFOA exposure.

Selected genes in the canonical *Wnt*/β-catenin pathway were examined for gene expression changes by RT-PCR. As with the evaluated genes in the *Ppa*r pathway, most of the gene expression changes in the *Wnt*/β-catenin pathway occurred at PND 7 (Figure 3-6, Table 3-6). Surprisingly, *Wnt2* was increased at PND 7 while Wif1 was not statistically altered at any time point evaluated; this did not agree with the array data where Wif1 was up-regulated by >15 fold. *Tgfβ3*r and β-catenin (*Ctnnβ1)* were reduced at PND 7; *Tgfβ3r* was down-regulated in arrays (-1.2). Lymphoid-binding enhancing factor (*Lef1*), which forms a complex with T-cell factor and *Ctnn*β1 to increase transcriptional activity, was increased at PND 7, suggesting that the pathway was activated (*35*). As *Ctnnβ1* is degraded when unbound to the *Lef/Tcf* co-activator complex, reductions in *Ctnnβ1* suggest that the protein was degraded and *Wnt* canonical pathway was not activated (*34*), providing a conflicting finding. Increased expression in frizzled receptor 2 (*Frzd2*) and *Tgfβ3* at PND 14 did not provide any further insight into this pathway. We did not explore the possibilities of the non-canonical *Wnt* pathways through RT-PCR analysis although array analysis suggests *Wnt* may have been activated in this manner. Further experiments are required to investigate the role of this candidate gene pathway in PFOA induced mammary changes.

As with *Ppar* and *Wnt* pathways, many gene expression changes occurred early in life at PND 7 for *Erα/Esr1* and endocrine related genes (Figure 3-6, Table 3-6). In agreement with microarray data, *Esr1* expression was reduced at PND 7. Progesterone receptor (*Pgr*) was increased at PND 7 along with hydroxysteroid dehydrogenase 11 beta 1 (*Hsd11β1*), one of the 6 genes altered at both PND 7 and 14 in the arrays. *Hsd11b1* is an enzyme that catalyzes the conversion of cortisol to cortisone and other steroids (*36*). In addition, *Hsd11β1* has been shown to regulate and be regulated by *Ppar* (*37, 38*). G coupled protein receptor 30 (*Gpr30*), a regulator of non-genomic *Erα* activation, was increased at PND

21 along with *Erα/Esr1* suggesting peri-pubertal activation of this signaling network. Amphiregulin (*Areg*), a growth factor ligand that binds to epidermal growth factor receptor (*Egfr*) and regulates *Erα* (*39*), was reduced at PND 21 by PFOA. *Erbb2*, androgen receptor (*Ar*), and *Pgr* gene expression was reduced in PFOA glands compared to controls, albeit non-significantly. Collectively, these data suggest that PFOA alters transcriptional activity of critical genes in the mammary specific paracrine hormones.

Microarray and RT-PCR results from this study are in accord with some RT-PCR data from mammary glands of CD-1 mice exposed to higher levels of PFOA (5 mg/kg) from GD 1-17 (*40*). A heat map of that data was created (Figure 3-7). White (68) also observed decreases in *Pparα* (PND 10) and non-significant changes in *Pparγ*; signal transducer and activator of transcription 5A (*Stat5A*) was reduced (PND 10) which matched our microarray results. These findings provide evidence that the gene expression changes are common to prenatal PFOA-induced changes to the mammary gland.

PFOA alters PPARγ and ERα protein levels in mammary glands

To determine whether PFOA-mediated gene expression changes translated to corresponding protein levels, Western blots of mammary whole cell lysates were evaluated. We decided to focus our analysis on the nuclear receptors PPARα, -βδ, γ, and ERα. Samples were selected to include mammary tissues collected from animals evaluated by microarray and/or RT-PCR analysis. Levels of the protein of interest were compared to vinculin for normalization. Vinculin was tested and showed to vary little between samples.

At PND 7, PPARγ protein levels were significantly increased by prenatal exposure in a dose-dependent manner for the two highest dose groups (0.1 and 1.0 mg/kg, Figure 3-8A). PPARγ levels were increased by 2.3 fold over controls in the 0.1 group (n=3) and 3.4 fold over controls in the 1.0 mg/kg group (n=3). Protein levels of PPARα and βδ also increased dose-dependently following prenatal PFOA exposure but did not reach statistical significance. Increases in PPARγ protein expression were not expected as array analysis and PCR data implicated inhibition of Pparγ. At PND 7, ERα protein levels

were also significantly increased in a dose dependent manner for the 2 highest dose groups. ERα levels were increased 1.8 fold over controls in both 0.1 and 1.0 mg/kg PFOA treated animals (n=3/treatment, Figure 3-8A). At PND 21, ERα protein levels were significantly reduced compared to controls in the 0.01 (-2.1 fold) and 0.1 mg/kg (-1.3) treatment groups (Figure 3-8B). Protein levels of ERα were increased at a time when gene transcripts were reduced and reduced when gene transcripts were increased. These data suggest PFOA alters translation of these proteins, a process that may have been a consequence of changes in genes associated with RNA post-transcriptional modification, which were not evaluated in these studies, but deserve further attention.

DISCUSSION

Low-dose prenatal PFOA exposure during the time of mammary bud development leads to morphological delays in the mammary gland characterized by reductions in epithelial longitudinal growth and number of terminal end buds which reflect the significant reductions in developmental scores Macon, Villanueva, Tatum-Gibbs, Zehr, Strynar, Stanko, White, Helfant and Fenton (*22*). In this study, we used mammary samples from the late gestation exposure study to characterize the molecular mechanisms underlying PFOA-induced delays. We identified *Ppar*, *Wnt*, and *Erα* and endocrine related genes as candidate gene pathways and determined expression changes using RT-PCR and Western blots. Notably, PFOA exposure statistically altered RNA and protein expression in all PFOA treatment groups for *Ppar*, *Wnt*, and endocrine related genes.

Microarray analysis of mammary glands in 1.0 mg/kg treatment group compared to controls identified changes in RNA post-transcriptional modification, lipid metabolism, and cholesterol biosynthesis as top networks and functions altered by PFOA. Gene changes in RNA post-transcriptional modification tended to be inhibited and may explain the dramatic decrease in the number of genes altered at PND 14 from arrays and selected genes evaluated by RT-PCR at PND 14 and 21. There was also

a great deal of variability in expression of genes within a treatment group that appeared to increase with age as large fold changes in expression were observed without statistical significance. In addition, reduction in RNA post-transcriptional modifications may also account for differences observed in protein and RNA expression of PPARγ and ERα. Consequently this data suggests RNA analysis, whether by microarray of RT-PCR, may not effectively describe molecular changes caused by PFOA.

Ironically, many of the genes that were altered at a greater magnitude in the microarray analysis were not found to be significantly altered in PCR analysis. For example, gap junction protein beta 2 (connexin 26, *cx26*) was up-regulated >30 fold at PND 7 in the array but was significantly down regulated in PCR analysis at PND 14 (data not shown). In addition, Wnt inhibitory factor 1 (*Wif1*) was up-regulated >15 fold in the array but was also not significantly altered through PCR analysis. It is possible that the fold changes in these genes were driven by the outlying samples in microarray analysis which were apparent from principal component analysis (Figure 3-2C). Although differences in RNA expression analysis differed from arrays to RT-PCR, the majority of gene expression changes were the same, at least for the selected genes that were evaluated.

Alterations in genes involved in lipid metabolism and cholesterol biosynthesis are more commonly associated with PFOA exposure and/or P*par* activation. Majority of array and RT-PCR data agreed with RT-PCR results from mammary glands of CD-1 mice prenatally exposed to 5 mg/kg PFOA (Figures 3-6, 3-7). Microarray data suggested *Pparα* and γ were inhibited following PFOA exposure in mammary glands, yet only *Pparα* RNA was significantly decreased (PND 14) from samples analyzed. Protein levels of PPARγ were unexpectedly increased at PND 7. Notably, PPARγ (also PPARα and βδ) bands appeared slightly higher than positive controls (>54kD), suggesting that PPARγ protein underwent post-transcriptional modification, such as phosphorylation. Increases in PPARγ phosphorylation have been reported to inhibit the nuclear receptors activity while PPARα phosphorylation generally increases transcriptional activity (*41*). If PFOA increased PPARγ phosphorylation thereby inhibiting its

transcriptional activity, this would explain observed decreases in the majority of *Pparγ* mediated genes in the array and RT-PCR analysis. In addition, we did not find changes in PPARα protein levels at any time-point. Protein levels of PPARα also remained unchanged following peri-pubertal PFOA exposures in mammary glands (*24*), suggesting that these affects in the mammary gland may be mediated by *Pparγ* rather than *Pparα*. PFOA has been shown to be a weak agonist of both *Pparα* and *Pparγ* (*33, 42*). In the liver, where *Pparα* expression far exceeds that of –γ, it has been shown that *Pparγ* target genes were altered following PFOA exposure (*25*) and that exposure affects RNA and protein levels (*43*). In the mammary gland, where *Pparγ* expression is more abundant than -α, it is likely that PFOA mediates the majority of effects via modulation of *Pparγ* transcriptional activity while a small subset is dependent on *Pparα*. In future studies, we will investigate the transcriptional activity of *Pparα* and γ through RNA analysis of target genes and protein analysis of phospho-proteins beyond the beginning of puberty.

Alterations in endocrine related genes were also found from our analyses. This was anticipated as others have found changes in timing of puberty (*14, 23, 24, 44*), other endocrine responsive tissues (*23, 24, 45*), as well as the mammary gland. Gestational exposure to PFOA increased protein levels of epidermal growth factor (EGF) in dams (*18*). Peri-pubertal exposure to PFOA resulted in reduced protein levels of AREG, EGFR, and ERα in Balb/C and C57Bl6 mice when morphological delays were observed (*24*) and increased when stimulatory effects were observed in C57Bl6 mice(*23*). Taken together, these data indicate that PFOA mediates effects through regulation of a similar set of genes regardless of timing of exposure or strain.

In addition, several enzymes involved in steroidogenesis were found to be altered by PFOA as determined by array or RT-PCR. As stated earlier, *Hsd11β1* was one of the few genes in the array that was statistically altered by PFOA at both PND 7 and 14; it was up-regulated in the array at PND 7 and 14 (4.39 and 3.10 respectively) and in RT-PCR analysis was up-regulated at PND 7 and down-regulated at PND 14 (Table 3-6). Elevated *Hsd11β1* may shift the initial conversion of cholesterol to steroids. In

addition, hydroxysteroid dehydrogenase 17 beta 11 and 12 (*Hsd17β11*, *Hsd17β12*) were down-regulated (-1.28) and unregulated (1.21) in arrays. *Hsd11b1* can also be regulated by *CEBPalpha/beta* (*46*). Collectively, this data suggest that changes in cholesterol biosynthesis following PFOA exposure may occur by altering catalytic enzymes ultimately leading to changes in steroid hormone production and steroid hormone receptor expression.

Wnt was another candidate gene pathway that was attempted to be validated by RT-PCR and Western blotting. Selected genes in the canonical *Wnt*/beta-catenin pathway were investigated. Significant changes in *Wif1* were not found from RT-PCR RNA analysis, although *Ctnnβ1* was down-regulated (-1.49 fold, PND 7). However *Myc*, a down-stream target of *Wnt*/beta-catenin pathway, was predicted to be an activated upstream regulator at PND 7 suggesting that this canonical pathway was activated. *Wnt* may also be activated through non-canonical means involving calcium (Ca^{+2}) and or cell polarity. Ca^{+2} signaling was the top listed canonical pathway altered at PND 7 in the arrays supporting the potential for activation of *Wnt* transcriptional activity. It is possible that *Wnt* may not be an important candidate pathway in PFOA-induced mammary effects, but further investigation is needed.

Interestingly, of the selected Top 10 Tox Lists altered by PFOA exposure in arrays, four were related to mitochondrial damage. In a recent study in our laboratory, we observed marked PFOA-induced morphological changes in mouse liver mitochondria which were evaluated by electron microscopy (*32*). Taken together, it is possible that PFOA may elicit effects in the mammary gland, and other tissues, through increased aberrant mitochondrial based modifications. Increased dysfunctional mitochondrion has been proposed to mediate aggressiveness of cancer cells (*47*).

In conclusion, results from this study provide evidence that prenatal PFOA exposure alters mammary morphology by modulating lipid metabolism and endocrine signaling via increased protein expression of PPARγ and modulation of ERα RNA and protein expression. Whether these early gene and protein changes persist beyond PND 21 in CD-1 mice deserves further investigation.

Table 3-1. Gene Primers for all studies

Gene	Forward Primer	Reverse Primer
Pparα	TGGCAAAAGGCAAGGAAG	CCCTCTACATAGAACTGCAAGGTTT
Pparβδ	CCGCCCTACAACGAGATCA	GCTCTCGGACTGTCTCCACTGT
Pparγ	CCCACCAACTTCGGAATCAG	AATGCTGGAGAAATCAACTGTGGTA
PparγCoa1	GATGACAGTGAAGATGAAAGTGATAAACT	GAAGGCGACACATCGAACAA
Fabp3	CCCCTCAGCTCAGCACCAT	GAAAATCCCAACCCAAGAATG
Ucp1	GGAGGTGTGGCAGTGTTCATT	TGGGCTTGCATTCTGACCTT
Adip	GACACCAAAAGGGCTCAGGAT	TGGGCAGGATTAAGAGGAACA
Adipr	ATAACGGGCCATCCATTTTTG	TGAAGCCTGGACGTACTTCCA
Aqp7	CTGGATGAGGCATTCGTGACT	GGCTCGGTCCCTTGAAGTG
Insr	GGTCTGATTGTGCTATATGAAGTGAGC	CGGACTCGAACACTGTAGTTTCC
Irs1	CGAGAGCTGTTTCAACATCAACA	CGCGGCAATGGCAA
Wnt2	GGCTCCTGTACTCGAGGACATG	GAGATAGTCGCCTGTTTTCCTGAA
Wif1	AAGCAAGTGTAAGTGCCCGAAA	CTCTCGACTGGCACTTGTTG
Frzd2	GCCTGTGGAAGCTGTTGGAT	GCGAGGAGAAGGGAAATAAAAC
Acvr1	CCCCACGGGAAGCTCAA	TGCAGCCGATATTGCTGATTA
Tgfβ3	TGTGTACGCCCCCTTTATATTGA	GGTTCGTGGACCATTTCC
Tgfβ3r	TAAGCGAAGGGATTATTAGCAAGGTA	CCAATGTGCTGGGTGTTCTG
Ctnnβ1	GGGCAACCCTGAGGAAGAA	AAAGCCTTGCTCCCATTCATAAA
Lef1	TCCCGCACTCAGTCTTCCA	AGCATCCGAGACAGCAAGAA
Ar	GGATGGGCTGAAAATCAAAA	TGAGCAGGATGTGGGATTCTT
ErbB2	AATCAACGAAGGCGACAGAA	CCGCATCTGAGCCTGGTT
Hsd11β1	GGGAAAATGACCCAGCCTATG	GGTGGAAAGAACCCATCCA
Hsd17β11	GACGAACAGGAGTGCGAACA	ATTGGTGCTTGGGTTCTTGATG
Hsd17β12	GCTGCCTGGCATGGTAGAA	CAACAATGGAACTGGGAGCAT
Tbp	CAGCCTTCCACCTTATGCTC	TGCTGCTGTCTTTGTTGCTC
Taqman Primers		
Gene		
Esr1	Mm00433149	
Errγ	Mm00516267	
Gpr30	Mm02620446	
Pgr	Mm00435628	
Tbp	Mm00446971	

Table 3-2. Western Blotting Antibody Dilutions

Primary Antibody	Primary Dilution	Secondary Dilution
PPARα	1:200	1:5,000
PPARβδ	1:200	1:5,000
PPARγ	1:100	1:5,000
ERα	1:750	1:20,000

Table 3-3. Genes that were altered by PFOA at both PND 7 and 14.

Gene	Name	Fold Change PND 7	PND 14
Bnc1	basonuclin 1	21.046	28.502
Hmgcs1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	1.446	-1.209
Hsd11ß1	hydroxysteroid (11-beta) dehydrogenase 1	4.388	3.104
LgalS7/LgalS7b	lectin, galactoside-binding, soluble, 7	76.664	68.439
Lypd3	LY6/PLAUR domain containing 3	27.306	44.784
Serpinb5	serpin peptidase inhibitor, clade B (ovalbumin), member 5	10.567	18.786

Note: Common genes that differentially expression in PFOA-exposed glands at PND 7 and 14..

Table 3-4. Top Molecular and Cellular Functions from Arrays

PND 7		PND 14	
Name	# Genes	Name	# Genes
RNA Post-Transcriptional Modifications	33	Amino Acid Metabolism	2
Lipid Metabolism	68	Molecular Transport	6
Small Molecule Biochemistry	101	Small Molecule Biochemistry	4
Vitamin and Mineral Metabolism	42	Carbohydrate Metabolism	2
Cellular Assembly and Organization	57	Cell Death and Survival	4

Note: The top molecular and cellular functions from arrays based on *p* values for each grouping. Note the dramatic decrease in gene number at PND 14.

Table 3-5. Selective PND 7 Predicted Upstream Regulators

Regulator	Molecule Type	z-score	p value
Myod1	transcription regulator	1.78	1.81E-10
Pparα	ligand-dependent nuclear receptor	-0.08	2.56E-09
Myc	transcription regulator	1.983	9.44E-09
dihydrotestosterone	chemical - endogenous mammalian	0.608	1.06E-08
beta-estradiol	chemical - endogenous mammalian	3.331	2.33E-05
Pparγ	ligand-dependent nuclear receptor	-0.779	6.86E-05
Erbb2	kinase	0.658	1.38E-04

Note: Selected Predicted Upstream Regulators at PND 7 from IPA of microarrays. Z-score indicates the predicted activation status of the regulators where a positive score indicates activation and a negative score indicates inhibition.

Table 3-6. Fold Induction of RNA following prenatal PFOA exposure for selected genes of candidate pathways

	PND 7			PND 14			PND 21		
GENE	0.01 mg/kg	0.1 mg/kg	1.0 mg/kg	0.01 mg/kg	0.1 mg/kg	1.0 mg/kg	0.01 mg/kg	0.1 mg/kg	1.0 mg/kg
Ppar genes									
Adip	1.66*	1.29	1.41*	-1.20	1.32	-1.22	-1.18	-1.48	-1.01
Adipr	1.24*	1.16*	1.42*	-1.23	1.19	-1.19	-1.29	-1.20*	-1.03
Aqp7	-1.27	1.22	-1.61*	1.22	-1.05	-1.12	1.11	1.06	1.10
Fabp3	-9.47*	-7.73*	-8.97*	-1.25	-1.09	-1.23	-1.36	-1.14	-1.78
Insr	-1.35	-1.19	-2.81	-1.15	1.57	1.73	-1.13	1.05	1.11
Irs1	-1.22	-1.26	-2.01*	-1.70	2.86	1.44	-1.06	1.48	1.14
Ppara	-3.26	-1.65	-3.43	-5.05*	-1.82	1.35	1.47	-1.22	-1.49
Pparγ	1.12	1.14	1.22	-1.26	-1.23	-1.02	1.02	1.09	-1.59
Pparβδ	-1.18	-1.12	1.15	1.39	1.41	-1.06	-1.12	-1.08	-1.02
PparγCoa1	-2.27*	-1.64	-1.94*	-1.24	-1.41	-1.06	1.21	-1.22	-1.16
Ucp1	-9.72	-6.07	-9.49	-5.38*	-2.33	1.35	-2.89	4.82	1.87
Wnt genes									
Ctnnβ1	-1.05	-1.25	-1.49*	-1.18	1.63	1.60	-1.29	-1.31	-1.07
Lef1	2.99*	2.38	3.17	NA	4.57*	4.34	-1.03	-1.26	-1.56
Wif1	1.14	1.02	-1.47	NA	NA	NA	-3.65	-3.57	-3.00
Wnt2	1.55*	1.15	1.87*	-1.26	1.03	-1.11	-1.50	-1.60	-1.51
Acvr1	-1.01	-1.06	-1.11	1.31	1.22	-1.20	-4.73	-4.68	-4.95
Frzd2	1.36	1.64	1.71	-1.03	1.95*	1.32	-1.34	-1.30	-1.30
Tgfβ3	-1.10	-1.25	-1.43	1.26	1.92*	1.11	-9.32	-8.97	-9.17
Tgfβ3R	1.15	1.07	-1.33*	-1.06	-1.04	1.04	-21.22	-14.63	-15.50
Erα/ Endocrine related genes									
Ar	1.23	-1.01	1.02	1.58	1.96	-1.13	-2.23	-2.08	-2.13
Areg	-1.37	-1.00	-1.07	NA	-2.01	1.38	-6.96*	-2.86*	-2.37
ErbB2	1.07	1.09	-1.27	-1.01	1.30	-1.23	-5.15	-3.27	-3.34
Esr1	-1.20	-1.06	-2.49*	-1.51	1.36	1.50	1.08	1.16*	1.22
Errγ	-3.45	-5.70	-3.33	NA	NA	NA	1.21	-1.10	1.23
Gpr30	-1.35	1.06	-1.91	NA	NA	NA	-1.01	1.45*	-1.33
Pgr	2.80*	1.95*	2.39	NA	NA	NA	-3.72	-1.97	-1.84
Hsd11β1	4.82*	3.25*	7.86*	-1.86*	-1.56	1.36	1.24	1.14	2.01

Note: Data are presented at SEM. Large fold changes without statistical significance are due to variability in expression within a treatment group. NA=Not analyzed. Genes with NA had either low levels of genes (Ct >35) or did not have sample duplicates that were outside of the range of acceptable deviation (SE>0.5).

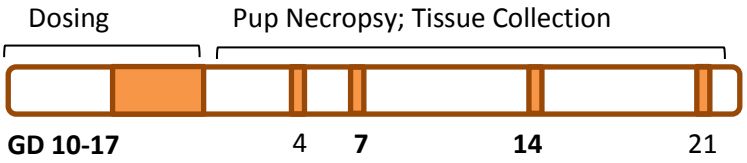

Figure 3-1. Schematic of study design for the late gestation study. Time-pregnant CD-1 mice were dosed with 0, 0.01, 0.1, or 1.0 mg PFOA/kg BW/day from gestation days (GD) 10-17. Mammary tissues from PND 7 and 14 from the control and highest PFOA group (1.0 mg/kg) were analyzed for gene expression changes using genome-wide microarrays.

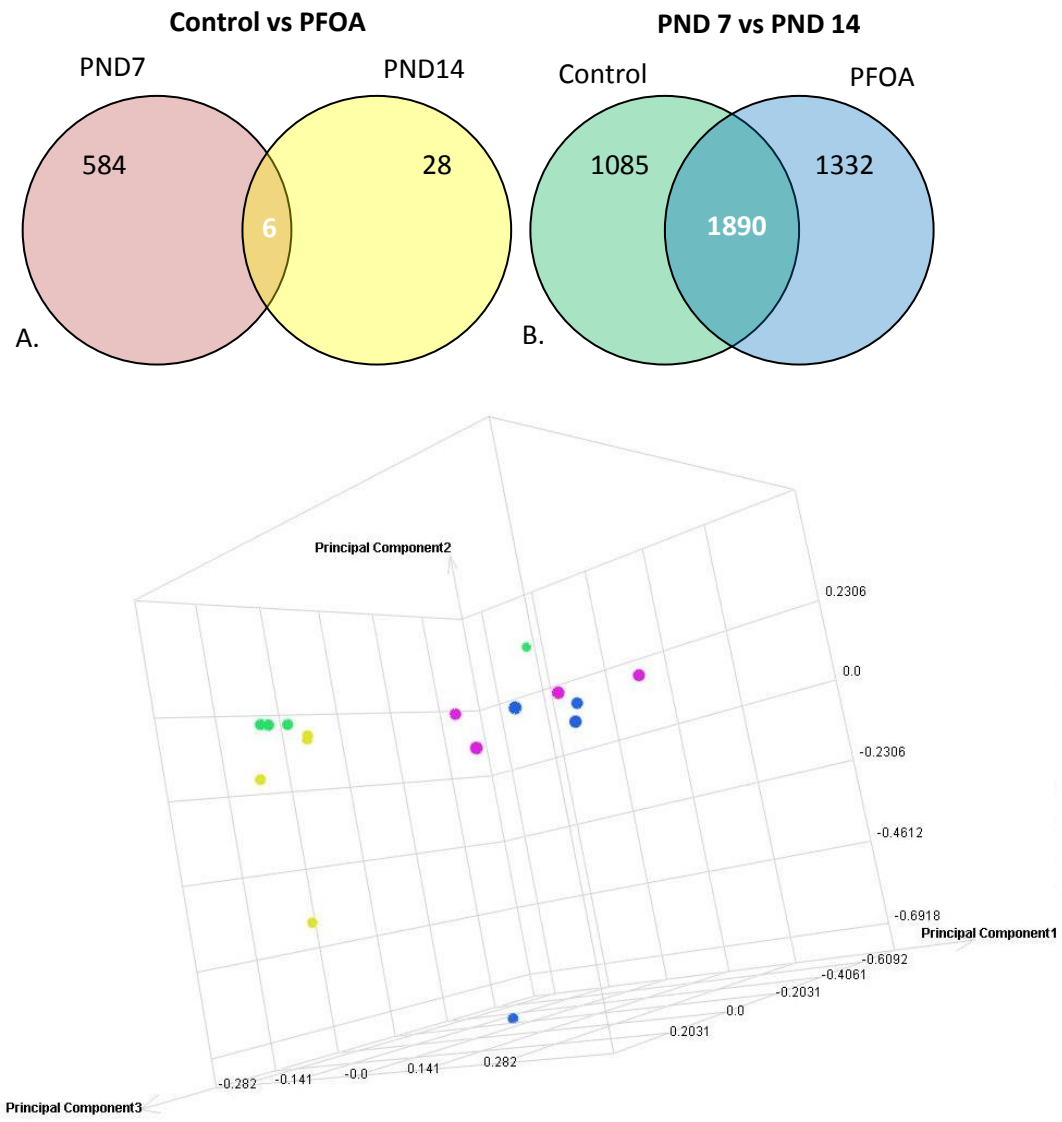

Figure 3-2. Overview of gene expression changes from microarray analysis. Venn diagram of altered gene expression from PFOA treated mammary glands (1.0mg/kg) compared to controls at PND 7 and 14 (A). Venn diagram of gene expression in mammary glands over time in controls and PFOA treated mice (B). Principal component analysis of mammary samples created in Rosetta Resolver (C). Green dots= PND7 Control glands; Yellow dots= PND 7 PFOA glands; Purple dots=PND14 Control glands; Blue dots= PND 14 PFOA glands. *n*=4/treatment/age.

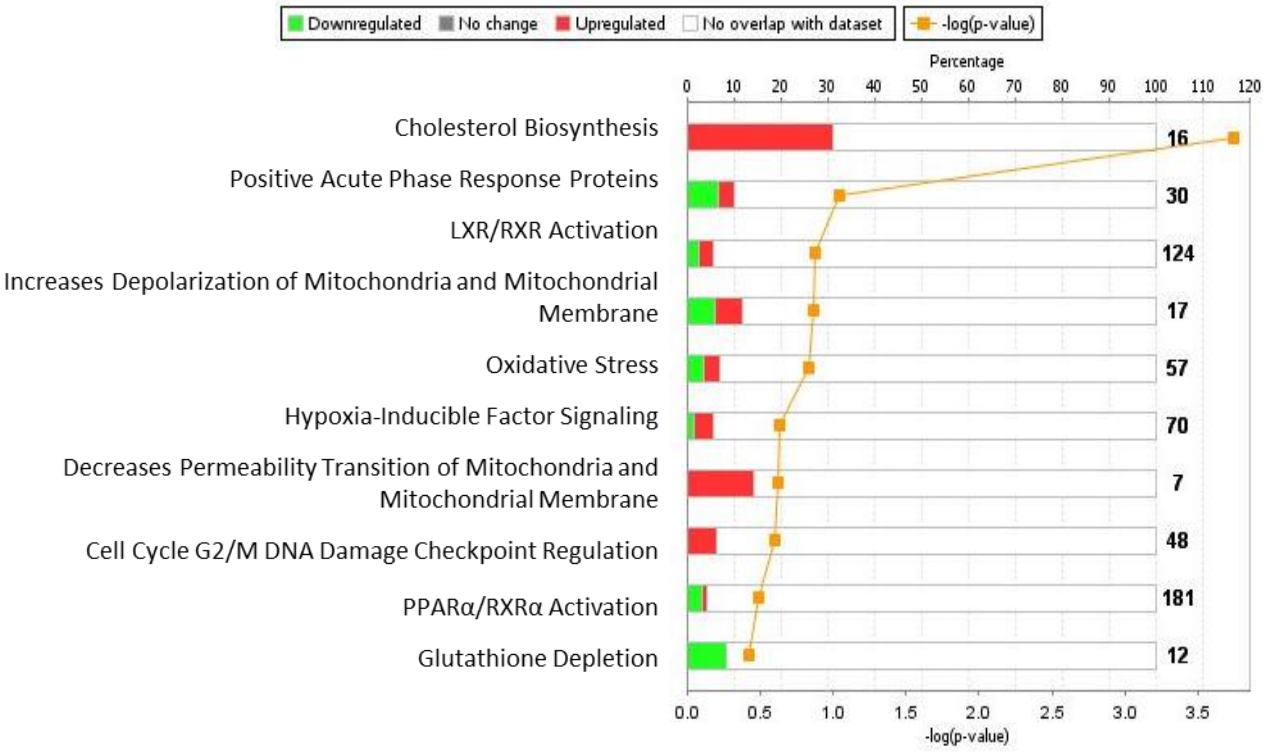

Figure 3-3. Ten Top Tox Lists at PND 7. Prenatal PFOA exposure caused gene alterations in the Top Tox Lists described above and organized according to p value. Groupings that were specific for other organs (i.e. cardiac hypertrophy) were removed from analysis. As indicated in the legend, red=percentage of up-regulated genes in group; green=percentage of down-regulated gene in group. The figure was generated with the use of IPA (Ingenuity® Systems, www.ingenuity.com).

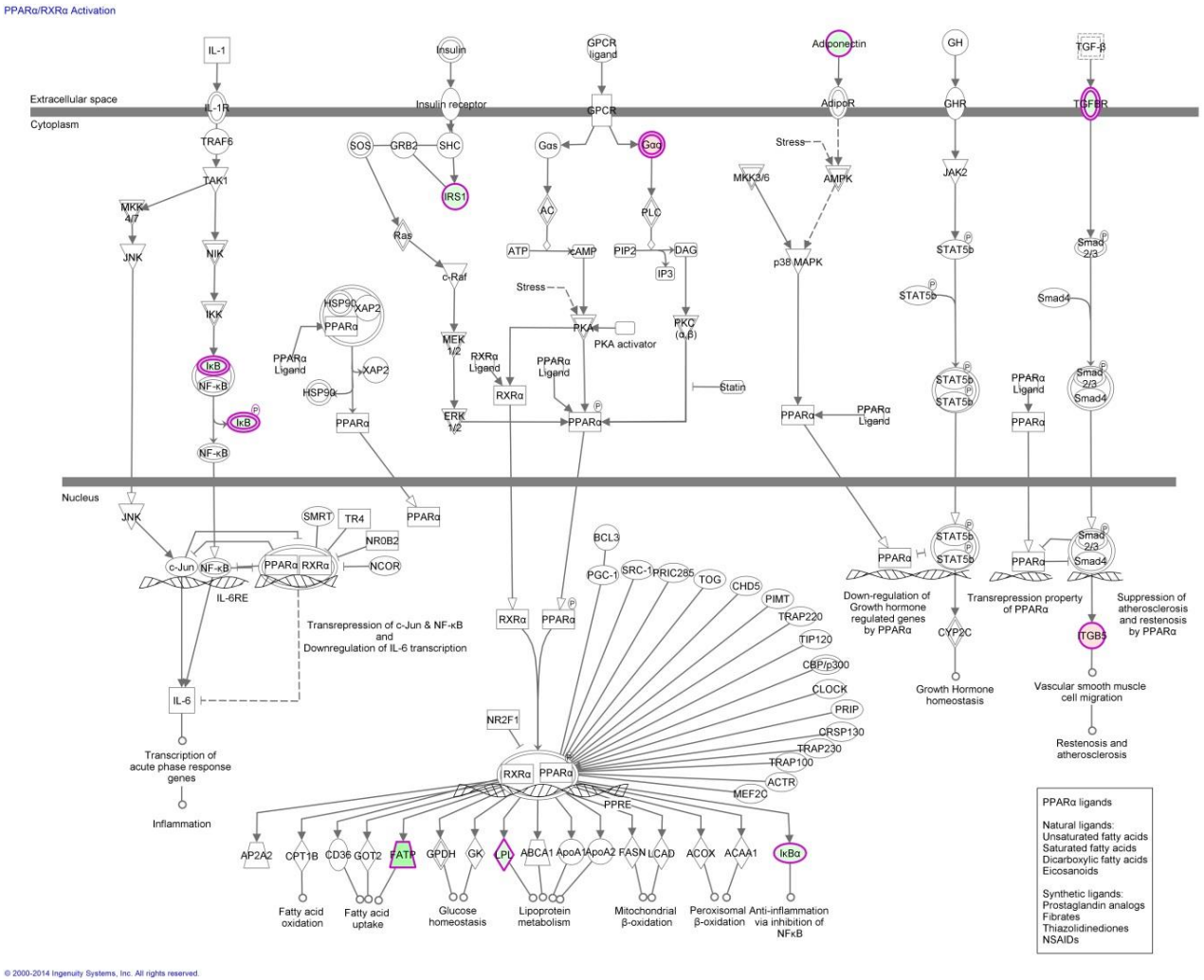

Figure 3-4. PPARα/Rxrα Activation Canonical Pathway Signaling altered by PFOA exposure at PND 7. All target genes of PPARα are downregulated suggesting that *Pparα* was downregulated in mammary tissue. Red genes= upregulated genes; Green= downregulated genes. Pathway analysis generated with IPA (Ingenuity® Systems, www.ingenuity.com).

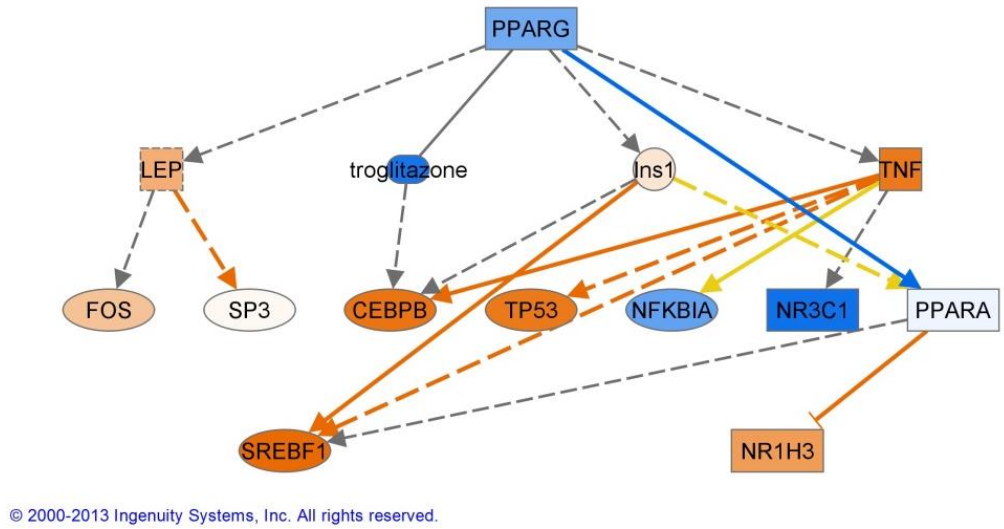

Figure 3-5. PND 7 Pparγ Predicted Upstream Regulator Pathway. Ingenuity produced predicted pathways for Pparγ. Blue coloring indicates predicted inhibition; Orange coloring indicates predicted activation. *Lep*= leptin; *Cebpb*= CCAAT enhancing binding protein beta; *Nr3c1*= Glucocorticoid Receptor; *Nr1h3*= Ligand X receptor; *Ins1*= insulin 1; *Tnf*= Tumor necrosis factor; *Sp3*= sp3 transcription factor; *Fos*= FBJ murine osteosarcoma viral oncogene homolog; *Nfkb*= nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; *Pparg*= peroxisome proliferator activated receptor gamma; *Ppara*= peroxisome proliferator activated receptor alpha; *Srebf1*= sterol regulatory element binding transcription factor 1. Genes were predicted and pathway was generated with the use of IPA (Ingenuity® Systems, www.ingenuity.com).

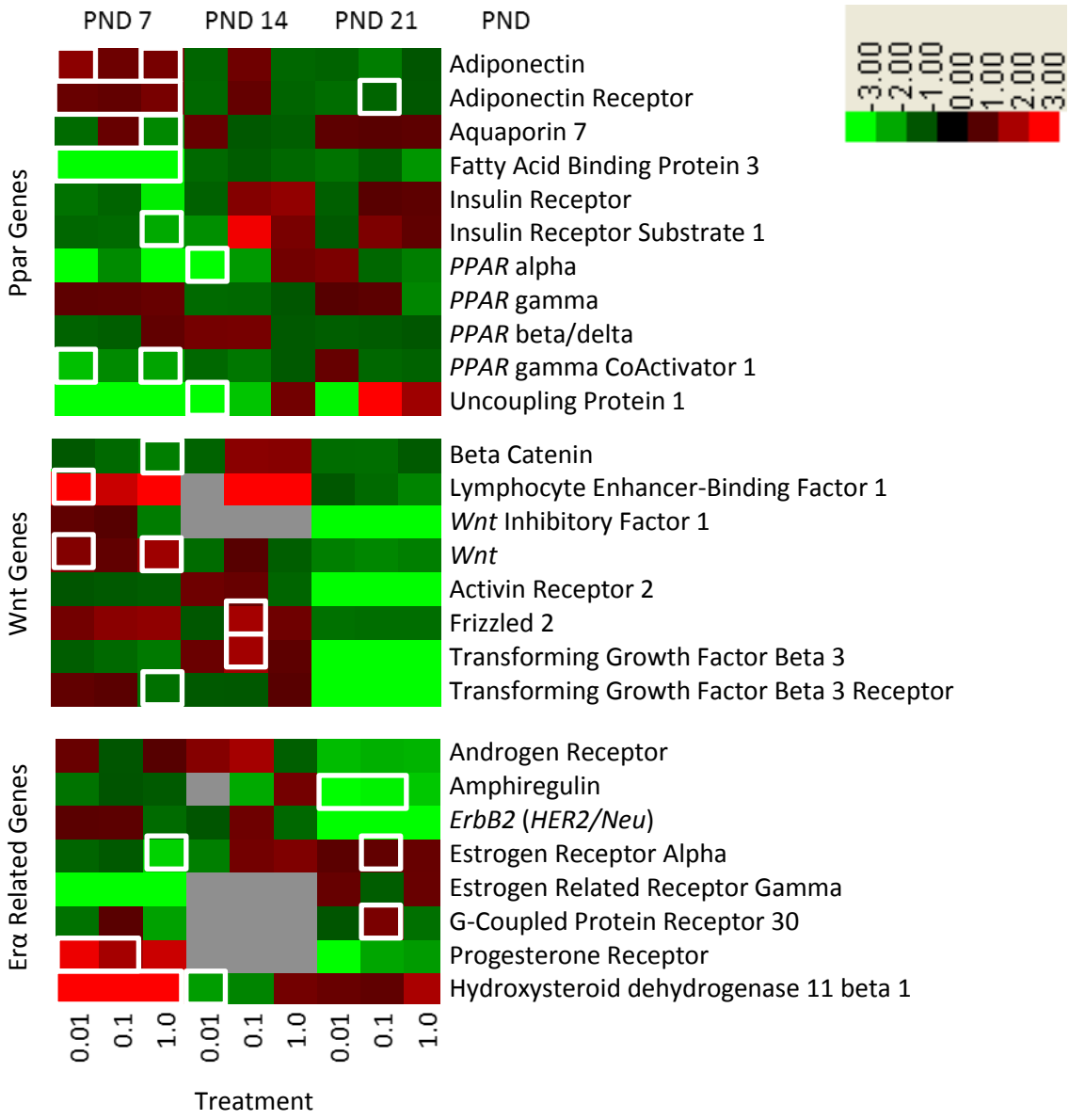

Figure 3-6. RT-PCR heat maps of fold change relative to controls for selected genes in Ppar, Wnt, and Erα pathways. Fold change was calculated by dividing the mean 2dct in treatment over mean 2dct of controls. White boxes indicate statistically significant changes by Student's T tests. Grey boxes indicate time-points were gene expression was too low to make comparisons (Ct>35) or when statistical comparisons were unable to be determined (n=1) due to removal of samples for analysis (duplicate SE > 0.5).

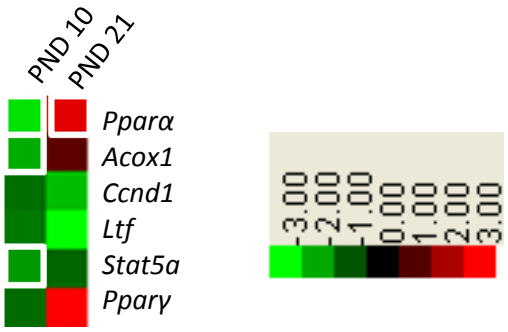

Figure 3-7. Heat maps of RT-PCR results of mammary gland from CD-1 mice prenatally exposed to 5 mg/kg PFOA from GD 1-17 at PND 10 and 21. These heat maps were modified from data reported in White 2008. White boxes indicate statistically significant changes by Student's t tests. We observed similar gene changes for Pparα, Pparγ from RT-PCR analysis and Stat5A from our microarray analysis.

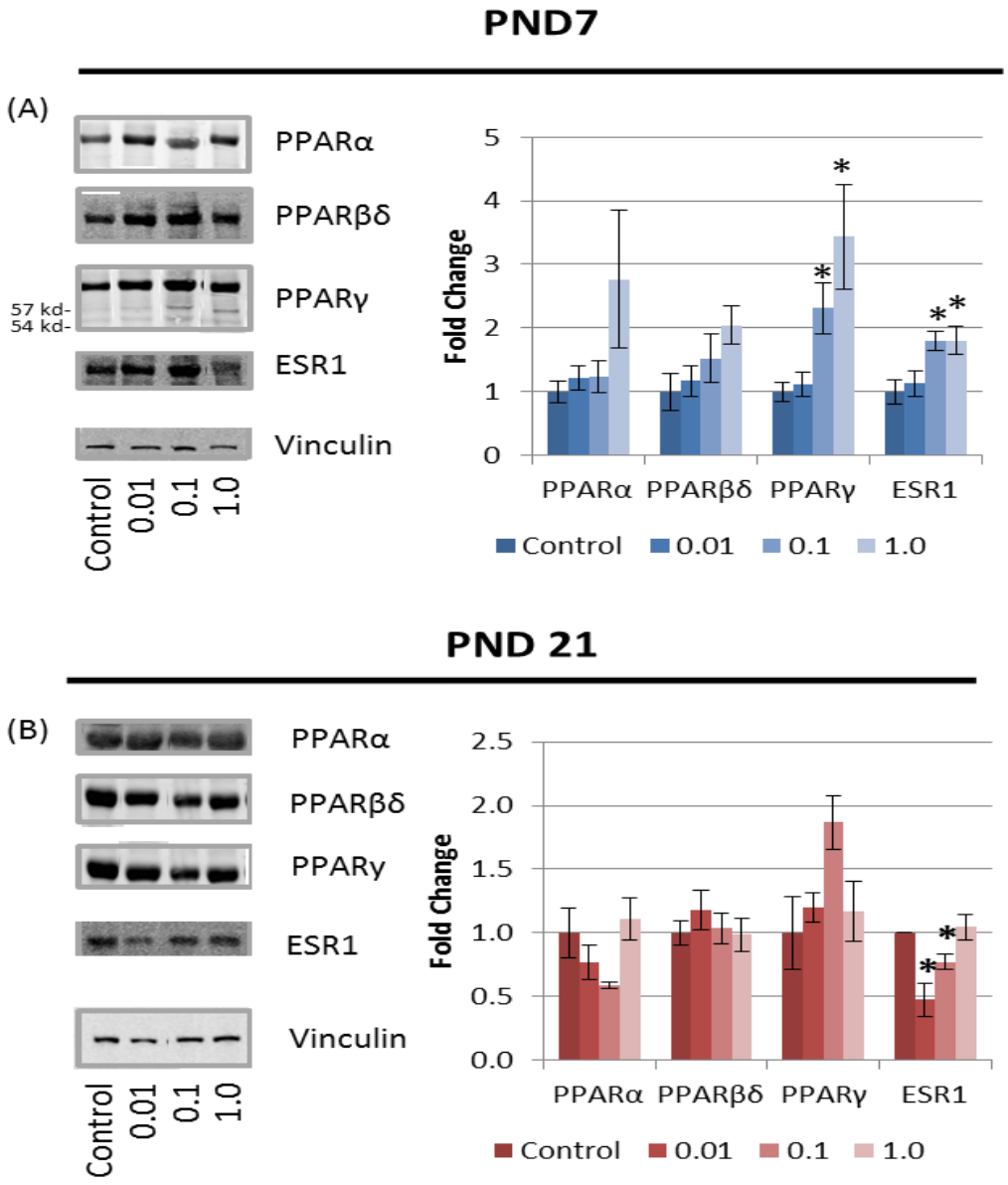

Figure 3-8. PFOA treatment alters protein expression in mammary glands of prenatally exposed mice. (A) Protein expression at PND 7. (B) Protein expression at PND 21. Representative blots of PPARα, PPARβδ, PPARγ, and ERα for each treatment group (n=3/treatment; left panel). Quantification of blots (right panel) are means ± SEM. Statistical significance by Student's T test; *p≤0.05

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CHAPTER 4

Mechanisms Associated with Prenatal PFOA-Induced Changes in Mouse Mammary Gland Development During Puberty and Early Adulthood[4]

OVERVIEW

Previous studies in our laboratory have shown that low-dose prenatal PFOA exposure delays mammary gland development in peri-pubertal CD-1 mice. These effects were driven by gene changes in lipid metabolism and endocrine signaling and are hypothesized to be mediated by peroxisome proliferator activated-receptor gamma (PPARγ) and estrogen receptor alpha (ERα/ESR1). To determine effects during and beyond puberty, time-pregnant CD-1 mice were dosed with 0, 0.01, 0.1, and 1.0 mg/kg PFOA from gestation days (GD) 10-17, as in previous studies, and mammary glands of female offspring were evaluated from neonatal to adult time-points. Adult glands of PFOA-treated animals had a distinctive abnormal appearance of disorganized growth with misdirected branching patterns, reduced side-branching, thickened ducts highlighted by increased collagen, and numerous active terminal end buds in comparison with controls. Epithelial estrogen receptor alpha (ERα) nuclear protein expression was reduced in glands from perinatal to adult time-points in the highest dose group (1.0 mg/kg). A dichotomous effect in nuclear protein expression at PND 56, as levels were increased in the lowest dose-group (0.01 mg/kg) and reduced in the highest dose-group (1.0 mg/kg). Protein levels of PPARγ and PPAR-alpha (PPARα) were reduced at PND 56 in the 0.1 and 1.0 mg/kg groups; ratios of phosphorylated PPARα to PPARα total protein were elevated in the lowest dose group compared to control. Changes in protein levels were most prominent for PPARγ, with over 20-fold reductions in expression. PFOA also

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altered serum sex steroid hormones, an effect that was most pronounced in androgens. Mammary epithelial transplant studies revealed PFOA mammary gland effects were driven by changes in the mammary stromal tissues. Collectively, these data suggest that prenatal PFOA exposure alters steroid hormone production and receptor expression leading to phenotypic features associated with risk factors for increased breast cancer. Results suggest that these effects are primarily mediated by Pparγ and other stromal paracrine factors that adversely influence mammary growth patterns.

INTRODUCTION

Perfluorooctanoic acid (PFOA) is a synthetic surfactant that has become a persistent environmental contaminant. PFOA is ubiquitous in the environment, as it is globally found in water, air, soil, aquatic and terrestrial wildlife, and in humans. Serum concentrations of PFOA in the general population have slowly decreased to 3.1 ng/ml (geometric mean) as a result of emission reductions (*1*). PFOA is slowly eliminated from the body (half-life 3.8 year (*2*)) as it is not readily metabolized. From laboratory animal studies, PFOA has been associated with a wide range of toxicities including liver, kidney, immune, endocrine disruption, and reproductive and developmental toxicity (*3-6*). As a developmental toxicant, PFOA has been shown to affect mammary gland development, maturation, and function (*7-12*).

The mammary gland is an important mammalian organ as it functions to provide nutritional sustenance. It is one of the few tissues that fully develops after birth. Normal mammary gland development involves cross-talk between mammary stromal cells and epithelium which coordinates the ebb and flow of proliferation and apoptosis to direct normal mammary ductal tree elongation and branching patterns. The mammary gland undergoes distinctive periods of rapid growth during different life stages: fetal, puberty, and pregnancy. Following pregnancy and/or lactation, the mammary gland is extensively remodeled through coordinated apoptosis.

In normal mammary gland development, during the pubertal expansion of the mammary ductal tree and fat pad, TEBs proliferate as ductal elongation and branching occurs. Once the ductal tree has grown to capacity, the TEBs differentiate and mature into terminal ends (TEs). Thus TEBs are abundant during puberty and decline approaching the end of adolescence. In the majority of animal models, TEB to TE ratio are relatively high at the beginning of puberty and relatively low at the end of adolescence. Since TEBs are highly proliferative, there are particularly influenced by endogenous growth cues and consequently highly susceptible to carcinogenic insults. An increase in the TEB: TE ratio or extending the presence of TEBS during adulthood is considered to be an unfavorable physiological characteristic indicative of pathology (*13*) .

We have previously characterized the early morphological changes following low-dose prenatal PFOA exposure (*9*). In a subsequent study, we found that early life mammary alterations were related to molecular changes in RNA post-transcriptional modification, lipid metabolism, and cholesterol biosynthesis that were mediated by peroxisome proliferator activated receptor (*Ppar*) and endocrine related genes. Studies in CD-1 and other strains of mice have also reported endocrine-related effects in the mammary gland following peri-pubertal PFOA exposure (*11, 12, 14*) such as delayed vaginal opening (VO), and changes in protein levels of estrogen receptor alpha (ERα) and amphiregulin (AREG). In this study, we investigated the morphological and molecular effects of mammary maturation after PND 21 to determine whether early life effects lingered. Timed-pregnant CD-1 mice were dosed with 0, 0.01, 0.1, and 1.0 mg/kg PFOA from gestation days (GD) 10-17. Mammary glands were collected at PND 7-56 and evaluated for growth patterns and gene and protein expression. We also evaluated puberty related endpoints and measured circulating serum sex hormones to investigate low-dose endocrine effects.

Although most diseases of the mammary gland are related to loss of function and neoplastic changes in the epithelium, current research has placed new focus on stromal tissues or the mammary microenvironment and its role in normal mammary gland development and carcinogenesis. As stromal-

epithelial interactions guide normal mammary gland development, aberrant signaling can lead to abnormal growth. A recent study by Naylor reported that stromal factors control epithelial protein expression (10). This technique was first described in 1950s, and was used to determine influences of mesencyhmal tissues on the structure and function of epithelium (11). In addition, this technique has been utilized by many to determine localized effects of chemicals to understand morphological changes (12). When chemicals mainly act by targeting stromal tissues, recombinants of treated epithelium into control cleared fat pad "rescue" the effect resulting in ductal trees with normal growth patterns. To determine whether prenatal PFOA exposure has a localized effect in mammary glands, mammary epithelial transplant surgeries were conducted. Recombination of control epithelium into "cleared" fat pads of 1.0 mg/kg treated mice and vice versa were compared to determine the effects of PFOA beyond puberty.

MATERIALS AND METHODS

Animals

Time pregnant CD-1 mice were obtained from the core animal facility at National Institute of Environmental Health Sciences (NIEHS) or purchased from Charles River Laboratories (Raleigh, NC). Pregnant dams were housed individually in polypropylene cages and received chow (LabDiet 5001, PMI Nutrition International LLC, Brentwood, MO or NIH 31 chow, Research Triangle Park, NC), and tap water *ad libitum*; both were known to contain PFOA below the levels of detection. Animal facilities were maintained on a 12:12 h light-dark cycle at 20°C–23°C and 40–50% relative humidity. All animal studies were approved by the NIEHS Animal Care and Use Committee.

Chemicals

Ammonium perfluorooctanoic (APFO, the ammonium salt of perfluorooctanoic acid (PFOA)) was purchased from Fluka or Sigma Aldrich (77262, Lot# 0001414284, Steinhiem, Switzerland). PFOA dosing

solutions were prepared in deionized water and were given at a volume of 10 µl solution/g body weight (BW).

Experimental Design

Two studies were conducted to determine the effects of prenatal PFOA exposure beyond puberty: The puberty study investigated PFOA effects on adult mammary gland effects and puberty related endpoints; the mammary epithelial transplant study investigated the effects of PFOA on the growth potential of the mammary gland.

Puberty Study: This study was performed with 3 blocks of animals. Block 1 animals were from NIEHS Core Facilities (53 dams); Blocks 2 (30 dams) and 3 (40 dams) were purchased from Charles River (Raleigh, NC). Time-pregnant animals from NIEHS or Charles River were transferred on gestation day (GD) 6 and were divided into 4 treatment groups. Each group was gavage dosed as described in Macon et al., (2011). Some dams (~20%) were not pregnant and a few were sacrificed before parturition due to dystocia. On postnatal day (PND) 4, male pups were culled to achieve normalized litters: In Block 1, all females from a litter were kept along with 2 males resulting in litter sizes of 6-11. In Blocks 2 and 3, litter size was equalized to 10 keeping as many females as possible and supplementing with males.

Mammary Epithelial Transplant Recombination Study: The mammary epithelial transplant study was performed with 2 blocks of timed-pregnant CD-1 mice (Block 1= 30 dams; Block 2= 20 dams) purchased from Charles River (Raleigh, NC). Timed pregnant dams arrived 1-3 days prior to exposure, consistent with other studies in our laboratory. In block 1 dam arrived on GD 7; Block 2 dams arrived on GD 9. In both blocks, dams were divided into 2 treatment groups (total *n*=25/treatment). Dams were given 1.0 mg PFOA/kg BW/ day or vehicle (deionized water) from GD 10-17. Two dams in the 1.0 mg/kg group were sacrificed due to dystocia, which was not attributed to chemical toxicity, as this pathology has been seen in both control and treated mice in other studies. Female offspring underwent mammary gland epithelial surgeries on PND 21 or 22 (See Mammary epithelial transplants recombination section

of methods). At least one female offspring from each treatment group did not undergo surgeries and served as an intact control.

Necropsy

In the puberty study, female offspring from Block 1 were necropsied on PND 7, 14, 21, 28, and 56+2 days by decapitation. Trunk blood was collected and immediately placed on ice. Body weights and liver weights were recorded. For necropsies on PND 7-21, one set of 4^{th} and 5^{th} mammary glands were removed for whole mount analysis and the contralateral set was frozen for RNA or protein analysis. Starting at PND 21, contralateral glands were also collected in histology cassettes and fixed in formalin for IHC processing. Female offspring from blocks 2 and 3 were necropsied on PND 7, 21, and 56+2 days by decapitation. In Block 2, body and liver weights were recorded. Mammary glands were collected and removed to be processed as whole mounts, frozen on dry ice and stored for RNA/protein analysis, or placed in cassettes and fixed in formalin.

For all blocks, female mice at PND 21 were only necropsied if they had not undergone vaginal opening. Following PND 21, female mice were necropsied only if animals were in, or transitioning to, the estrus stage of the estrous cycle. Animals were weaned at 3 weeks of age and sexes were separated. After weaning, cages of control male mice were kept and strategically placed on racks with females or bedding from control male cages was liberally placed in female cages each week in an attempt to normalize female cyclicity with male pheromones.

Puberty Endpoints

Beginning at PND 20, female offspring were monitored for vaginal opening (VO) and age and weight at VO was recorded. Vaginal smears were taken from female mice that had reached VO by flushing the vagina with small amounts of deionized water or phosphate buffered saline (PBS). Vaginal smears were evaluated on a Leica DM2000 light-microscope (Leica Microsystems) to determine stage of estrous cycle based on historical images from Goldman, 2007 (*15*). The age at which cornified epithelial

cells were first present in the vaginal smear was considered to be age at first estrus. Vaginal smears were collected until a full normal estrous cycle was observed. We defined a normal cycle as 4-6 days with 2-4 days of diestrus, 2-3 days of estrus, and 0-1 days of proestrus, with # diestrus days ≥ estrus days. Data was analyzed for age and weight at VO, age and weight at first estrus, and age and weight at first full normal cycle.

Mammary Gland Preparations

Mammary glands whole mounts were prepared and stained with carmine alum as described in Macon *et al.,* 2011(*9*). Images of whole mounts were evaluated on a Leica DM2000 or Z16 APO with a Leica DFC295 Camera (Leica Microsystems, Frankfurt, Germany) and given a developmental score based on growth characteristics as previously described in Macon et al., 2011(*9*).

Real Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from frozen mammary tissues using the modified trizol/chloroform method described in Chapter 3. Approximately 1µg RNA was reverse transcribed using the Superscript III. 25 ng of cDNA was run in duplicate on 7900HT Fast Real Time PCR System (Applied Biosystems). Primers used were described in Chapter 3 (Listed in Appendix I).

Western Blotting

Whole protein lysates were extracted from frozen tissues using RIPA lysis buffer (R0278, Sigma Aldrich) with protease (Sigma Aldrich) and phosphatase inhibitor cocktails (Thermo Scientific, Rockford, IL 1862495) and quantified using BCA (Pierce BCA Protein Assay Kit, Thermo Scientific). Cell lysates containing ~25 µg of protein were loaded onto a sodium dodecyl sulfate-polyacrylamide 10% pre-cast TGX Bio Rad gel, transferred to nitrocellulose membranes, blocked in bovine serum albumin or milk for 1 hr, probed with appropriate primary antibody overnight at 4°C, incubated with peroxidase goat anti-mouse secondary antibody, developed by Pierce chemiluminescence, and visualized and analyzed using Carestream software as described in Chapter 3. All primary antibodies were rabbit polycolonal and

purchased from Abcam (Cambridge, MA; PPARα phospho Serine 12 rabbit polyclonal at 1:500 dilution) or Santa Cruz (PPARα H-98 (sc-9000), 1:500 dilution; PPARγ H-100 (sc-7196), 1:500 dilution); PPARγ phosphor Serine 112 (sc-28001-R), 1:500 dilution; NEU C-18 (sc-284), 1:500 dilution; vinculin H-300 (sc-5573), 1:250 dilution). Goat anti rabbit HRP secondary antibody was purchased from KLP (074-1506; Gaithersburg, MD, 1:10,000 dilution).

Histology and Immunohistochemistry

Formalin-fixed mammary glands were transferred to 70% ethanol after ~24 hour fixation. The Histology Core at NIEHS embedded tissues in paraffin and cut the tissues into 5 µm sections. Sectioned tissues were deparaffinized in xylene, rehydrated with ethanol, and blocked with hydrogen peroxide. Some sections required pressure heating for antigen retrieval. Protein blocking was performed using the appropriate serum or Dako Protein Blocking Reagent (Dako; Carpenteria, CA) followed by Avidin-Biotin Blocking Kit (Vector Laboratories; Burlingame, CA). Tissue sections were incubated with primary antibody (Mouse anti ER 1:50 diluion, Beckman Coulter, Fullerton, CA; Mouse anti PR 1:150 dilution, Vector Laboratories; Rat anti Ki-67 1:80 dilution, Dako) followed by a matching secondary antibody (Horse anti-mouse 1:1000 dilution, Vector Laboratories; Rabbit anti-rat 1:500 dilution, Vector Laboratories). Negative control tissue sections were incubated with a non-specific immunoglobin or non-immune serum for each antibody. Labeling incubation was done using a RTU Vectastain Kit (Vector Laboratories). Slides were developed with 3-diaminobenzidine (DAB) chromagen (Dako), counterstained with Modified Harris Hematoxylin (Richard-Allan Scientific; Kalamazoo, MI), dehydrated in ethanol, cleared in xylene and coverslipped. TUNEL staining of was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Cat# S7101, Millipore, Billerica, MA) according to the manufacturer's recommendations. Blocking was done by immersing the sections in 3% H_2O_2 for 15 minutes; after which heat-induced epitope retrieval was performed using a citrate buffer pH6.0 (Biocare Medical, CA) in the Decloaker® pressure chamber for 5 minutes at 120°C. TdT enzyme incubation was performed at a 1:3

dilution for 15min at 37°C. --Staining was visualized using 3-diaminobenzidine (DAB) chromagen (DakoCytomation, Carpenteria, CA) and counterstained with hematoxylin. The slides were dehydrated through graded ethanol, cleared in xylene, and coverslipped.

Steroid receptor immunohistochemically (IHC) stained sections were scored based on the Quickscore method as described by Detre, 1995 (*16*). With this method, sections are given a score according to the amount of nuclear cells stained (1-6 scale where 1=0-4%, 2=5-19%,3=20-39%, 4=40-59%, 5=60-79%, 6=80-100%) as well as the intensity of the staining (0-3 scale where 0= none, 1=weak, 2=moderate, or 3=strong staining). Sections were scored blindly by two staff and the two scores were averaged. Mean scores for treatment groups were calculated for each time point and analyzed for statistical significance.

To determine proliferative or apoptotic index of tissues, mammary sections were evaluated for staining of Ki67 or TUNEL, respectively. Three images from each section were taken at 20 X magnification using a Leica DM2000 (Leica Microsystems). Whenever possible, images were taken from epithelial structures of both the 4^{th} and the 5^{th} gland. The total number of stained epithelial cells was counted and compared to the total number of epithelial cells in each image. For TUNEL staining, positive cells were defined as cells with strong nuclear staining and apoptotic appearance. Mean values for each treatment group were averaged and analyzed for statistical significance.

Steroid Hormone ELISA

Trunk blood was spun down to obtain serum which was snap frozen on dry ice, and stored at -80°C until analyzed. For Blocks 1 and 2, serum samples of female mice were analyzed by ILS (ILS INC., Research Triangle Park, NC). Plates and standards to detect mouse serum estradiol and progesterone were set up according to manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). Assays were performed in 96 well, 4 spot plates, that were pre-coated with estradiol and progesterone antibodies. Serum samples were added to the plate followed by conjugated antibodies with an

electrochemiluminescent compound. ELISAs were detected on a SECTOR 2400 imager (Meso Scale Discovery). Samples were run in duplicate and analyzed for coefficients of variance (CV). Due to low sample volume, one sample was not run in duplicate, but was included for analysis. For all others, duplicates that were within ± 20% CV were acceptable. Limit of Detection (LOD) for estradiol was 6 pg/ml and 0.07 ng/ml for progesterone. Values for samples that were below the LOD were reported as the value calculated by dividing the LOD by the square root of 2 (LOD/√2) for statistical analysis. Blocks 3 serum samples were analyzed at NIEHS using customized plates pre-coated with estradiol, progesterone, testosterone, and dihydroepiandrosterone (DHEA) (Meso Scale Discovery). LOD = estradiol 5 pg/ml; progesterone 0.07 ng/ml; testosterone 0.02 ng/ml; DHEA 2.7 ng/ml. Samples were run in duplicate. Since LODs for estradiol and progesterone were similar among plates, data from all blocks were combined for those hormones.

Serum PFOA Dosimetry

Serum was analyzed for PFOA concentration as described in Macon *et al*., 2011(*9*). As in previous studies, when samples were measured below the limit of quantification (LOQ), values were calculated as the LOQ divided by the square root of 2 (LOQ/√2).

Mammary epithelial transplants recombination

Mammary epithelial transplant methods were modified from Brill *et al*. (14). Mice were anesthetized with isofluorane, injected with buprenorphine HCl (sub-cutaneous at 0.1 mg/kg), and the abdominal area was shaved and cleaned with iodine. Small incisions were made around the 4th nipple, then the mammary fat pad was pulled through the incision, the lymph node identified, and the area distal to the lymph node was removed, clearing the 4th gland of epithelium. This process was repeated on the contralateral 4th gland. The mammary tissue containing the epithelium was kept in cold sterile PBS and trimmed into small pieces. A small pocket was made in the cleared mammary fat pad. One piece of excised tissue from the same animal was immediately placed into the pocket and mammary

tissue from a donor was placed into the pocket of the contralateral mammary fat pad. Incisions were closed with surgical thread and glue. Mice were allowed to recover in heated cages and were monitored for 2 days to ensure proper closure of sutures. Surgeries were conducted over a 2-day period. After 6 weeks, mice were necropsied and mammary glands were collected and prepared as whole mounts.

Statistical Analysis

For RT-PCR analysis, data was analyzed in Microsoft Excel and statistical significance was determined with Students t tests. All other statistical analysis was conducted with Statistical Analysis System (SAS Enterprise; SAS Institute, Inc., Cary, NC). Data was evaluated by age and treatment using general linear model analysis of variance (ANOVA). All block data was combined for analysis; no consistent block difference in body weight X treatment were found. Difference between PFOA-treated groups and controls were evaluated by Dunnett's post hoc test. In accordance with previous developmental studies conducted in our laboratory, dam or litter was used as the unit of measure to determine statistical significance. As in previous studies (*9*), when measurements from more than one pup from a litter was taken, values from both or more pups were averaged, and the mean was used for statistical significance. Significance was accepted for p-values ≤ 0.05.

For body weight indices, outliers were removed from analysis based on whole body weights and analyzed by Grubb's test (http://graphpad.com/quickcalcs/grubbs1/). Data from ELISAs were analyzed for outliers based on progesterone levels, as this hormone had the greatest variability within treatment groups. At PND 56, samples from one litter in the 0.01 mg/kg group were removed from all analyses based on severely abnormal mammary gland whole mounts and histology sections of litters.

RESULTS

PFOA elevates relative liver weights

Body weights were recorded on days of necropsy to determine overt toxicity in female offspring. Body weights and net body weights can be found in Table 4-1. With all blocks combined (n=8-28/treatment), prenatal PFOA exposure reduced pup body weights at 3 timepoints; PND 7 (0.1 mg/kg, p=0.03), PND 21 (0.1 mg/kg p=0.02), and 56 (1.0 mg/kg p=0.05). As hepatomegaly is a common effect of PFOA, net body weights were calculated (body weight minus the liver weight) to remove the influence of liver weight effects. PFOA also reduced net body weights at PND 7 (0.1 mg/kg, p=0.03) and there was a significant treatment trend for PND 21 and 56 (F= 0.003 and 0.01, respectively).

Liver weights (Table 4-1) were elevated in PFOA treated animals in the highest dose group (1.0 mg/kg) at PND 7 ($p < 0.0001$) and a significant treatment trend in increased liver weights was observed at PND 14 and 21 (F=0.01 and 0.004, respectively). Relative liver weights (liver:body weight) were also elevated but only in the highest treatment group (1.0 mg/kg) which recapitulated what we observed in our previous study for late-gestational exposures (8). Relative liver weights were significantly increased in this group from PND 7-21 (p= 0.004-<0.0001). These transient effects on liver weights were not observed beyond PND 21.

Low-dose prenatal PFOA does not alter timing of puberty or sexual maturation

Studies by Lau *et al*. (2006) and Yang *et al*. (2009) have shown that full gestational or peripubertal PFOA exposure, respectively, delays timing of puberty in 3 strains of female mice when assessed by vaginal opening and or age at first estrus at ≥2.5mg/kg. To determine whether low-dose PFOA exposure delayed timing of puberty, we monitored female mice from PND 20 and recorded age and weight at vaginal opening (VO), first estrus, and normal cycling. This data can be found in Table 4-2. No differences were detected in any puberty endpoint measured. These data suggest that low-dose,

late-gestation PFOA exposure has no overall effect on sexual maturation end points that are controlled by the hypothalamic-gonadal axis.

Prenatal PFOA exposures led to aberrant mammary growth that persisted beyond puberty

Although other pubertal indices were unaltered following low-dose prenatal PFOA exposure, mammary glands of PFOA-exposed groups displayed characteristics of delayed or aberrant growth from neonatal to adult time-points. Carmine stained whole mounts were evaluated for developmental growth patterns in comparison with controls and assessed a qualitative developmental score (n=14-28/treatment). As observed in our previous study (8), in the present more robust evaluation low-dose prenatal PFOA exposure results in dose-dependent reductions in developmental scores. Developmental scores for glands can be found in Table 4-3. Neonatal glands (PND 7) of PFOA-exposed mice appeared smaller in size and had reduced branching. At PND 21, as before (8), treated glands had fewer terminal end buds (TEBs), reduced branching, and reduced epithelial growth. These delays persisted and manifested into an aberrant phenotype by PND 56. Adult glands were characterized as disorganized, with misdirected epithelial branching patterns, thick epithelial ducts, increased active TEBS, and reduced side branching (Figure 4-1A). Reductions in epithelial ductal elongation observed at earlier time-points had resolved except in the highest dose group (1.0 mg/kg) whose ductal tree had not reached the perimeter of the fat pad. In Figure 4-1B, a higher magnification of a representative gland from control and 1.0 mg/kg group highlight those features. The disorganized and misdirected growth patterns are distinctive outcomes of prenatal PFOA exposure and have been observed in previous studies in our laboratory with longer and/or higher levels of exposure (8, 9).

To gain more insight into the morphological changes induced by PFOA exposure in young adulthood, histological sections were assessed for differences. Hemotoxylin and Eosin (H&E) stained sections of mammary glands were thought to have increased collagen surrounding epithelial ducts, a theory which we validated with Massons Trichrome (Figure 4-1B). It is likely that increased collagen

accounted for the thickened ductal appearance observed in the whole mounts. Increased collagen density has also been observed in a prenatal PFOA restricted exposure study; this effect was much more pronounced at 18 months of age in mice (9).

Mammary sections from PND 21 and 56 glands were also evaluated for changes in proliferation and apoptosis using Ki67 and TUNEL staining. As we observed delayed growth patterns, we theorized there would be evident reductions in Ki67. Stained sections were evaluated for positive cell staining compared to total cells as described in the Material and Methods Section. At PND 21, there was a significant treatment trend for reductions in the proliferative index (F=0.02) as shown in Figure 4-2. As animals approached adulthood, the proliferative index was significantly reduced in the 1.0 mg/kg group by 4.9 fold (p=0.009, Figure 4-2).

Epithelial cell apoptotic index was also determined by evaluating TUNEL staining. Overall, there were very few positively stained epithelial cells that met the criteria in all treatment groups (<3%). However, at PND 21 TUNEL staining was statistically reduced in the two highest treatment groups by >3.0 fold (0.1 and 1.0 mg/kg p<0.04, Figure 4-2). At PND 56, reduction in apoptotic index approached statically significance (F=0.056). These data show that PFOA reduces proliferation and apoptosis in mammary epithelium; however, due to low basal levels of apoptosis in controls it is difficult to determine whether this effect has any biological significance.

PFOA-induced RNA expression alters few genes in adolescent mammary glands

In a previous study (Chapter 3), microarray and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed PFOA-induced changes in *Ppar*, *Wnt*, and endocrine related genes in mammary glands at PND 7-21. To determine whether PFOA induced changes in specific gene expression pathways beyond puberty, we evaluated mammary tissues from PND 21 -56. A heat map of RT-PCR gene expression changes for the *Ppar*, *Wnt* and *Esr1* gene families are illustrated in Figure 4-3. PND 7-14 values are from samples reported in Chapter 3; PND 21-56 are values from mammary samples in this

study. There were few dose and/or dose/time-dependent gene changes found in the tissues evaluated. At PND 21, PFOA exposure significantly up-regulated expression of adiponectin (*Adip*), adiponectin receptor (*Adipr*), and G-protein coupled receptor 30 (*Gpr30*). At PND 56, there was significant PFOA-induced up-regulation of activin receptor (*Acvr*), insulin receptor substrate 1 (*Irs1*), and *Wnt*. In addition, we also found up-regulation of androgen receptor (1.4) and down-regulation of *ErbB2* (-2.0) at PND 28 in the 0.1 mg/kg group (data not shown). Although we did not find a great deal of significant gene expression changes, when grouped together, we noticed a trend in gene expression over time for the *Ppar* pathway with down-regulation of genes in early life and up-regulation of genes in adolescent and early adult mammary tissues. This shift in expression suggests there may be a compensatory effect for *Ppar* related genes. Similar effects in gene changes have been observed in a wide range of tissues following prenatal PFOA exposure (*17*).

Prenatal PFOA exposure has dichotomous effect on PPARs and Paracrine related proteins

We reported that prenatal PFOA exposure reduced PPARγ and ERα protein levels at PND 7 and increased ERα protein levels at PND 21 (Chapter 3). In addition, changes in protein levels were opposite or in absence of gene changes. IHC analysis from the puberty study revealed ERα and PGR epithelial nuclear expression was altered in the absence of gene changes in adolescent and early adult glands.Using whole cell lysates, we investigated the protein levels of the tyrosine receptor kinase proto-oncogene, ERBB2, PPARα, and PPARγ at PND 56. As we were unable to determine transcriptional effects of PFOA exposure with RT-PCR, we also investigated protein levels of phosphorylated-PPARα serine 12 and phosphorylated PPARγ serine 112 to gain insights into the transcriptional activity of PPARs (Figure 4-4).

We observed a dichotomous trend in protein level expression at PND 56 in mammary tissues for ERBB2, PPARα, and Phospho-PPARγ following prenatal PFOA exposure; protein levels were increased at the lowest dose (0.01 mg/kg) and reduced at the two higher doses (0.1 and 1.0 mg/kg). This trend did

not always produce statistically significant effects, as was found for ERBB2. Proteins were statistically reduced for PPARα protein levels by over 13 fold in the highest dose group (1.0 mg/kg, -13.2). PPARγ protein levels were reduced by over 20 fold in the highest dose groups. These data suggest that PFOA mediates effects in the mammary gland, in part, by dramatically reducing translation of PPARα and more notably, PPARγ.

Phospho-PPARα serine 12 protein levels were reduced following prenatal PFOA exposure. Levels were statistically reduced in the 0.1 mg/kg group (-3.1). However, when we compared the ratio of Phospho-PPARα to PPARα, ratios were increased in the 0.01 mg/kg group (4.3 fold) suggesting a dichotomous effect in protein expression. Mitogen activated protein kinase (*MAPK*) mediated phosphorylation of PPARα at serine 12 is associated with increased transcriptional activity (*18*). These data further suggest there was a dichotomous effect in PPARα transcriptional (increased, 0.01 mg/kg) and translational activity (reduced, 1.0 mg/kg). We expected to observe increased protein levels as PPARα appeared highly expressed in both PFOA-treated and control glands from IHC (Appendix II). Phospho-PPARγ protein levels were also reduced at PND 56. As shown in Figure 4-5, protein bands in the two highest treated groups were exceedingly reduced (0.1 mg/kg) or not present at all (1.0 mg/kg); levels were reduced by over 15 fold. Phosphorylation of PPARγ at serine 112 is reported to reduce transcriptional activity of the receptor (*18*). However, due to severely reduced levels of PPARγ in the 0.1 and 1.0 mg/kg group, levels of phospho-PPARγ serine 112 were negligible. These data provided further evidence that prenatal PFOA exposure mediated effects via modulation of PPARγ protein expression levels.

Prenatal PFOA exposure alters expression of ERα and PGR

We previously observed changes in estrogen receptor alpha (Erα) RNA and protein levels in whole mammary tissues following prenatal PFOA exposure in mice (Chapter 3). To determine whether this effect altered protein expression specifically in epithelial cells, immunohistochemical (IHC) staining

of mammary gland sections for ERα were evaluated. To quantify the levels of ERα, sections were evaluated for epithelial nuclear expression and given a quickscore. Epithelial ERα expression was reduced in a dose-dependent manner at PND 21; ERα was statistically reduced in glands of 1.0 mg/kg group (Figure 4-5 A-B). These reductions in ERα at PND 21 are in accord with our previous data from whole mammary cell lysates evaluated with Western blots (Chapter 3), although we also observed reductions in 0.01 and 0.1 mg/kg groups. At PND 56, there was a dichotomous effect as epithelial ERα levels were elevated in glands of the lowest dose (0.01 mg/kg) and statistically reduced in the highest dose group (1.0 mg/kg).

We also evaluated expression levels of progesterone receptor (PGR) as we were unable to evaluate protein levels with Western blot analysis due to low expression within all samples. PGR expression levels were also reduced in mammary sections at PND 21 as quickscores for nuclear epithelial expression were statistically reduced in glands of 1.0 mg/kg group (Figure 4-4 A-B). In addition, as with ERα, at PND 56 we observed a dichotomous effect in PGR nuclear epithelial protein expression. PGR levels were statistically elevated in glands of the 0.01 mg/kg and similar to controls in the two higher dose groups (0.1 and 1.0 mg/kg). Collectively these data suggest that PFOA not only reduces circulating androgens (endocrine effect), but also alters sex hormone receptor expression in epithelial ducts of mammary glands (paracrine effect). PFOA-induced RNA expression altered few genes in early adult mammary glands

PFOA reduces circulating androgens

As changes in sex hormone receptor expression levels were found in mammary epithelium at PND 21, as described in Chapter 3, but not in puberty endpoints, we investigated the levels of serum hormones. We measured levels of estradiol (E2), progesterone (P), dihydroepiandostrone (DHEA), and testosterone (T) by ELISA. Circulating levels of E2 and progesterone were not affected following prenatal PFOA exposure in comparison with controls which are shown in Table 4-4. Comparison of hormone

concentrations over time revealed that the change in estradiol levels were different in the 1.0 mg/kg group when compared to controls. As shown in Figure 4-6A, E2 concentrations in controls decreased over time yet were relatively stable in the 1.0 mg/kg group. This finding suggests that PFOA alters normal hormone production in female mice.

Circulating levels of DHEA and T in serum were also determined in the last block of animals for this study. This was based on updated analysis of microarray data which predicted dihydroxytestosterone as an activated regulator of PFOA gene alterations in mammary tissues (Chapter 3). Serum T levels were statistically reduced in the two highest groups (0.1 and 1.0 mg/kg) at PND 21. DHEA levels were statistically reduced at PND 56 in a dose-dependent manner (Table 4-4). In addition, the change in hormones over time was different for both T and DHEA (Figure 4-6 C-D). These finding provide evidence that PFOA disrupts the endocrine system and may have anti-androgenic-like properties.

Serum PFOA Concentrations

To correlate prenatal PFOA-induced effects with internal dosimetry, serum was analyzed for PFOA concentrations. Table 4-5 shows PFOA serum concentrations for all treatment groups from PND 7-56. Control samples had low to <LOQ levels of PFOA. Similar results in controls were observed in a previous study (*9*). Serum PFOA concentrations decreased dramatically from PND7. PFOA concentrations approached control background levels by PND 28 in the 0.01 mg/kg group and by PND 56 in the 0.1 mg/kg group (Table 4-5).

Mammary Epithelial Transplant Recombination

In the Puberty Study, we determined that low-dose PFOA exposure alters mammary gland maturation by altering endocrine and paracrine signaling. More focus has been placed on stromal cells and their role in normal mammary gland growth and development of disease. A recent study by Naylor reported that stromal factors control epithelial protein expression (*19*). To determine whether prenatal

PFOA exposure has a localized effect in mammary glands, mammary epithelial transplant surgeries were conducted. This technique was first described in 1950s to determine influences of mesencyhmal tissues on structure and function of epithelium (*20*). In addition, this technique has be utilized by many to determine localized effects of a chemical to explain morphological changes (*21*). When chemicals act through mainly by targeting stromal tissues, recombinants of treated epithelium into control cleared fat pad "rescue" the effect resulting in ductal trees with normal growth patterns; recombinants of control epithelium into treated cleared fat pad result in ductal trees with stunted, aberrant growth patterns that mimic treated abnormal patterns.

Mammary epithelial transplant surgeries were conducted as described in the Methods. In this study, animals served as their own control and experimental sample as illustrated in Figure 4-7A. Using this technique it was found that stromal tissues heavily influenced growth patterns of the epithelial ductal tree. In Figure 4-7A, representative pictures of whole mount recombinants of treated epithelium in control fat pad (PFOA/Control; left side) and recombinants of control epithelium in treated fat pad (Control/PFOA; right side) are shown. Recombinants transplanted to the 4^{th} gland took on the appearance of the 5^{th} gland regardless of treatment (Figure 4-7A). Morphological qualities of recombinants were recorded and graphed in Figure 4-6B to identify influences of tissue specific compartments. Epithelial ductal tree of recombinants of PFOA-treated fat pads were more likely to have thick ducts and reduced side branching, characteristics associated with prenatal PFOA induced mammary gland effects. Recombinants of treated epithelium into treated fat pads (PFOA/PFOA) were more likely to have areas of darkly staining foci or potentially hyperplastic epithelia (evaluated from whole mounts). The influence of the stromal tissues was more apparent from the graphed comparison of transplant characteristics based on control or PFOA-treated fat-pads. Treated stromal tissues reduced side branching and were more likely to affect appearance of thickened ducts and potential epithelial

hyperplasia. These data suggest that prenatal PFOA exposure directly affects mammary stromal tissue to influence mammary epithelial growth patterns.

DISCUSSION

We have previously reported (Chapter 3) that prenatal PFOA exposure delays early mammary gland development and that these delays involved lipid metabolism, cholesterol biosynthesis, and specifically Ppar, and endocrine related genes. Herein two studies were conducted to 1) determine the latent effects of prenatal PFOA exposure and 2) determine the tissue specific influences of the phenotypic effect. We determined that prenatal PFOA exposure has latent effects on mammary gland maturation which were characterized by disorganized, misdirected growth patterns, reduced side-branching, thickened ductal appearance due to increased collagen density, increased active TEBs in adult glands, and reduced developmental scores. PFOA exposure resulted in a dichotomous trend in protein expression levels at PND 56 as levels tended to be increased in the 0.01 mg/kg group and were reduced in the two higher doses (0.1 and 1.0 mg/kg). Decreased PPARα and PPARγ protein levels were found and T and DHEA serum levels were reduced. We conclude that prenatal PFOA exposure has persistent effects on mammary gland maturation that are mediated by paracrine and endocrine hormone/receptor disruption and reductions in PPARα and PPARγ protein. Results from mammary epithelial transplants suggest the stroma may be mediating changes induced by PFOA exposure. Notably, PFOA concentrations for all treatment groups overlapped with serum PFOA levels reported to be found in children living in a highly contaminated community in the Ohio River Valley (*22*). Importantly, PFOA levels reached human relevant concentrations by PND 56 in all dose groups, and we observed morphological abnormalities, gene expression, protein phosphorylation and translation, and steroid receptor reduction in mammary tissue at these times/internal doses that resulted from prenatal exposure.

Relative liver weights were elevated only in the 1.0 mg/kg group as previously described in Macon *et al*., (2011(*9*)). Elevated liver weights are indicative of hepatomegaly, a common PFOA-induced liver effect. As we previously observed mammary gland morphological (8), RNA expression and protein level changes (Chapter 3) in all dose groups and across a longer span of time, this finding provides further evidence that the mammary gland is more sensitive than the liver in regards to responses following prenatal PFOA exposure.

It was found that thickened epithelial ducts observed in whole mounts were related to increased collagen density evaluated by Massons collagen staining of IHC sections. Microarray results from Chapter 3 indicated PFOA-induced up regulation of collagens (*Col11a1*, 6.29; *Col12a1*, 1.6; *Col1a1*, 1.19) at PND 7. Increased collagen density in mammary tissues is often associated with increased risk for breast cancer in women (*23*). However recent data suggests that organization of collagen is more associated with increased mammary tumor risk (*24*), that is, collagen that is static and organized to inhibit movement is associated with early pregnancy mammary changes, a protective risk factor for breast cancer. Therefore, additional data is needed to further characterize mammary collagen deposition following prenatal PFOA exposure.

Prenatal PFOA treatment increased active TEBs at PND 56. The extended presence of TEBs is another morphological characteristics associated with increased risk for mammary tumor development (*13, 25, 26*). It is possible that decreased proliferative and apoptotic indices contributed to the extended presence of TEBs and decreased the rate of differentiation. In addition, we also observed a reduction of side branching in glands from PFOA-treated animals. Morphological reductions in side-branching is a characteristic of nulliparous mammary glands (*27*). As nulliparity is a risk factor associated with increased breast cancer risk, PFOA-induced reductions in side-branching may indicate increased susceptibility for the development of tumors.

Protein levels of ERα and PPARγ were reduced at PND 56. In a previous study, we reported increased protein levels of both at PND 7 (Chapter 3). Reduced expression of ERα and PPARγ in early adulthood from elevated neonatal expression supports our findings that there is a compensatory effect in RNA and, with this study, protein levels. In addition, reductions at early adulthood in PPARγ also provide evidence that at PND 7, PPARγ transcriptional activity was reduced, likely via phosphorylation. Following PFOA exposure, changes in proteins expression levels were also observed in the absence of changes in RNA expression. These data suggest prenatal PFOA exposure affects transcription and/or translation of genes. Translation changes may have occurred as a result of epigenetic changes such as methylation or histone deacetylation.

As the changes in PPARγ protein levels were altered at the greatest magnitude (3.4 fold at PND 7; -27.8 fold at PND 56) in both studies (Chapter 3 and 4), we conclude that Pparγ plays a role in low-dose, prenatal PFOA exposure effects in the mammary gland. Pparγ is an important human relevant mediator of mammary epithelial growth and carcinogenesis. Higher expression of PPARγ protein has been associated with better prognosis and more differentiated breast tumors (*28*). In breast cancers, high PPARγ protein was positively associated with ERα and Ki67 expression and negatively associated with tumor size (*29*). Mice heterozygous for *Pparγ* were found to have increased mammary adenocarcinomas following carcinogenic administration (*30*). Also agonists of Pparγ have been shown to have anti-carcinogenic properties (*28*), and are currently being used in clinical trials as a potential therapeutic agent (*31*). These finding provide further evidence that prenatal PFOA may potentially increase susceptibility to the development of mammary tumors over a life-time. There are few epidemiological studies that have found positive associations between PFOA exposures and increased breast cancer risk, however a recent case-control study of Inuit women found that women who had breast cancer were more likely to have higher circulating levels of perfluorinated compounds, polychlorinated biphenyls (PCBs), and organo-chlorine pesticides (OCPs), collectively (*32*). Interestingly,

a recent study found reductions *Pparγ* in adipocytes/stromal cells led to increased tumor incidence and reduced latency (*33*) while no effects are observed in selected deletion of *Pparγ* in epithelium. Mammary epithelial transplant studies revealed that PFOA effects in the stromal tissues were propelling the morphological changes observed. Therefore, in future studies, we will determine the spatial and temporal expression levels of PPARγ as well as other proteins.

Changes in circulating steroid hormones that are generally described as anti-androgenic effects were also observed. Testosterone levels were reduced at PND 21 while DHEA levels were reduced at PND 56. In females, testosterone is a precursor for the production of estradiol via the enzyme aromatase. Biegel et al., (1995) concluded that PFOA-induced reductions in serum testosterone and elevation in serum estradiol in adult exposed male rats was due to increased levels, but not increased activity, of aromatase(*34*). As there were significant decreases in testosterone yet no difference in estradiol levels, these data suggest that PFOA may increase amount and/or activity of aromatase as well. DHEA is a peroxisome proliferator and has been shown to alter expression of PPARα and –γ (*35, 36*). The connection between DHEA and protein levels of PPARs and the androgen receptor in PFOA-treated mammary tissues deserves further study.

In conclusion, several paracrine and endocrine targets of prenatal PFOA exposure have been identified in treated mammary tissues. These effects occurred at low and human-relevant exposures and appear to persist from birth to early adulthood. Importantly, results collectively suggest that PFOA is a potential modulator of breast cancer risk factors as PFOA exposures increased mammary collagen density, delayed epithelial differentiation, altered serum hormones, and altered protein indicators of prognosis.

Table 4-1. Body weight indices

Body Weights (g)

Treatment	PND 7	PND 14	PND 21	PND 28	PND 56
Control	4.83 ± 0.07	9.47 ± 0.222	13.69 ± 0.25	21.63 ± 0.52	29.27 ± 0.56
0.01 mg/kg	4.64 ± 0.08	8.98 ± 0.15	13.78 ± 0.20	22.20 ± 0.53	29.52 ± 0.52
0.1 mg/kg	**4.70 ± 0.10***	9.59± 0.26	**12.98 ± 0.29***	21.19 ± 0.43	28.18 ± 0.45
1.0 mg/kg	4.64 ± 0.08	9.08 ± 0.12	13.28 ± 0.31	21.46 ± 0.56	**27.48 ± 0.41***

Net Body Weights (g)

Treatment	PND 7	PND 14	PND 21†	PND 28	PND 56†
Control	4.71 ± 0.08	9.12 ± 0.21	13.17 ± 0.35	20.46 ± 0.48	27.91 ± 0.72
0.01 mg/kg	4.47 ± 0.10	8.66 ± 0.14	13.13 ± 0.17	20.96 ± 0.50	28.04 ± 0.68
0.1 mg/kg	**4.58± 0.14***	9.22 ± 0.25	12.57 ± 0.39	19.98 ± 0.43	26.49 ± 0.60
1.0 mg/kg	4.51 ± 0.09	8.69 ± 0.12	13.07 ± 0.33	20.24 ± 0.54	26.00 ± 0.51

Liver Weights (g)

Treatment	PND 7	PND 14†	PND 21†	PND 28	PND 56
Control	0.158 ± 0.005	0.349 ± 0.014	0.715 ± 0.025	1.168 ± 0.042	1.511 ± 0.079
0.01 mg/kg	0.146 ± 0.005	0.326 ± 0.010	0.732 ± 0.019	1.247 ± 0.041	1.480 ± 0.050
0.1 mg/kg	0.164 ± 0.007	0.361 ± 0.014	0.712 ± 0.028	1.208 ± 0.025	1.395 ± 0.036
1.0 mg/kg	**0.212 ± 0.007***	0.393 ± 0.010	0.785 ± 0.028	1.224 ± 0.032	1.371 ± 0.051

Relative Liver Weights

Treatment	PND 7	PND 14	PND 21	PND 28	PND 56
Control	0.032 ± 0.001	0.037 ± 0.001	0.051 ± 0.001	0.054 ± 0.001	0.051 ± 0.002
0.01 mg/kg	0.032 ± 0.001	0.036 ± 0.001	0.053 ± 0.001	0.056 ± 0.001	0.050 ± 0.001
0.1 mg/kg	0.035 ± 0.001	0.038 ± 0.001	0.053 ± 0.001	0.057 ± 0.001	0.050 ± 0.001
1.0 mg/kg	**0.045 ± 0.001***	**0.043 ± 0.001***	**0.056 ± 0.001***	0.057 ± 0.001	0.050 ± 0.001

Note: Body weight indices of female offspring over time. Data are presented as mean ± SEM. *n* for PND 7=25-29; PND 14=10-14; PND 21=23-28; PND 28=8-13; PND 26=20-23. Significant effects compared to controls by Dunnett's,*p≤0.05, **p<0.01, ***p<0.01. Significant treatment trend by Dunnett's, †F≤0.05.

Table 4-2. Puberty Endpoints

	Control	0.01 mg/kg	0.1 mg/kg	1.0 mg/kg
VO (age)	25.5 ± 0.3	25.9 ± 0.7	25.7 ± 0.7	25.7 ± 0.5
VO (weight)	17.6 ± 0.4	17.8 ± 0.5	16.0 ± 1.0	17.2 ± 0.3
Estrus (age)	26.1 ± 0.3	26.6 ± 0.5	27.1 ± 0.6	26.5 ± 0.5
Estrus weight	18.1 ± 0.47	18.2 ± 0.3	17.1 ± 0.8	17.8 ± 0.4
VO to Estrus	0.65 ± 0.20	0.64 ± 0.3	1.39 ± 0.43	0.71 ± 0.15
Cyclicity	42.8 ± 1.7	45.6 ± 2.5	41.4 ± 1.6	41.8 ± 1.6
Cyclicity weight	25.2 ± 0.8	25.6 ± 0.8	23.2 ± 0.5	23.8 ± 0.4
Estrus to Cyclicity	16.8 ± 1.7	19.2 ± 2.6	14.0 ± 2.0	15.3 ± 1.5

Note: Puberty endpoints of a subset of female mice following prenatal PFOA exposure. Data are presented as mean ± SEM. Controls, n= 8; 0.01 mg/kg, n= 7; 0.1 mg/kg, n= 6; 1.0 mg/kg, n= 8. No significant effects were found compared to controls by Students t tests. VO= vaginal opening; Estrus= first estrus.

Table 4-3. Mammary Gland Developmental Scores

	PND 7	PND 21	PND 56
Control	2.86 ± 0.16 (19)	2.86 ± 0.13 (26)	2.93 ± 0.14 (26)
0.01 mg/kg	2.46 ± 0.20 (17)	2.41 ± 0.13 (26)	2.58 ± 0.14 (19)
0.1 mg/kg	**2.24 ± 0.22 (15)***	**2.35 ± 0.15 (24)***	**2.34 ± 0.17 (22)***
1.0 mg/kg	**1.93 ± 0.14 (14)****	**2.16 ± 0.14 (24)****	**2.07 ± 0.15 (18)*****

Note: Mammary gland developmental scores from female offspring across time. Data presented at mean ± SEM. Significant effects compared to controls by Dunnetts,*p<0.05, **p<0.01, ***p<0.01.

Table 4-5. Serum PFOA Concentration

	Control	0.01 mg/kg	0.1 mg/kg	1.0 mg/kg
PND 7	6.7 ± 1.1 (5)	149.5 ± 11.7 (4)*	1113.5 ± 57.2 (4)*	9163.5 ± 629.7 (3)*
PND 14	4.9 ± 1.2 (4)	95.0 ± 13.3 (3)*	747.7 ± 38.2 (4)*	6448.8 ± 328.3 (5)*
PND 21	< 5, LOQ (5)	29.3 ± 12.5 (4)*	201.0 ± 27.1 (5)*	2250.0 ± 170.8 (5)*
PND 28	< 5, LOQ (5)	8.0 ± 1.0 (5)*	64.0 ± 12.8 (5)*	1249.4 ± 227.6 (5)*
PND 56	< 5, LOQ (5)	< 10, LOQ (5)*	13.1 ± 1.9 (5)*	57.9 ± 18.6 (5)*

Note: Serum PFOA Concentrations in a subset of female mice across time. Data are presented as mean ± SEM (*n*). Significant treatment effect compared to controls, *p<0.05 by Dunnetts. PND= Postnatal day; LOQ= limit of quantification. Values below LOQ were calculated as described in Materials and Methods.

Table 4-4. Serum Sex Hormones

	Treatment	Estradiol (pg/ml)	Progesterone (ng/ml)	DHEA (ng/ml)	Testosterone (ng/ml)
PND 21	Control	16.97± 2.45 ()	1.61 ± 0.25	98.85 ± 18.88	0.18 ± 0.02
	0.01 mg/kg	17.35 ± 3.51	1.22 ± 0.17	67.27 ± 3.34	0.12 ± 0.01
	0.1 mg/kg	13.35 ± 2.94	1.54 ± 0.33	71.28 ± 9.23	0.12 ± 0.02*
	1.0 mg/kg	12.31 ± 2.37	1.08 ± 0.12	91.09 ± 13.73	0.12 ± 0.01*
PND 28	Control	16.15 ± 2.57	1.07 ± 0.12	-	-
	0.01 mg/kg	14.95 ± 7.75	0.91 ± 0.11	-	-
	0.1 mg/kg	14.83 ± 4.55	0.99 ± 0.11	-	-
	1.0 mg/kg	17.64 ± 3.67	0.73 ± 0.06	-	-
PND 56	Control	8.70 ± 1.44	2.29 ± 0.31	86.82 ± 8.00 ()	0.11 ± 0.01
	0.01 mg/kg	7.22 ± 1.79	2.16 ± 0.60	64.72 ± 6.22 ()*	0.14 ± 0.02
	0.1 mg/kg	6.65 ± 2.03	1.30 ± 0.17	57.61 ± 3.08 ()*	0.11 ± 0.01
	1.0 mg/kg	15.20 ± 3.10	1.49 ± 0.27	47.21 ± 4.06 ()*	0.09 ± 0.01

Note. Serum sex steroid hormones in female mice across time. Serum collected from mice was analyzed for hormones using ELISA. Data are presented as mean ± SEM (*n*). Data were analyzed by ANOVA with Dunnetts, *p=0.05. DHEA= dihydroepiandostrane.

Table 4-5. Serum PFOA Concentration

	Control	0.01 mg/kg	0.1 mg/kg	1.0 mg/kg
PND 7	6.7 ± 1.1 (5)	149.5 ± 11.7 (4)*	1113.5 ± 57.2 (4)*	9163.5 ± 629.7 (3)*
PND 14	4.9 ± 1.2 (4)	95.0 ± 13.3 (3)*	747.7 ± 38.2 (4)*	6448.8 ± 328.3 (5)*
PND 21	< 5, LOQ (5)	29.3 ± 12.5 (4)*	201.0 ± 27.1 (5)*	2250.0 ± 170.8 (5)*
PND 28	< 5, LOQ (5)	8.0 ± 1.0 (5)*	64.0 ± 12.8 (5)*	1249.4 ± 227.6 (5)*
PND 56	< 5, LOQ (5)	< 10, LOQ (5)*	13.1 ± 1.9 (5)*	57.9 ± 18.6 (5)*

Note: Serum PFOA Concentrations in a subset of female mice across time. Data are presented as mean ± SEM (*n*). Significant treatment effect compared to controls, *p<0.05 by Dunnetts. PND= Postnatal day; LOQ= limit of quantification. Values below LOQ were calculated as described in Materials and Methods.

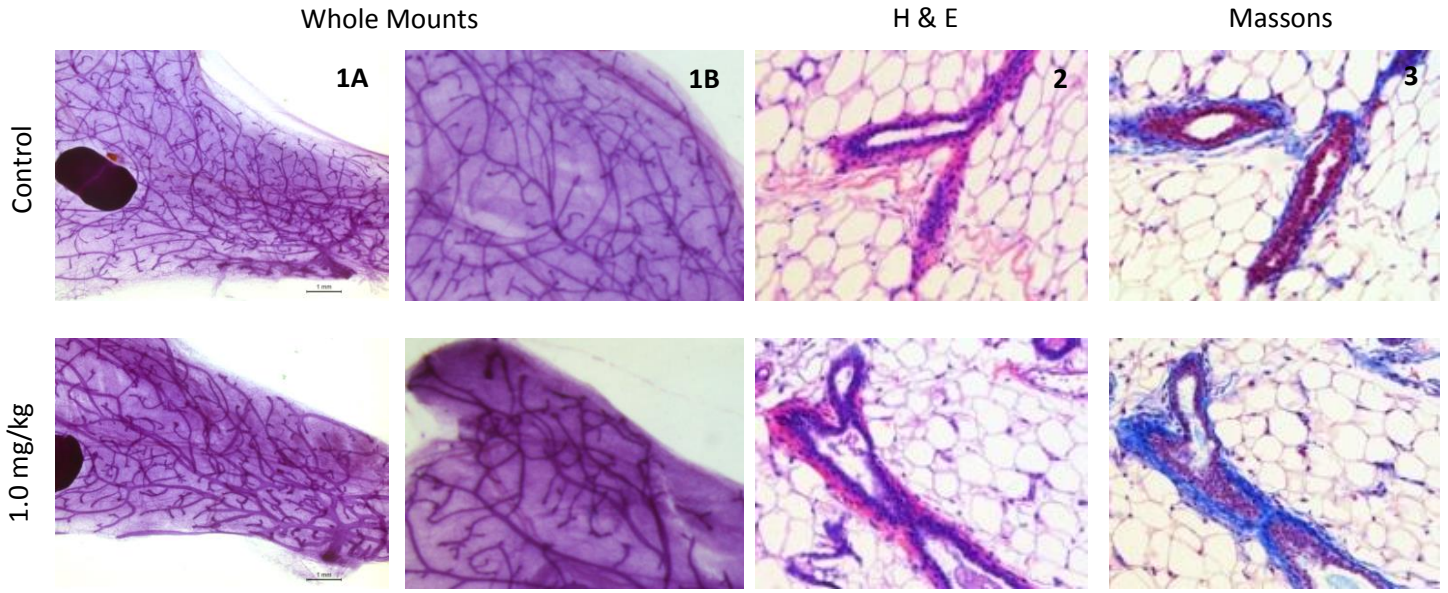

Figure 4-1. Prenatal PFOA exposure alters mammary gland maturation at PND 56. Images are representative of each treatment group. Whole mount mammary glands of PFOA-treated groups demonstrated disorganized, misdirected growth patterns, thick ducts, reduced side-branching, and increased active terminal end buds (TEBs) in early adulthood. Lower power magnification of whole mounts (1A) Higher power magnification of whole mounts (1B). Histology sections of (2) H&E and (3) Masson trichrome show thick ducts are due to increased collagen deposition.

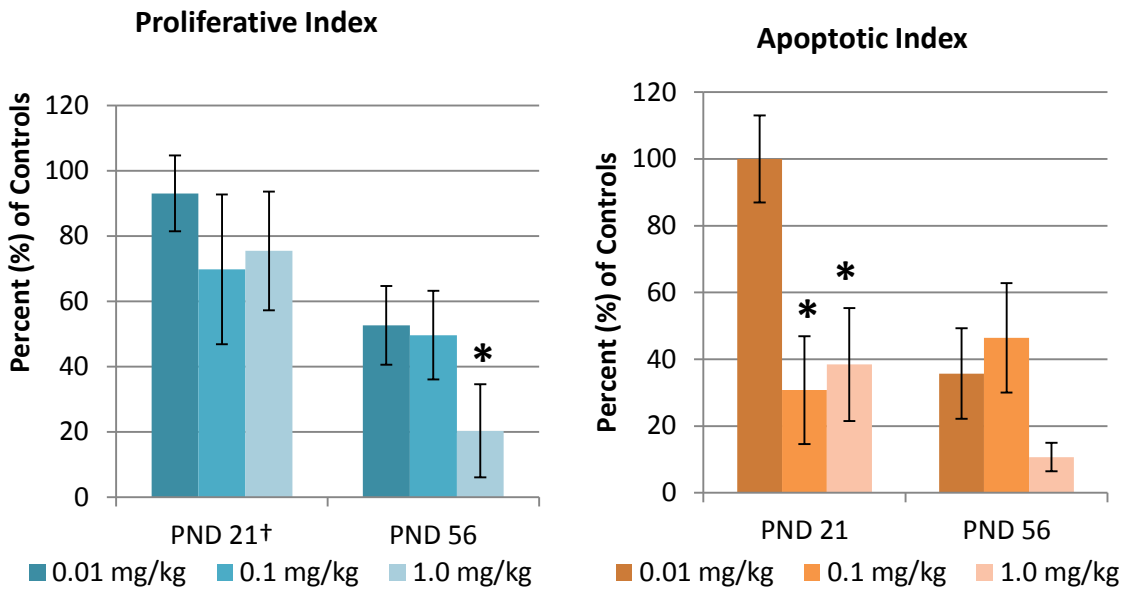

Figure 4-2. Epithelial proliferative and apoptotic indices of PFOA-treated mammary glands. Proliferative index is indicative of epithelial Ki67 staining. Apoptotic Index is indicative of epithelial TUNEL incorporation/staining. Data presented as percent staining compared to controls ± SEM. Data were analyzed by ANOVA with Dunnetts, *p≤0.05. Treatment effect by least square means, †F≤0.05.

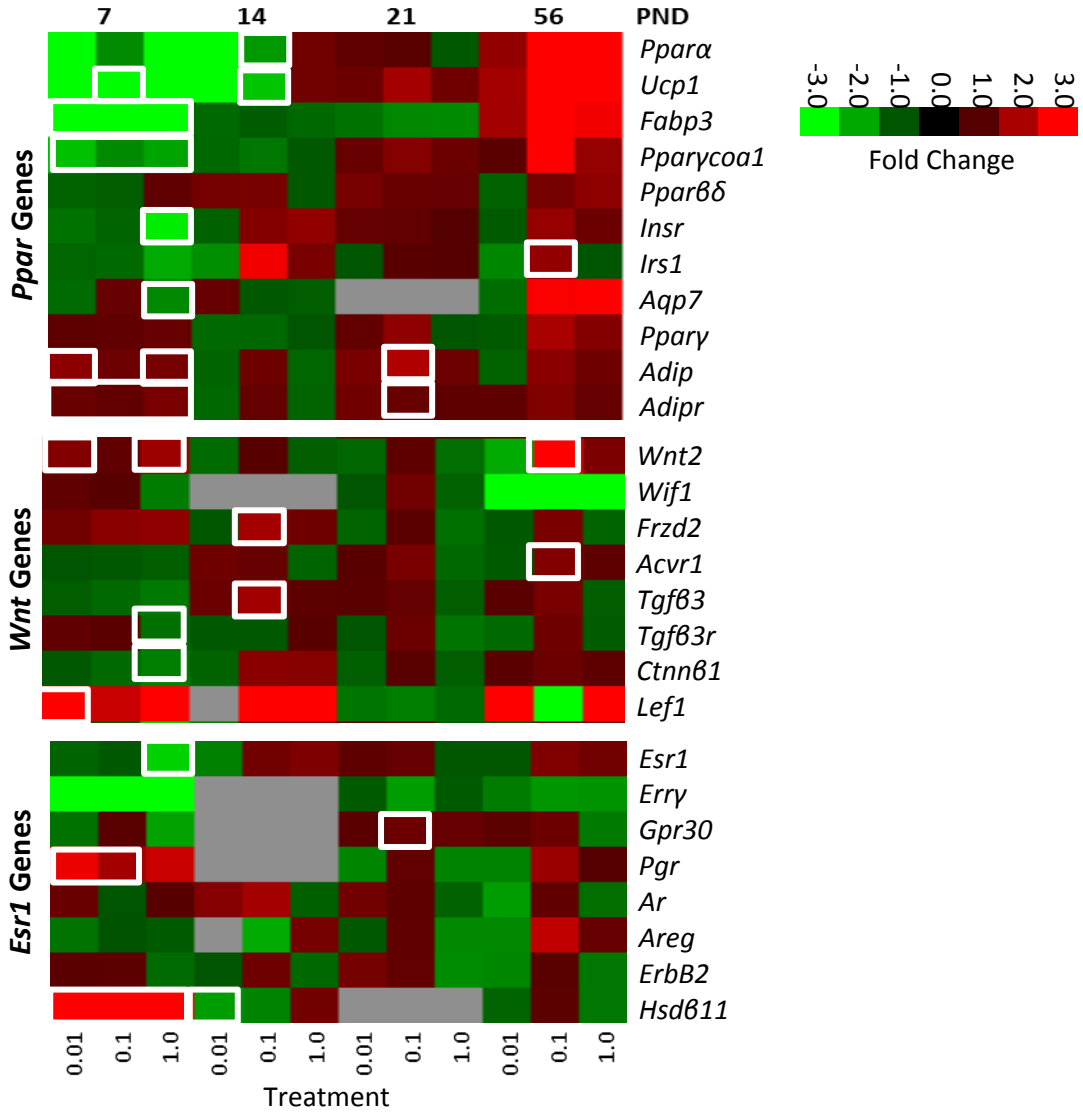

Figure 4-3. Altered gene expression in PFOA treated mammary glands by PCR analysis. The relative amount of each transcript was normalized to the amount of *Tbp* transcript in the same sample. Fold change was calculated using $2^{-\Delta ct}$; the results are expressed as the ratio of the value of control at each respective time-point (n=2-6). Red or green correspond to average up- or down-regulation, respectively. White outlined boxes indicate statistically significant change from controls, $p < 0.05$ by student's t test.

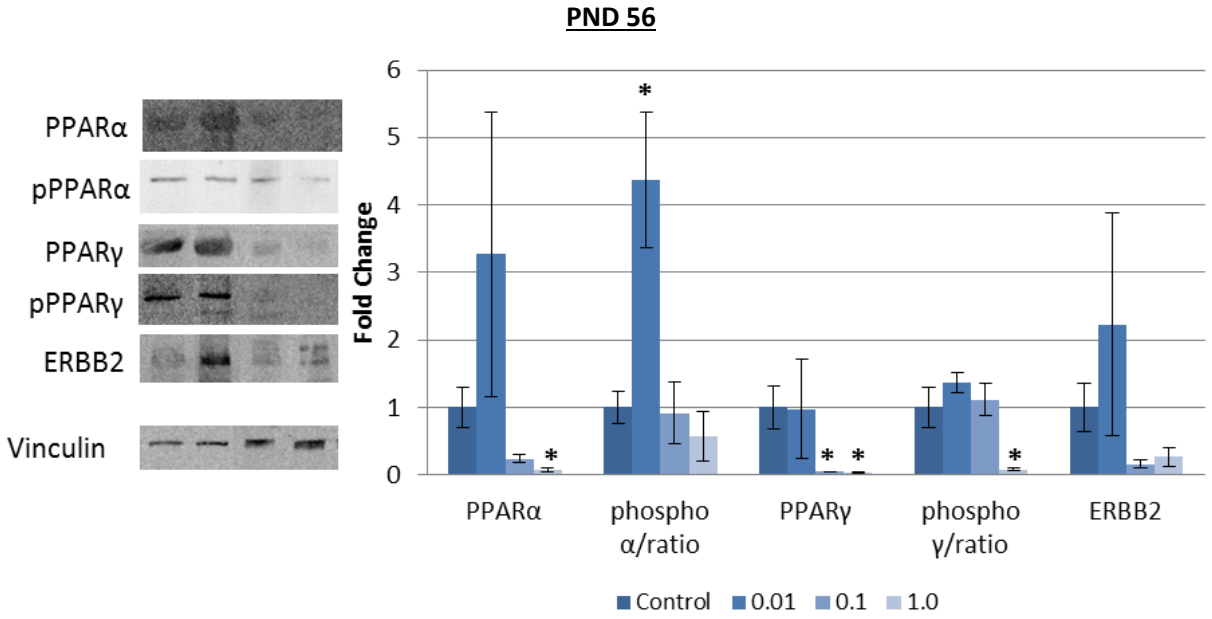

Figure 4-4. PFOA has latent effects on protein expression levels. (A) Representative blots of PPARα, PPARγ and ERBB2 for each treatment group. Levels of phospho-PPARα Serine 12 and phosphor-PPARγ Serine 112 were compared to their respective receptors and graphed (n=3/treatment). Quantification of blots (right panel) are means ± SEM. Statistical significance by Student's t test; *p≤0.05

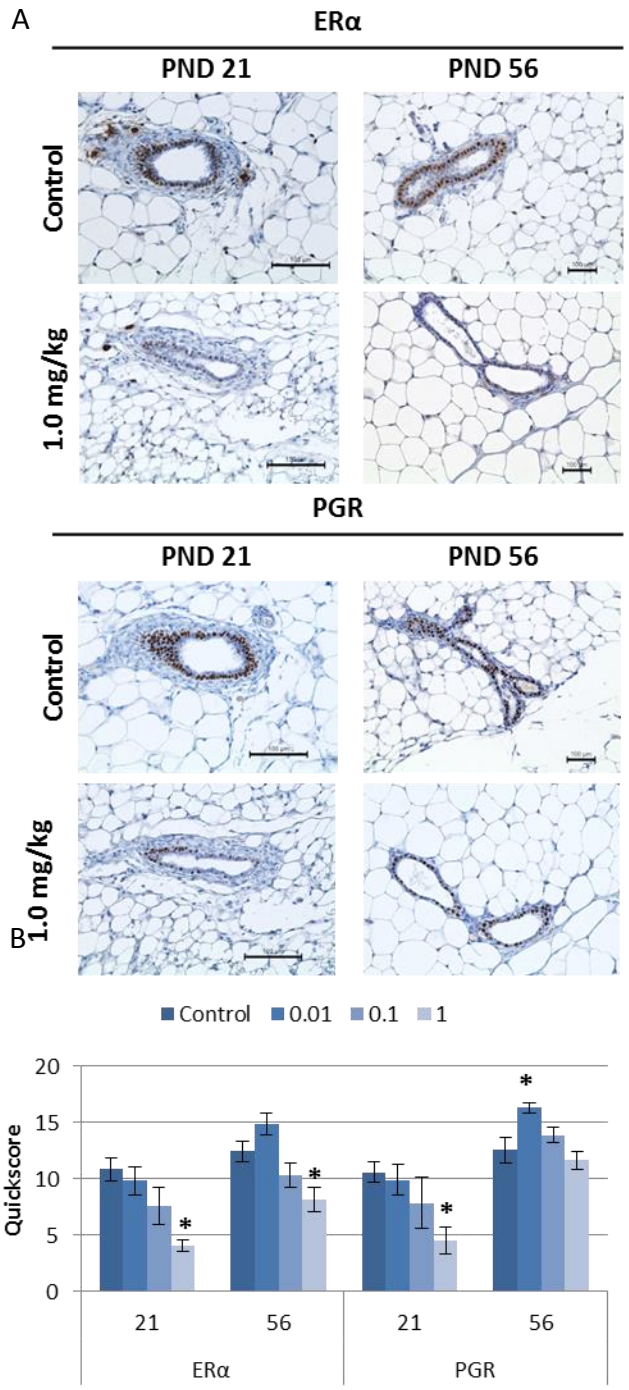

Figure 4-5. IHC stained sections for ERα and PGR. Representative images for each treatment group. (A) PFOA reduced ERα expression at PND 21 and 56 in the 1.0 mg/kg group. (Upper panel) PFOA reduced PGR expression at PND 21. PGR quickscores were increased in the 0.01 mg/kg group at PND 56. (B) Quickscores for all treatment groups. Data presented as mean ± SEM. Data were analyzed by ANOVA with Dunnetts post hoc test, *p≤0.05.

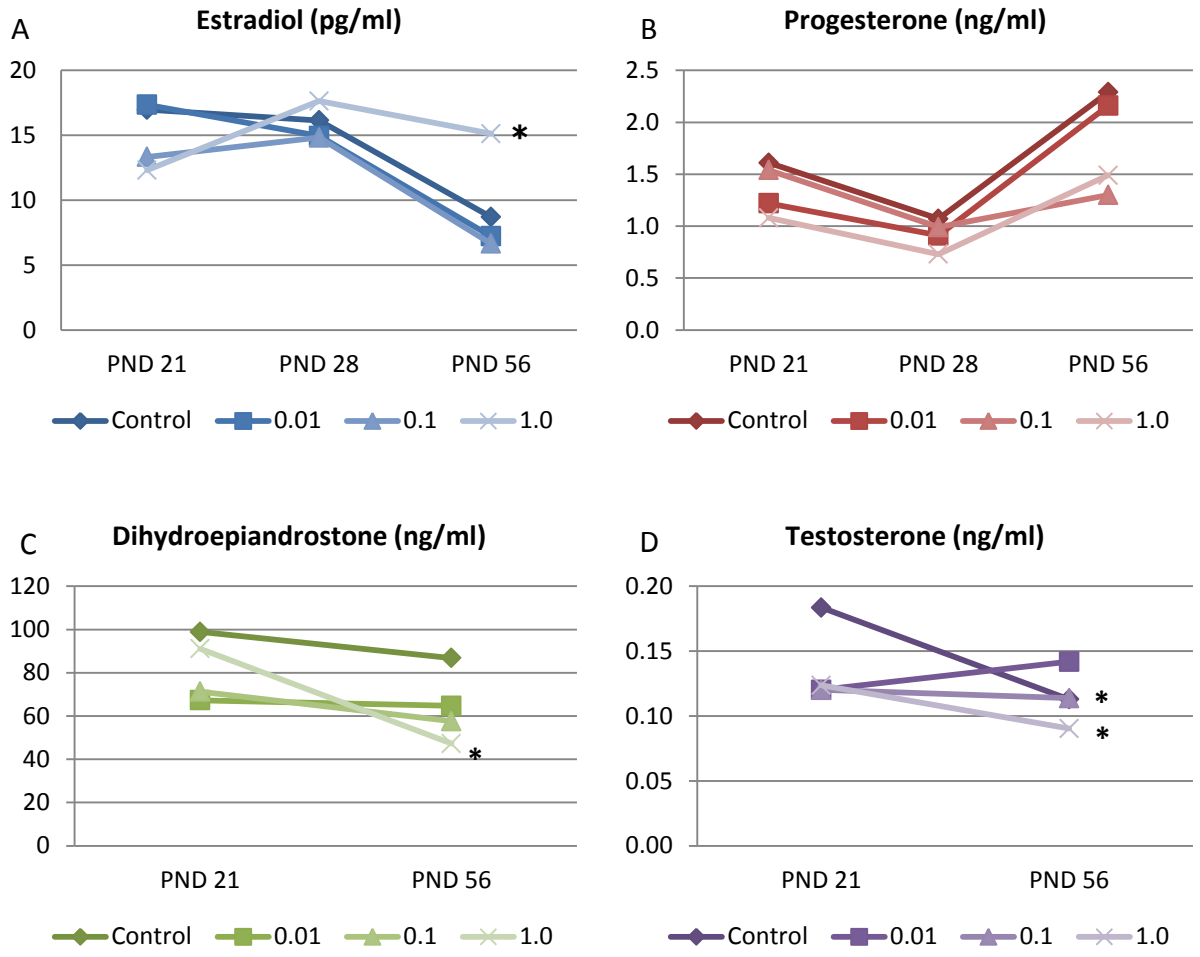

Figure 4-6. Serum hormone concentration. Serum collected from whole blood was analyzed for sex steroid hormones by ELISA n=18-22 for estradiol and progesterone; n=6-9 for testosterone and dihydroepiandrostone (DHEA). Data are presented at mean. Comparison of hormones over time (A-D). Significant effects compared to controls by Dunnetts, *p≤0.05. PFOA altered the change in hormones for estradiol (A), DHEA (C), and testosterone (D).

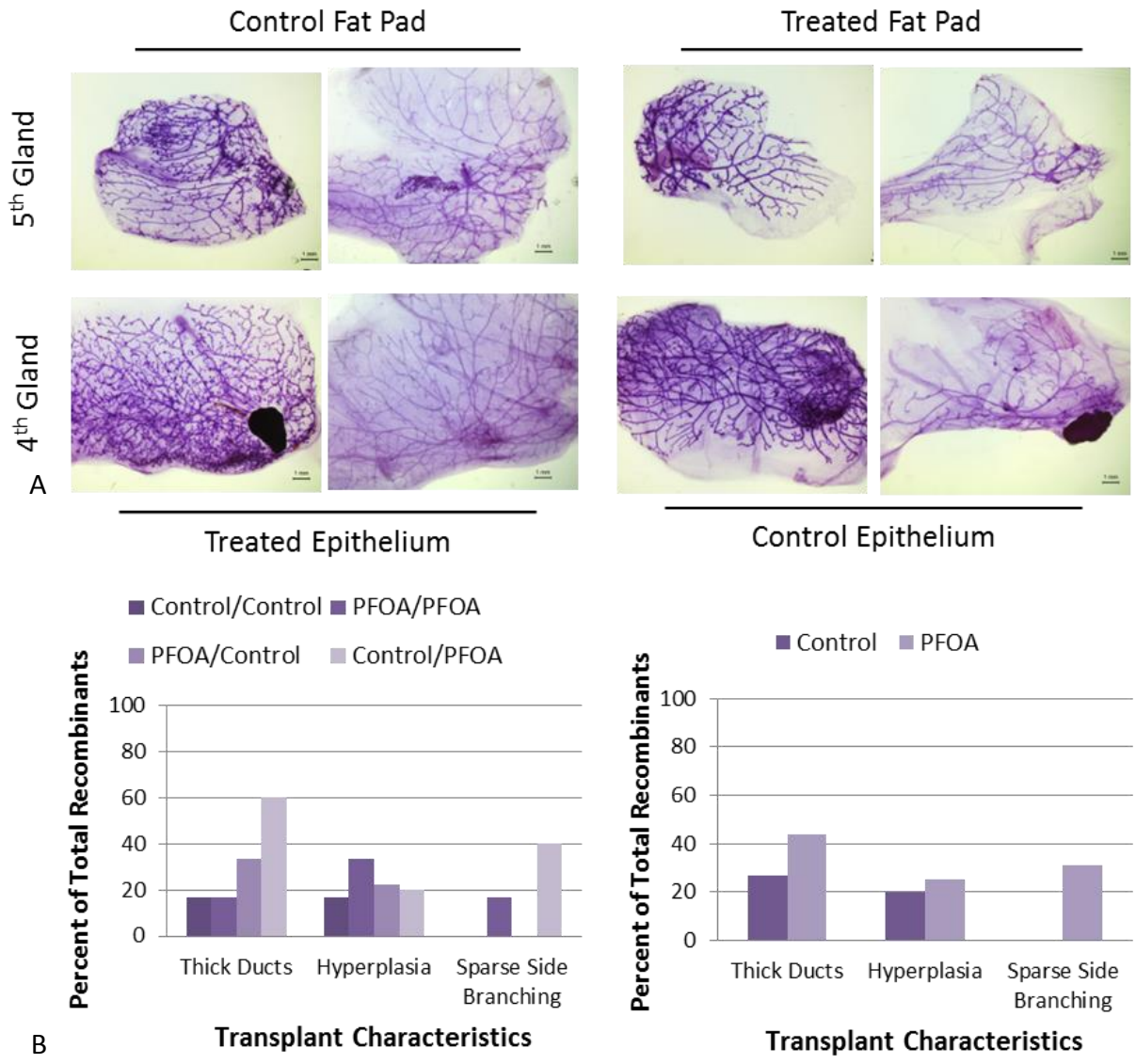

Figure 4-7. Mammary epithelial transplant recombinants of control and PFOA-treated compartments. (A) Representative images of recombinants of treated epithelium in control fat pads (right panel) and control epithelium in treated fat-pads (left panel). 5th glands are endogenous tissues; 4th glands are recombinants. (B) Overall transplant characteristics of total successful transplants for all combinations (left panel) and recombinants of fat pads (right panel).

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CHAPTER 5

Conclusions and Future Perspectives

SUMMARY OF RESULTS

This research project has produced data that describes the molecular and protein signaling pathways triggered by prenatal PFOA exposure which results in mouse mammary gland developmental delays and abnormal morphological effects. Low-dose prenatal PFOA exposure elicits effects in the mammary gland that present in early life as smaller mammary ductal trees with reduced branching density, longitudinal growth, change in longitudinal growth, and number of terminal end buds (TEBs). In early adulthood, PFOA-treated mammary glands appears to have distinctive disorganized and mis-directed growth patterns, reduced side-branching, thickened epithelial ducts due to increased collagen deposition, and increased number of active TEBs. PFOA-induced paracrine effects in the mammary gland included significant down-regulation of peroxisome-proliferator activated receptor gamma (PPARγ), PPAR-alpha (PPARα), estrogen receptor alpha (ERα), and progesterone receptor (PGR) proteins. Analysis of RNA and protein expression indicated a compensatory trend in RNA expression from early life to early adulthood and a dichotomous effect in changes in protein levels. The morphological and protein expression changes found following prenatal PFOA exposure in mice are similar to risk factors associated with increased breast cancer risk and/or poor prognosis in women (*1-8*).

PFOA altered mammary-specific endocrine related receptors and circulating hormone levels from adolescence through young adulthood. β-estradiol and dihydroxytestosterone were predicted upstream regulators of gene expression changes by microarray analysis. Notably, changes in Erα and Pgr

protein expression levels were observed in the absence of changes in RNA expression levels or changes in levels of circulating estradiol (E2) or progesterone (P4). Although others have noted delayed onset of vaginal opening with varied exposure paradigms and higher doses than those used here (*9, 10*), there were no differences in puberty-related endpoints that are related to the hypothalamic-gonadal axis. However, reductions in circulating testosterone (T) and dihydroepiandrostone (DHEA) were found at PND 21 and 56, respectively. These data, in addition to the significant changes in ERα and PGR protein, provide further evidence that PFOA has endocrine disrupting properties and can mediate paracrine receptor expression in mammary epithelial cells. Reductions in levels of DHEA have been associated with increased risk for premenopausal breast cancer (*11*). In addition, there may be sex-specific effects following prenatal PFOA due to its anti-androgenic like properties. As we were interested in changes in female mammary gland growth patterns, we did not investigate effects in male offspring. Data produced from this study indicate characterizations of male effects are warranted. Endocrine related effects have previously been reported at PFOA levels used in the CD-1 mouse studies described herein; Hines and colleagues (2009,(*12*)) reported increased body weights and serum insulin and leptin at mid-life ages following low dose prenatal exposures (0.01-0.1 mg/kg PFOA). Activation of Pparγ has been shown to decrease serum leptin, thus observed reductions in Pparγ may account for increases in serum leptin and insulin reported by Hines and coworkers (12). This data collectively suggest that low-dose prenatal PFOA exposure causes systemic disruption of the endocrine system.

Studies in a limited number of 129S1/SvlmJ *Pparα* wild type (WT) and knockout (KO) mice treated prenatally with PFOA demonstrated non-significant differences in mammary glands of KO PFOA-treated animals compared to controls (Appendix III). The differences at PND 56 were more marked in KO mice than in WT mice, which suggest that *Pparα* signaling may be protective against the mammary effects induced by prenatal PFOA exposure. PFOA-induced effects observed in 129 WT and *Pparα* KO

animals were not as pronounced as effects observed in CD-1 mice, indicating that there is strain specific sensitivity to PFOA-induced mammary effects as previously reported by Yang and colleagues (2010 (*10*)).

Due to the prenatal lethality of *Pparγ* KO, it would be difficult to find an appropriate KO model to definitively confirm that prenatal PFOA-induced mammary effects are dependent on *Pparγ* (*13*). Experiments with Pparγ agonist and antagonists or *Ppar* pan agonists may also be used, however others have had difficulties recapitulating PFOA effects with Pparα agonists possibly due to relative quick elimination of these compounds in comparison with the slow elimination of PFOA (*14*). Pparγ signaling/expression is also associated with other types of cancers (*6*). If *Pparγ* mediates PFOA-induced effects in other tissues, findings presented in this study may aid in the understanding of those effects.

It is important to note that abbreviated dosing exposures were utilized to minimize influence of confounding toxicities. Yet humans are likely to be exposed to variable levels of PFOA *in utero* and after birth/throughout life. In fact, work by White and coworkers (*15*) demonstrated that the addition of 5 ppb PFOA in the water supply of CD-1 mice increased the mammary developmental defects seen following prenatal 1 mg PFOA/kg exposures. Differences in the study design utilized and real-life exposures lengths should be considered when assessing the risks of this health outcome.

Most notably, gene, protein, and hormone level differences were observed in the lowest PFOA dose group of 0.01 mg/kg. When all mammary gland scores were combined from all studies, PFOA-treated glands in this group were significantly reduced at PND 21 (Table 5-1). Changes observed in the 0.01 mg/kg group were often contrary to those in the two higher dose groups, particularly at PND 56. This resulted in dichotomous trends for difference in protein expression levels when compared to controls (Figure 5-1). At PND 56, PGR nuclear protein expression in epithelium was increased and PPARα transcriptional activity was likely increased in comparison with controls in this treatment group. As shown in Figure 5-1, protein levels in the highest dose groups (0.1 and 1.0 mg/lg) tended to decrease over time while levels in the lowest treatment group (0.01 mg/kg) appeared to overcorrect for PFOA

effects (Figure 5-1). It is possible that there are temporal differences in when this effect manifests depending on dose and thus severity of molecular effects. Indeed, western blots revealed ERα protein levels were increased non-significantly at PND 7 in the 0.01 mg/kg group, but were significantly decreased at PND 21 compared to controls, and at the greatest magnitude compared to other treatment groups (Chapter 3). It is also possible that mice may recover from effects in the lowest exposure group and the compensatory changes noted may indicate initiation of the recovery process in the 0.01 mg PFOA/kg group.

Of great importance, serum PFOA concentrations were below level of quantification at PND56 although we still observed differences. This suggests that while measurements of serum PFOA concentrations help to determine previous exposures, serum levels do not necessarily correlate to odds or risks of effect. These results may explain why few studies find positive associations for effects in the breast and other tissues from collected epidemiological data and highlights the need for a complementary biomarker of PFOA exposure that better correlates with effects. We propose fatty acid binding protein 3 (FABP3), aka mammary gland derived growth inhibitor, as a potential biomarker to assess PFOA exposure and related effects in mammary tissues. Analysis of mammary gland scores along with RT-PCR data at PND 56 indicated that glands with poor development, as assessed by low mammary scores, were found to have significantly lower levels of FABP3 (-3.0 fold) in comparison with glands that had the best development, regardless of treatment. FABP3 is considered a tumor suppressor gene, and high levels are associated with terminally differentiated mammary epithelial cells (hyunh 1997). Lack of epithelial differentiation is associated with increased susceptibility to cancer and potentially, PFOA exposure. Utilization of FABP# as a biomarkers would require analysis of mammary tissues. A more non-invasive methods would be more optimal.

IMPACT OF FINDINGS

Data produced from this project has already influenced local regulatory levels of PFOA in North Carolina drinking water; The NC Scientific Advisory Board included data presented in Chapter 2, along with works conducted by others, in their assessment of PFOA health effects and subsequent recommendation to lower the states' drinking water maximum allowable levels for PFOA. In California in 2008, the State Senate approved a bill to ban the use of PFOA in food packaging; however, the bill was vetoed by then Governor Schwarzenegger in lieu of a more comprehensive review. Hopefully data produced from this project will serve as part of that review for current or future assessment in CA or other states. In the near future, the International Agency for Research on Cancer will review data to assess the risks of PFOA exposure in relation to the development of cancer. Findings produced from this project may aid in the assessment of PFOA's impact on human health. In addition, data produced from this study may be of importance to clinicians as PPARγ agonists are being extensively used to treat a variety of diseases, most notably type II diabetes and most recently breast cancer (*4*). There was a compensatory effect in RNA and protein expression levels following prenatal and lactational exposures to a weak agonist, a potential undesirable consequence for patients that require extended use of medications and an effect that should be considered.

As mentioned before, numerous known and speculative risk factors for breast cancer may be adversely modified by prenatal exposure to PFOA. Following prenatal PFOA exposure, mammary protein levels and circulating hormone were after PFOA serum concentrations reached background levels. Latent morphological changes in mammary glands observed included more TEBs and thickened ductal appearance. On the molecular level PFOA altered steroid hormones and receptors. Reductions in levels of DHEA have been associated with increased risk for premenopausal breast cancer in women (*11*). Additionally, reductions in PPARγ have been associated with mammary tumor formation and responsiveness to chemotherapeutics due to reduced ERα expression (*16*). The fact that low-dose

prenatal PFOA exposures altered paracrine and endocrine factors similar to known and speculative factors associated with breast cancer underscore the need to incorporate the mammary gland in standard regulatory evaluations for toxicants.

FUTURE PERSPECTIVES

In Chapter 4, mammary epithelial transplant surgeries were conducted to determine the growth potential of each compartment (stromal or epithelial) of PFOA-treated mammary glands. Recombinants of control and PFOA-treated tissues indicated stromal tissues dictated and directed growth patterns of the epithelial ductal tree. This suggested that PFOA directly affected the mammary stromal tissues to alter epithelial ductal growth patterns. In an effort to analyze mammary compartments independently, mammary tissue cell type isolation experiments were conducted to determine unique signaling pathways involved in prenatal PFOA-induced mammary effects. As described in Appendix IV, mammary adipocytes and epithelial cells were separated and isolated from whole mammary glands of control and PFOA treated mice. Those separated samples will be analyzed in the near future. Yet, evaluation of histological and IHC sections revealed a trend in the abnormal appearance of adipocytes from PFOA treated mammary gland; white adipocytes appeared smaller in size (Appendix III) compared to controls. Additionally, during collection of mammary glands during necropsy it was noted that PFOA-treated glands tend to be grossly thinner compared to controls, suggesting a decrease in fat cells or lipid components. Along with latent reductions in *Pparγ* (a known mediator of adipocyte differentiation), this data suggested that prenatal PFOA exposure affected adipogenesis in the mammary fat pad.

A review of current literature related to adipocyte influences on mammary gland development and carcinogenesis provided scientific basis for current and proposed projects. Ablation or reductions in white adipose tissue has a profound effect on mammary gland development. White adipose tissue KO mice have mammary glands with severely retarded growth. Mammary glands of A-ZIP/F1 transgenic

mouse models lacking white adipose tissue had smaller epithelial tree with thick ducks highlighted by pleiotropic epithelial cells and increased surrounding collagen and fibroblasts (*17*), some features that parallel appearance of prenatal PFOA-treated glands. The FAT-ATTAC mouse or "fat apoptosis through targeted activation of caspase 8" mouse developed by Scherer and colleagues, provides a unique tool to investigate the temporal influences of white adipocytes by administration of an analog that dimerizes with the adipocyte specific caspase 8 to initiate apoptosis of adipocytes (*18*). In females, FAT-ATTAC mice have been used to study the influence of mammary gland development (*19*). Targeted apoptosis of mammary gland starting at 2 weeks of life stunted the growth of the mammary ductal tree, significantly reduced numbers of TEBs, reduced secondary side branches, and reduced proliferation and apoptosis in TEBs of treated mice, features that parallel prenatal PFOA–induced mammary glands. In addition, targeted apoptosis of white adipose fat beginning at 7 weeks of life led to accelerated mammary gland maturation which parallels the stain-specific stimulatory growth patterns observed in C57Bl/6 mice following peri-natal PFOA exposures of 5 mg/kg (*10*). No changes in circulating estradiol were observed with either treatment in the FAT-ATTAC mouse, a "non" effect found in Chapter 4 and previously reported by others (*19, 20*). Take together these data suggest that PFOA-induced effects in the mammary gland are mediated by changes in adipogenesis. In addition, effects observed in PFOA studies mimic those observed in white adipose tissue KO and knockdown (KD) studies. The similarities have been summarized in Table 5-1.

Histological analysis of glands confirmed changes in mammary fat pad. White adipocytes were disorganized in their arrangement and appeared hypotrophic. Previous studies by Hines and colleagues reported increased total brown adipose tissue (BAT) weights in PFOA-exposed animals (11). To determine whether differences in BAT existed in mammary glands of PFOA-treated mice of current studies, IHC of uncoupling protein 1 (UCP1) was evaluated. UCP1, previously considered as brown adipocyte marker, revealed abundance of UCP1 expression in brown and surprisingly white adipose cells

(Appendix III, Figure 1). Evidence of "beige" or "bright" adipocytes has recently been described (*21-23*); Beige adipocytes are white adipocytes that acquire classical phenotypic and functional properties of brown adipocytes. Common traits of white adipocytes include large cell size and lipid droplets while brown adipocytes are multi-vacuolated with increased mitochondria and vascularization (*23*). Under stimuli, e.g. cold exposure or *Pparγ* activation, white adipocytes differentiate into 'beige' adipocytes; chronic *Pparγ* exposures result in increased 'beige' adipocytes and down-regulation of PPARγ levels *in vitro* (*21*), effects that are similar to observed prenatal-PFOA induced mammary changes.

For all the aforementioned reasons, the role of adipogenesis in prenatal PFOA-induced mammary effects will be investigated using SABiosciences adipogenesis arrays. Mammary adipocytes isolated from whole mammary glands in Block 3 of the Puberty study described in Chapter 4 will be used. Isolated cells from control and 1.0 mg/kg mammary gland from PND 7 and 21 will be analyzed for gene expression changes related to adipogenesis of WAT and BAT. The array to be utilized includes genes for non-canonical and Wnt/β- signaling as both pathways have been reported to modulate adipogenesis (*22, 24*). This array will help determine whether change in non-canonical and/or canonical signaling is involved in prenatal PFOA-induced mammary changes as this pathway was not fully explored in the current project.

Wnt signaling can be influenced by glucocorticoid levels (*22*). Hydroxysterioid 11 beta dehydrogenase 1 (*Hsd11β1*), an enzyme involved in the synthesis of glucocorticoids, is one of the few genes altered by PFOA at PND 7 and 14 from the array analysis. As this gene is not included in the adipogenesis array, future studies may wish to determine expression differences for Hsd11β1 in isolated mammary adipocytes from PFOA-treated and control glands. It is possible that observed differences for this gene in whole mammary tissues microarray analysis were driven by changes specific to the adipocytes. Lastly, future studies may wish to determine the role of mitochondrial dysfunction in prenatal PFOA induced changes in the mammary gland. Changes related to mitochondrial dysfunction

were involved in PFOA-induced gene changes in the mammary gland from array analysis, yet this pathology was not pursued. Recent analysis of prenatal PFOA-treated livers reported increased cellular mitochondria with abnormal morphology (*25*), which suggest PFOA may potential mediate effects in other tissues beyond the liver via this mechanism of action. In addition, Pparγ activation increases mitochondrial biogenesis, which is a part of the trans-differentiation process from white adipocytes to 'beige' adipocytes (*21, 23*). A recent paper by Santidrian and colleagues has noted the critical function of mitochondria in breast tumor progression (*26*). Connections between Ppar, mitochondria, and cancer was recently reviewed (*27*). Based on array data from this project and data in the literature, future studies should explore changes in mitochondrial dysfunction in prenatal PFOA-induced mammary changes.

Although it is concluded that prenatal PFOA-induced mammary gland changes are dependent upon *Pparγ*, mammary effects are also thought to be mediated by *Pparα*, albeit to a lesser extent. PPARα protein levels were reduced at PND 56 (Chapter 4). As many of the gene targets of Pparγ and Pparα are can also be regulated by Pparδ, this subtype may also mediate some PFOA mammary effects. PFOA structurally resembles the natural fatty acid capyrlic acid, aka octanoate. Caprylic acid is a medium chain fatty acid (MCFA) and has been shown to be a weak pan-Ppar agonist (*28*). However, that study showed that the ligand binding domain (LBD) for Pparγ can be occupied by 3 molecules of MCFA and MCFAs were partially selective for Pparγ compared to other subtypes. MCFAs were shown to attenuate adipogenesis initiated by rosiglitazone, a potent Pparγ agonist (*28*). PFOA may act similar to caprylic acid on Pparγ by competitively binding to the LBD to inhibit or minimize effects of endogenous/exogenous ligands. In would be interesting to administer PFOA and rosiglitazone, or another Ppar agonists, concomitantly to observe if effects are attenuated or synergistically additive.

To determine if low-dose prenatal PFOA exposures influence susceptibility for the development of breast cancer, future studies in mice are warranted. A study of this nature would decisively answer

whether PFOA exposures alone can influence breast cancer risk. Yet, as with other single chemical exposure studies, those results would not accurately portray “real world” health outcomes as humans are exposed in mixtures and chemicals can synergistically interact to manifest effects.

With the assured emergence of environmental exposures to new chemicals as an unintended consequence from the phase-out of PFOA and other endocrine disrupting compounds, non-invasive biomarkers of breast disease and standardized, objective measures are needed to assess hazards and risk for the development of disease. Future investigations may seek to identify serum or urinary biomarkers that could predict mammary gland effects mediated by PFOA and/or like chemicals.

Table 5-1. Collective Mammary Gland Scores from all Studies

	PND 7	PND 21	PND 56
Control	2.93 ± 0.12 (27)	2.89 ± 0.12 (32)	2.92 ± 0.14 (26)
0.01 mg/kg	2.48 ± 0.17 (21)	**2.39 ± 0..12 (30)****	2.58 ± 0.14 (19)
0.1 mg/kg	**2.20 ± 0.19 (17)****	**2.31 ± 0.14 (28)****	**2.34 ± 0.17 (22)***
1.0 mg/kg	**2.03 ± 0.12 (19)*****	**2.03 ± 0.13 (28)*****	**2.07 ± 0.15 (18)*****

Note: Mammary gland scores from all studies combined. Data presented as mean ± SEM (n). Significant differences compared to controls analyzed by ANOVA and Dunnetts post hoc tests, *p<0.05, **p<0.01, *p<0.001.

Table 5-2. Toxicities of WAT KO or KD mice or PFOA

	PFOA Effects	WAT KO/KD
Hepatomegaly	+	+
Anti-inflammatory Response	-	-
Enlarged Kidney	+	+
Enlarged Spleen	-	+
Increased Neonatal Mortality	+	+
Estradiol	No change	No change
Mammary Gland Delays	+	+
Thick Mammary Ducts	+	+
Reduced Terminal End Buds	+	+

Note: Toxicity of PFOA and WAT KO or KD Studies (*1-3*)

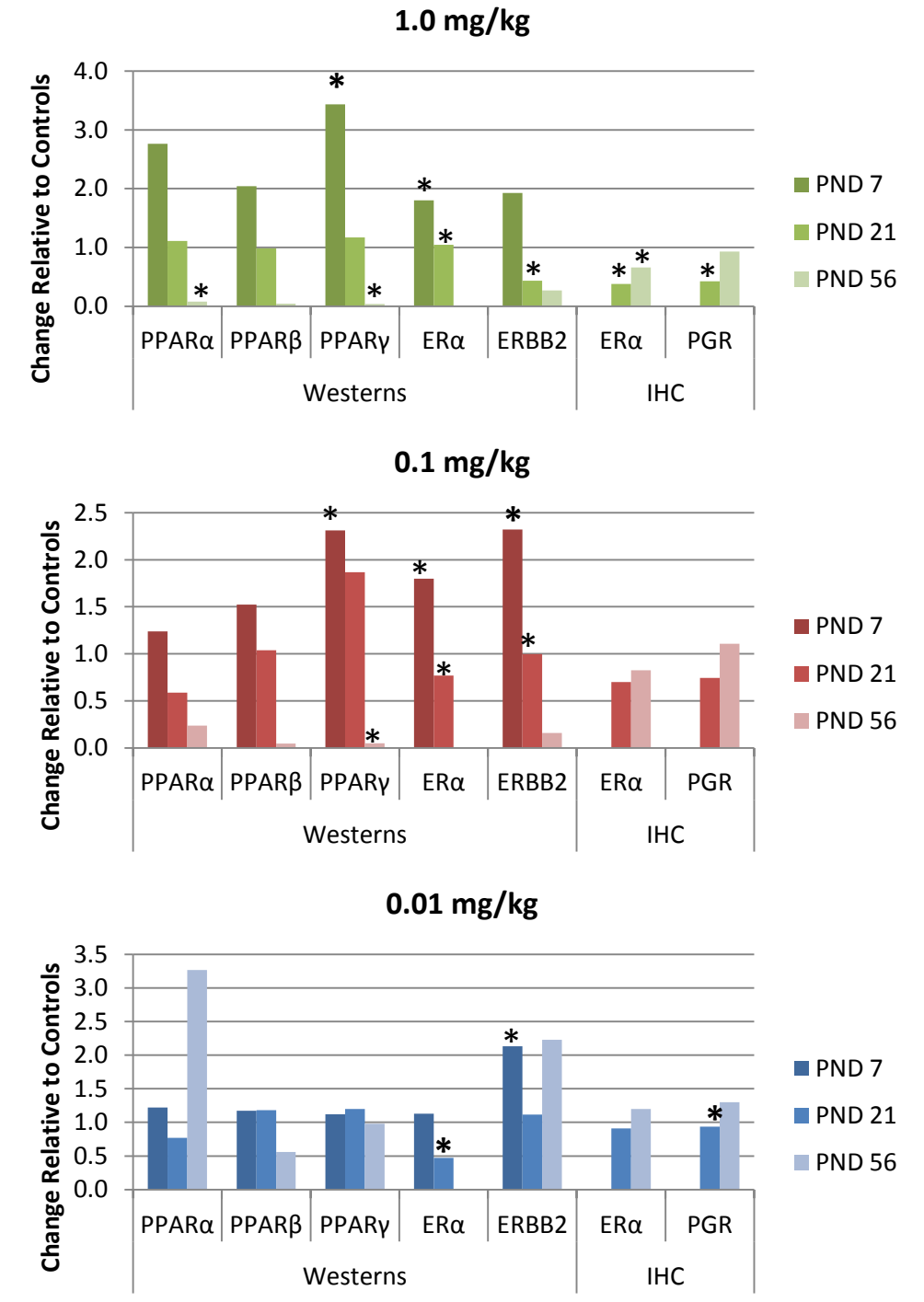

Figure 5-1. Trend in protein fold change by treatment group. Protein levels of 0.1 and 1.0 mg/kg group tended to decrease over time; protein levels of 0.01 mg/kg group appeared to rebound. Mean fold change of protein compared to controls. Significant effects compared to controls by Students t tests. *p≤0.05

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APPENDIX I

RT-PCR Gene Primers

Table AII-1. Gene Primers for all studies

Gene	Forward Primer	Reverse Primer
Pparα	TGGCAAAAGGCAAGGAAG	CCCTCTACATAGAACTGCAAGGTTT
Pparβδ	CCGCCCTACAACGAGATCA	GCTCTCGGACTGTCTCCACTGT
Pparγ	CCCACCAACTTCGGAATCAG	AATGCTGGAGAAATCAACTGTGGTA
PparγCoa1	GATGACAGTGAAGATGAAAGTGATAAACT	GAAGGCGACACATCGAACAA
Fabp3	CCCCTCAGCTCAGCACCAT	GAAAATCCCAACCCAAGAATG
Ucp1	GGAGGTGTGGCAGTGTTCATT	TGGGCTTGCATTCTGACCTT
Adip	GACACCAAAAGGGCTCAGGAT	TGGGCAGGATTAAGAGGAACA
Adipr	ATAACGGGCCATCCATTTTTG	TGAAGCCTGGACGTACTTCCA
Aqp7	CTGGATGAGGCATTCGTGACT	GGCTCGGTCCCTTGAAGTG
Insr	GGTCTGATTGTGCTATATGAAGTGAGC	CGGACTCGAACACTGTAGTTTCC
Irs1	CGAGAGCTGTTTCAACATCAACA	CGCGGCAATGGCAA
Wnt2	GGCTCCTGTACTCGAGGACATG	GAGATAGTCGCCTGTTTTCCTGAA
Wif1	AAGCAAGTGTAAGTGCCCGAAA	CTCTCGACTGGCACTTGTTG
Frzd2	GCCTGTGGAAGCTGTTGGAT	GCGAGGAGAAGGGAAATAAAAC
Acvr1	CCCCACGGGAAGCTCAA	TGCAGCCGATATTGCTGATTA
Tgfβ3	TGTGTACGCCCCCTTTATATTGA	GGTTCGTGGACCATTTCC
Tgfβ3r	TAAGCGAAGGGATTATTAGCAAGGTA	CCAATGTGCTGGGTGTTCTG
Ctnnβ1	GGGCAACCCTGAGGAAGAA	AAAGCCTTGCTCCCATTCATAAA
Lef1	TCCCGCACTCAGTCTTCCA	AGCATCCGAGACAGCAAGAA
Ar	GGATGGGCTGAAAATCAAAA	TGAGCAGGATGTGGGATTCTT
ErbB2	AATCAACGAAGGCGACAGAA	CCGCATCTGAGCCTGGTT
Hsd11β1	GGGAAAATGACCCAGCCTATG	GGTGGAAAGAACCCATCCA
Hsd17β11	GACGAACAGGAGTGCGAACA	ATTGGTGCTTGGGTTCTTGATG

Hsd17β12	GCTGCCTGGCATGGTAGAA	CAACAATGGAACTGGGAGCAT
Tbp	CAGCCTTCCACCTTATGCTC	TGCTGCTGTCTTTGTTGCTC

Taqman Primers	
Gene	
Esr1	Mm00433149
Errγ	Mm00516267
Gpr30	Mm02620446
Pgr	Mm00435628
Tbp	Mm00446971

Note: Gene primers used for studies in Chapters 3 and 4. Primers designed in Primer Express 3.0 were validated for efficiency using serial dilutions of cDNA and melting curves of primer products. Primers had efficiencies of 1.8-2.2 and only one product on the melting curve.

Appendix II.

Comparisons of PFOA Treated Mammary Glands from Wild-type and Knock-out *Pparα* Mice

INTRODUCTION

The aim of this study was to determine the role of peroxisome proliferator activated receptor alpha (*Pparα*) in perfluorooctanoic acid (PFOA) induced mammary gland delays. In vitro studies show that PFOA is a weak agonist of Pparα (*1*). Many have utilized the potent Pparα agonist Wy 14,643 and/or Pparα knock-out (KO) animals to determine its involvement in liver toxicity (*2-4*). From those studies, it was determined that many PFOA-induced liver effects, particularly the formation of PFOA-induced liver tumors, are dependent on *Pparα* activation. However, there is increasing evidence that there are *Pparα*-independent effects in the liver (*2-6*). Others have investigated PFOA effects in the mammary gland with *Pparα* KO mice, but the role of *Pparα* in PFOA-mediated mammary gland effects remains unclear (*7, 8*). To determine the role of *Pparα* following prenatal PFOA exposure, we utilized pregnant *Pparα* KO and wild-type (WT) animals and evaluated the development of mammary glands of female offspring. We originally hypothesized that PFOA-induced mammary gland effects were independent of *Pparα*, however results from our previous studies reported in Chapter 3 and 4, suggest *that Pparα* may have a minimal role in this effect.

MATERIALS AND METHODS

Animals

Male and female adult 129 SvImJ (referred to as 129 WT) and 129S4/SvJae-*Ppar*αtm1Gonz/J (referred to as *Pparα* KO) mice were purchased from Jackson Laboratories. Animals were allowed to acclimate to the environment for weeks and then were bred to increase female animal numbers. Additional male and female adult 129 WT and *Pparα* KO mice were generously donated by Dr. Abbott of the US Environmental Protection Agency. Both sets of animals were transferred to Alion Contracting facilities (Durham, NC). Genetically confirmed strains were kept in separate rooms at all times. Breeding, dosing, and animal experiments were carried out at the Alion facilities. Breeder male mice were singly housed; female mice were housed with litter mates.

Chemicals

Ammonium perfluorooctanoic (APFO, the ammonium salt of perfluorooctanoic acid (PFOA)) was purchased from Fluka (Steinhiem, Switzerland) or Sigma Aldrich (77262, Lot# 0001414284, Steinhiem, Switzerland). PFOA dosing solutions were prepared in deionized water in advance and were given at a volume of 10 µl solution/g body weight (BW) by the animal staff at Alion.

Experimental Design

A pilot study of 129 WT and Pparα KO pregnant dams were dosed with PFOA using the same dosing levels as in the late gestation exposure study (0.01, 0.1, and 1.0 mg/kg; Chapter 2 (*9*)). Mammary glands were removed on PND 7-21 and evaluated for development based on our scoring method described in Macon *et al*. 2011 (*9*). Difference between PFOA treated glands and their respective controls were unremarkable. We did note that mammary glands from 129 WT and *Pparα* KO controls were much less developed in comparison with CD-1 control mice of the same again. Thus, the experimental design was altered to evaluate glands at a more mature time of PND 56.

In a another set of animals, male and female mice within a strain were placed in cages in the evening and allowed to breed. In the morning, male mice were removed from cages and females were checked for copulatory plugs. Plug positive animals were considered to be GD 0. Plug positive females were housed individually and divided into 2 groups within each strain. 129 WT plug positive females were gavage dosed with vehicle (deionized water) or 0.6 mg PFOA /kg of body weight (BW)/ day from GD 2-18. *Pparα* KO plug positive females were dosed with vehicle or 1.0 mg/kg BW/day from GD 2-18. These doses were chosen as 129 WT mice are more sensitive to PFOA-induced developmental effects and have increased prenatal and postnatal loss of offspring at 1.0 mg/kg/day; similar effects were seen in *Pparα* KO mice at 3.0 mg/kg (*10*). We also lost many litters (from prenatal loss and postnatal maternal cannibalism), which was likely due to the stress of oral gavage dosing. Therefore we shifted our dosing time from GD 1-17 to GD 2-18, as 129 mice have gestational lengths of 20 days, in attempt to decrease feta loss. Plug positive females were weighed every 2 days to determine appropriate dosing amounts and as an indicator of pregnancy maintenance. Plug positive mice that were not pregnant and dosed with deionized water (controls) were placed back into the breeding pool as there was a limited supply of animals. Non-pregnant dosed animals were euthanized. All pups were kept in their respective litters; there was no equalization. Weaning occurred at 4 weeks of age. At weaning male and female offspring were separated and singly housed.

Necropsy

Female offspring were sacrificed on PND 21 and 56 by decapitation as approved by the Animal Safety Protocol. A greater percentage of the litters were kept until PND 56 to ensure there would be appreciable differences in mammary gland growth patterns. Body weights and liver weights were recorded. One set of 4^{th} and 5^{th} mammary glands were collected and mounted on slides to be prepared as whole mounts as described in Macon *et al*., 2011 (*9*). The contralateral 4^{th} and 5^{th} mammary gland was collected in tubes, placed on dry ice, and stored at -80°C.

Statistical Analysis

Pair-wise comparison of weights and developmental mammary gland scores within a strain were calculated in Microsoft Office Excel using Student's t-tests. P values ≤0.05 were considered statistically significant. For the *Pparα* KO mice at PND 21, for all measurements other than body weights n=1 and statistical significance could not be determined.

RESULTS

Body weights were increased, albeit non-significantly, in the PFOA treated 129 WT mice compared to vehicle controls at PND 21. Absolute and relative liver weights were significantly increased in PFOA treated 129 WT mice compared to controls at PND 21, an indication of hepatomegaly and liver toxicity. Due to low n, we could not determine the significance of absolute or relative liver weights, although it appears that relative liver weights were increased in PFOA treated *Pparα* KO mice relative to controls (0.048g/g vs 0.041g/g, respectively).

At PND 56 *Pparα* KO mice had reduced body weights, liver weights, and net body weights compared to controls (Table A II-1). This suggests that PFOA can have an overt toxicity in mice independent of *Pparα*.

Some differences were observed in mammary glands of female mice. PFOA-induced effects in 129 WT mice appeared more severe; however there was wide variability in mammary gland development of 129 WT controls. Variability in control mammary gland growth made it difficult to attribute mammary gland effects to PFOA exposure. At PND 56, mammary glands of PFOA-treated *Pparα* KO mice did appear to be altered by PFOA exposure as reflected by developmental scores, albeit scores were not statistically significant (Table A II-2; Figure A II-1).

DISCUSSION

From these low-powered studies in 129 WT and *Pparα* KO mice, we were able to gain insights into the role of *Pparα* KO in PFOA-induced mammary gland effects. It was difficult to interpret our results, despite great efforts on our part and our contract laboratory, as we were unable to produce enough offspring to produce a robust study. However, we were able to produce results in *Pparα* KO mice exposed to PFOA (Figure AII-1). We observed attenuated mammary gland changes in *Pparα* KO mice at PND 56 suggesting that prenatal PFOA-induced mammary effects are dependent on *Pparα* but are not required, that is, *Pparα* has a minimal role in mediating PFOA-induced effects in the mammary gland.

Table AII-1. Weight Indices of Female Offspring

Treatment	Strain	Age	Weight (g)	Liver (g)	Net (g)	Relative Liver (g/g)
Control	WT	21	9.19 ± 0.70 (4)	0.408 ± 0.004 (3)	9.47 ± 0.15 (3)	0.0414 ± 0.001 (3)
0.6 mg/kg	WT	21	11.95 ± 0.82 (2)	0.585 ± 0.045 (2)*	11.37 ± 0.78 (2)*	0.0489 ± 0.000 (2)*
Control	KO	21	8.52 ± 0.44 (2)	0.330 (1)	7.75 (1)	0.0408 (1)
1.0 mg/kg	KO	21	9.05 ± 0.39 (2)	0.450 (1)	8.99 (1)	0.0477 (1)
Control	WT	56	18.62 ± 0.70 (6)	0.761 ± 0.025 (6)	17.86 ± 0.68 (6)	0.0410 ± 0.001 (6)
0.6 mg/kg	WT	56	19.10 ± 0.47 (4)	0.732 ± 0.039 (4)	18.37 ± 0.43 (4)	0.0382 ± 0.001 (4)
Control	KO	56	19.65 ± 0.40 (6)	0.883 ±0.039 (6)†	18.77 ± 0.39 (6)	0.0449 ± 0.002 (6)
1.0 mg/kg	KO	56	17.45 ± 0.57 (3)*	0.708 ± 0.038 (3)*	16.75 ±0.54 (3)*	0.0405 ±0.001(3)

Note: Body weights, net weights, liver weights, and relative weights for female offspring from the 129 WT and *Pparα* KO study. Data presented as mean ± SEM (n). Significant difference compared to respective strain controls by Students t test, *p≤0.05. Significant differences of *Pparα* KO mice compared to 129 WT mice by Students t test, †p≤0.05.

Table AII-2. Mammary Gland Scores

	WT		KO	
	Control	0.6 mg/kg	Control	1.0 mg/kg
Week 3	2.50 ± 0.42 (4)	2.50 ± 0.50 (2)	3.13 ± 0.38 (2)	3.25 ± 0.75 (2)
Week 8	2.69 ± 0.37 (6)	2.28 ± 0.46 (4)	3.23 ± 0.25 (6)	2.33 ± 0.54 (3)

Note: PFOA mammary gland scores for Pparα WT and KO female offspring. Data presented as mean ± SEM (n). Data analyzed for significant differences by Students' t test; none were found.

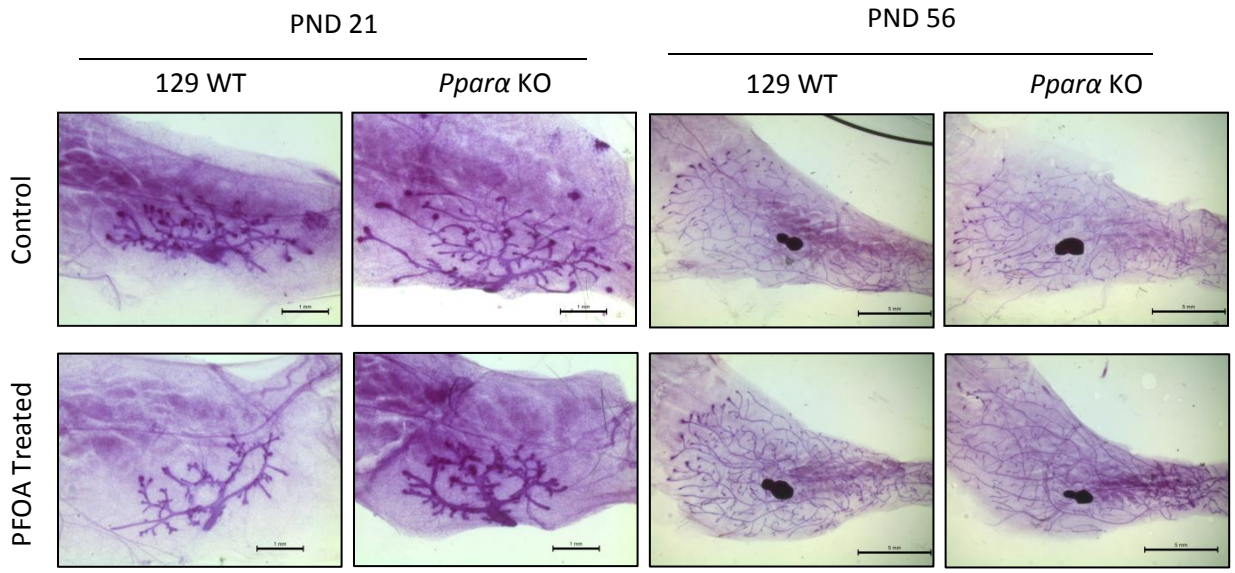

Figure AII-1. PFOA induced mammary gland morphological effect in 129 WT and *Ppar*α KO mice at PND 21 and 56. (PND 21 129 WT n=2-4; PD 21 *Pparα* KO n=2; PND 56 129 WT n=4-6; PND 56 *Pparα* KO n=3-6. Differences were detected in PFOA treated *Pparα* KO mice compared to controls but did not produce significant effects.

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APPENDIX III.

Immunohistochemical Staining for Current and Future Perspectives

Immunohistochemical (IHC) staining of mammary gland sections at PND 56 was conducted to determine the expression levels of various proteins. Although IHC staining was useful, expression of some protein were difficult to interpret, and were not included or shown in chapters of the dissertation but may be more useful in future studies. The expression of peroxisome-proliferator activated receptor alpha (PPARα), PPARγ, and uncoupling protein 1 (UCP1) were investigated in mammary sections to assess differences in adipocytes protein expression.

PPARγ was only detected in the nuclei of adipocytes. It appeared that there was less expression in PFOA-treated glands but it was difficult to assess differences in staining with any certainty. Western blots of whole cell lysates were used to more accurately quantify differences in expression levels.

PPARα was ubiquitously detected in the cytoplasm and nuclei of adipocytes and epithelium of all cells. Due to the strong intensity of staining throughout sections, it was difficult to assess differences in staining. As with PPARγ, Western blots of whole cell lysates were used to more accurately quantify differences in expression levels.

UCP1 was initially used to determine differences in brown adipose tissues (BAT) of the mammary gland. Increases in whole body BAT weight were increased in mice prenatally exposed to PFOA (*1*). We did detect increases in Ucp1 expression levels in PFOA-treated glands due to expression in white adipocytes. These 'beige' adipocytes are indicative of transdifferentiation of white adipose tissues (WAT) and metabolic adaptation.

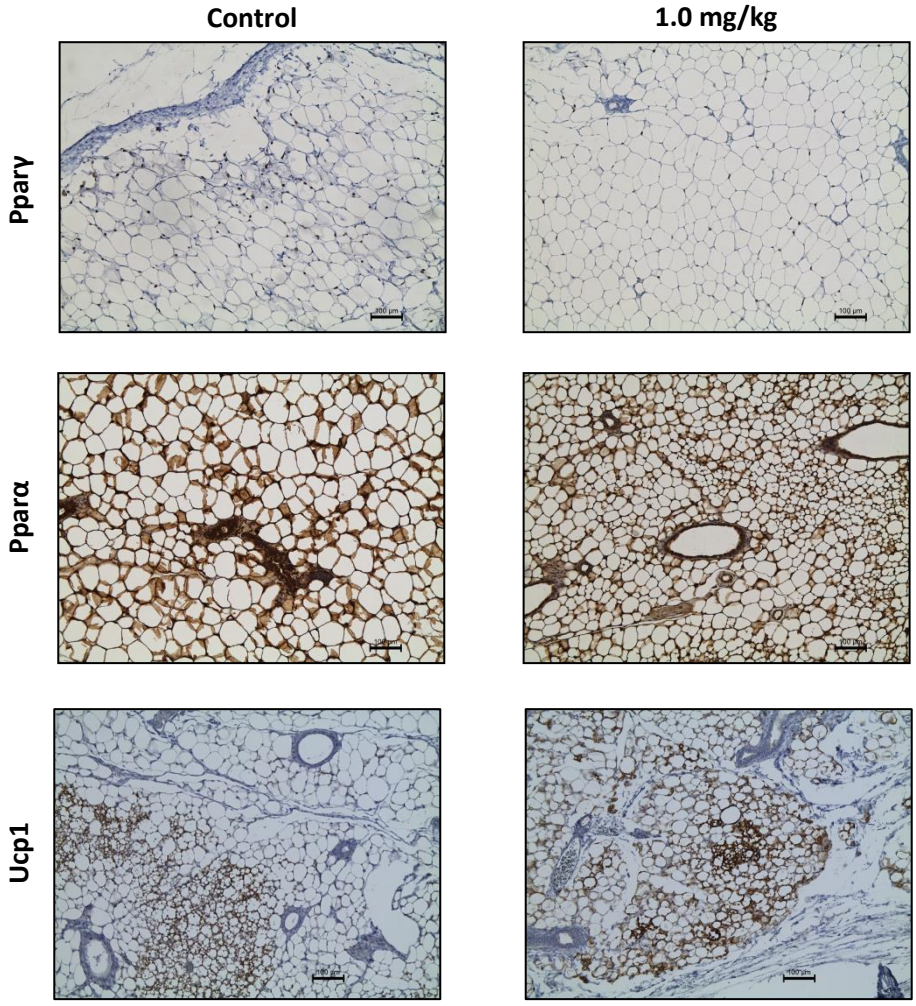

Figure A III-1. IHC sections for PPARγ, PPARα, and UCP1 at PND 56. Images are representative of each treatment group. Differences in section were difficult to appreciate through qualitative assessments. Notice adipocytes in all PFOA-treated sections are smaller in comparison with control sections.

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APPENDIX IV

Isolated Mammary Gland Adipocytes from PFOA Treated Animals

INTRODUCTION

Normal mammary gland development and differentiation involves complex coordination of many interactions including: branching morphogenesis, stromal-epithelial interactions, programmed cell death, cell proliferation, immune cell influences, extracellular matrix-remodeling and hormonal influences (*1*). The majority cells of the MG are epithelial cells, adipocytes, fibroblasts, macrophages, and eosinophils (*2*-*7*), the latter four comprising what many refer to as the stroma. Stromal tissues of the mammary glands can heavily influence normal branching patterns and outward epithelial growth into the mammary fat pad, serving as a framework for the structure and function of the tissue. This was apparent in the results that we obtained from our mammary epithelial transplant recombinants as the growth patterns of transplanted epithelium paralleled that of the intact gland. However, one of the characteristics of all glands transplanted into PFOA-treated fat pads were the reductions in side branching. Naylor and colleagues (2002) reported that stromal factors are responsible for side-branching patterns as well as sex hormone receptor expression (*8*). These results suggested that the morphological effects of PFOA in the mammary gland are regulated by the stromal tissues rather than the epithelium. Given that there is a cooperative relationship between mammary epithelium and stromal cells, it was advantageous to separately analyze mammary samples by cell type. We hypothesized that PFOA directly affected mammary stromal cells to indirectly affect epithelial growth patterns. To determine the

localization of PFOA effects, we separated mammary adipocytes, fibroblasts, and epithelial cells from whole glands.

MATERIALS AND METHODS

We isolated mammary specific tissue types using a protocol modified from Smalley (2010,(*9*)) and Sleeman (2006, (*10*)) to determine the tissue specific molecular changes involved in PFOA-induced mammary gland alterations. Mammary tissues removed during necropsy were washed in ethanol and placed in Leibowitz medium (L15, Invitrogen; Carlsbad, CA) containing 10% fetal bovine serum, minced with sterile scissors, digested in collagenase digestion mix for 1 hour at 37°C, and spun down to pellet the epithelial organoids. The top layer containing adipocytes was collected and immediately placed on dry ice and stored at -80°C. The pelleted epithelial fragments were resuspended in L15, centrifuged, washed, incubated in red cell lysis buffer (Sigma; St. Louis, MO). The epithelial fragments were then plated in tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) adhering the fibroblasts to the plastic flask for 1 hr at 37°C 5% CO_2/5% O_2. The flasks were shaken, rinsed, and the medium was transferred to a tube to collect the epithelial sample and stored at 4°C overnight. Tissue culture flasks were rinsed with trizol and fibroblast enriched samples were placed on dry ice and stored at -80°C.

The following day, epithelial cell enriched samples were washed in versene and resuspended in Joklik's medium (Sigma-Aldrich), incubated at 37° C for 15 min, resuspended, trypsinized, incubated with DNase (Sigma-Aldrich) for 5 minutes at 37C°. Samples were filtered through 40 μm cell strainer to obtain single cell suspension then pelleted and resuspended in LI5/10% FBS. Filtered cells were counted on a Beckman Coulter Cell Counter (Beckman Coulter). Samples were then incubated with fluorescent labeled primary CD 24 (FITC rat anti-mouse), and CD 45 (PE-Cy5 Rat anti-mouse; BD Pharmigen) antibodies: high DAPI accounted for dead cells, CD45 bound to leukocytes, and low CD24 staining for

basal cells and high CD 24 for luminal epithelial cells. In addition control samples were stained with IgG FITC and IgG Pe Cy5. Stained cells were transferred to the Flow Cytometry Core facilities at NIEHS. Using gated analysis on a Beckson Dickinson LSR II Flow Cytometer we were able to characterize our isolate mammary epithelial cell suspensions.

RESULTS

Using the isolation technique, adipocytes and epithelial cells were isolated from whole mammary gland tissues. In anticipation of low cell numbers from mammary isolations at PND 7, collected mammary adipocytes and epithelial cells were pooled from controls and PFOA-treated samples and plated in 6 well plates to determine if any viable cells were collected. Images of the cell cultures can be found in Figure A IV-1 and provide evidence that we were able to separate specific cell types with this isolation technique.

Isolated mammary epithelial samples were analyzed for cell type specific markers with flow cytometry. A sample from PND 21 is shown in Figure A IV-2. There were no remarkable differences found between control and treated samples with flow cytometry.

DICUSSION

If this study were repeated, more mammary tissues would be collected per sample to reduce process timing and increase percentage of sample viability. In future studies, mammary adipocytes and epithelial samples collected in these studies will be used to determine unique gene and/or protein expression profiles.

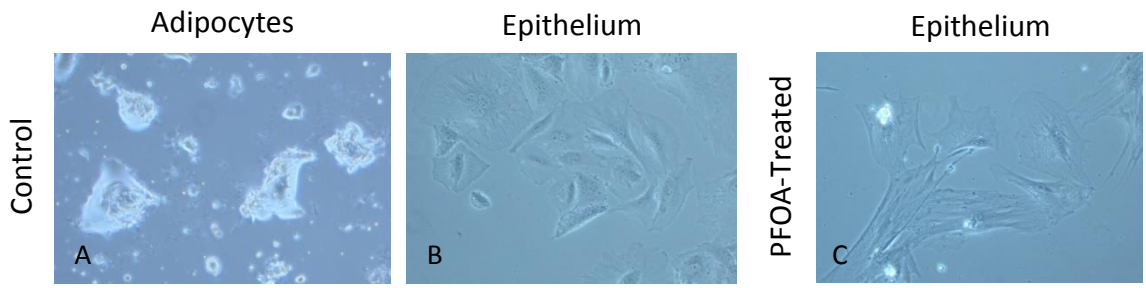

Figure A IV-1. Proof of concept for mammary epithelia cell isolations. Images of cell culture of mammary adipocyte and epithelial cells at PND 7. Cell culture provides evidence that we were able to separate specific cell types from mammary isolation techniques.

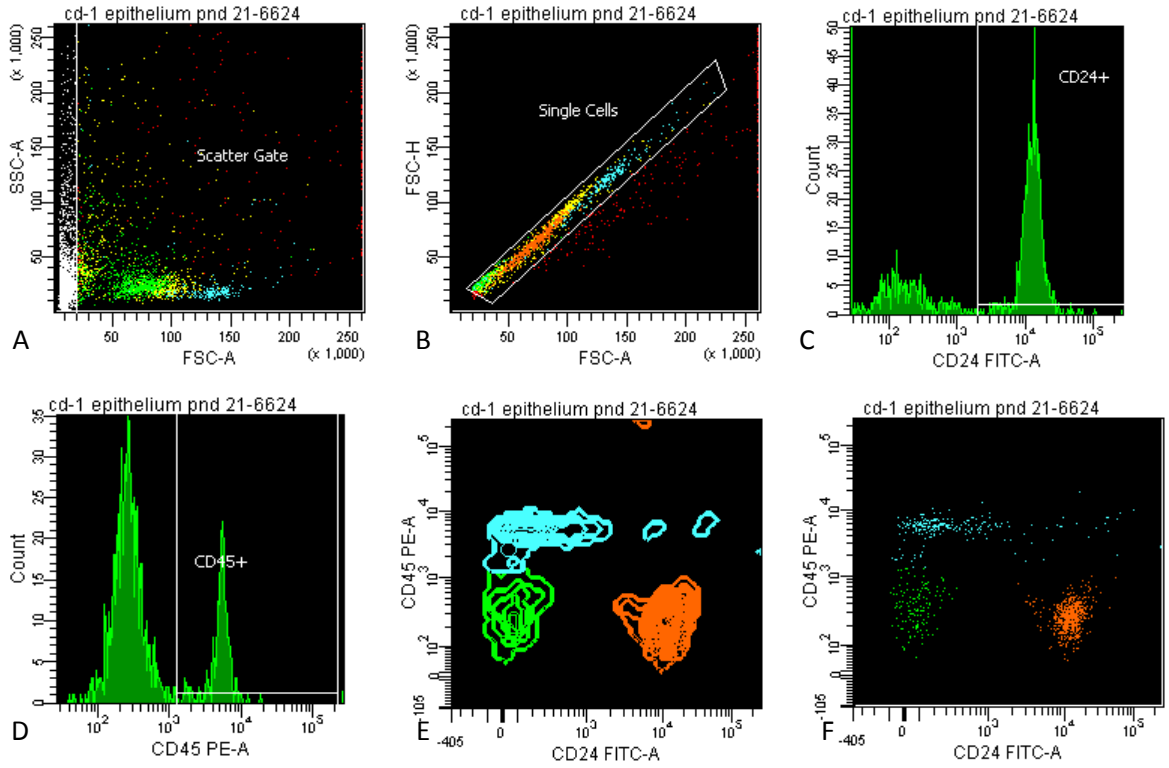

Figure A IV-2. Flow cytometry of mammary epithelial cell isolation at PND 21. Fluorescent staining showed high contamination of lymphocytes due to high CD 45 stained cells (light blue). Majority of epithelial cells isolated were luminal (high CD 24). Images indicate the ability to obtain single-cell suspension (A-B), and staining of CD 24 and CD 45 (C-D). Epithelial cell enriched samples had relative higher amounts of luminal cells (high CD 24) compared to basal cells (low CD24), as expected (E-F).

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APPENDIX V.

Laser Capture Microdissection of Mammary Epithelial Cells

INTRODUCTION

To determine the cell specific molecular signaling of prenatal PFOA exposure of mammary glands, we proposed to use laser capture microdissection to isolate mammary epithelial cells for further gene/protein characterizations.

METHODS

Mammary glands removed during necropsy were placed in a cyromold filled with OCT compound (Sakura Fine Technical; Tokyo, Japan), frozen on dry ice, and stored at -80°C. Under the guidance of the Laser Capture Microdissection Core Facility at NIEHS (RTP, NC) the frozen glands were sectioned into 8 µm sections onto a PEN foil membrane using a cyrostat at -20 °C. Tissues sections were placed on a slide and kept on dry ice for immediate use or stored at -80°C. Slides were fixed in graded ethanol, stained with cresyl violet, dehydrated in graded ethanol and xylene. Air dried slides were cut with a laser on the Molecular Machines and Industries CellCut Instrument (Molecular Machines and Industries; Zurich, Switzerland) and Leica Microsystems AS/LMD instrument (Leica Microsystems; Frankfurt, Germany) to isolate specific cell types. Total RNA was isolated from laser dissected tissues using Arcturus PicoPure Kits (Applied Biosystems; Foster City, CA) according to the manufacturer's suggestions. Quality of RNA was determined using RNA Pico chips (Agilent; Waldbronn, Germany).

Figure V-1 illustrates the successful removal of epithelial cells from sections using this technique. Figure V-2 shows RNA bioanalyzer results from isolated mammary adipocytes and epithelial cells.

RESULTS

Structures in frozen mammary sections were challenging to differentiate. Due to the difficulty to distinguish mammary ductal epithelium from endothelial and lymphoid vessels or the ability to separate ductal epithelium and surrounding stroma, this technique was abandoned for this project.

DICSUSSION

As the focus of the project has centered more on investigation the role of mammary stromal tissues, laser capture microdissection could be used in future techniques to determine gene expression differences in adipocytes, especially in very early tissues (PND 1-4). Current mammary cell-type isolation techniques separate mature adipocytes from other mammary types, as the mature, lipid-rich adipocytes disperse to the top layer following centrifugation while pre-adipocytes settle into the organoid pellet with the epithelial cells. Therefore, laser capture microdissection would allow the collection of both pre and mature adipocytes for a more robust analysis.

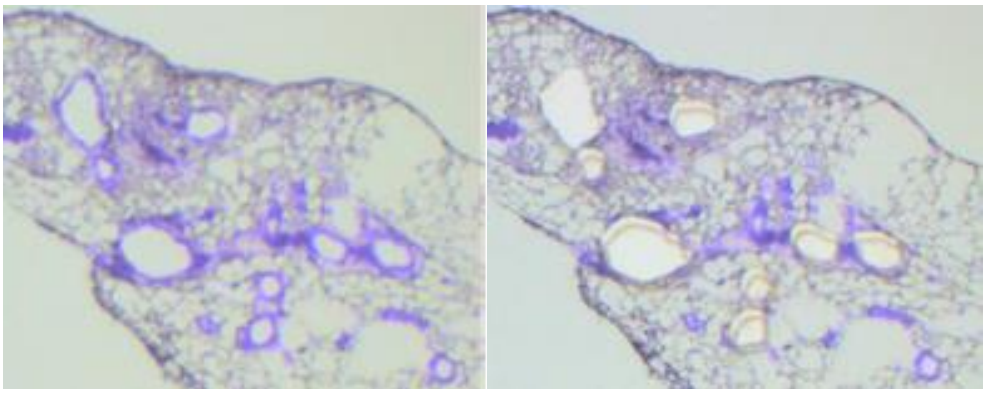

Figure A V-1. Frozen OCT section of mammary tissues used for laser capture microdissection. Image of sections before capture (Left panel) and after capture of desired cells (Right panel).

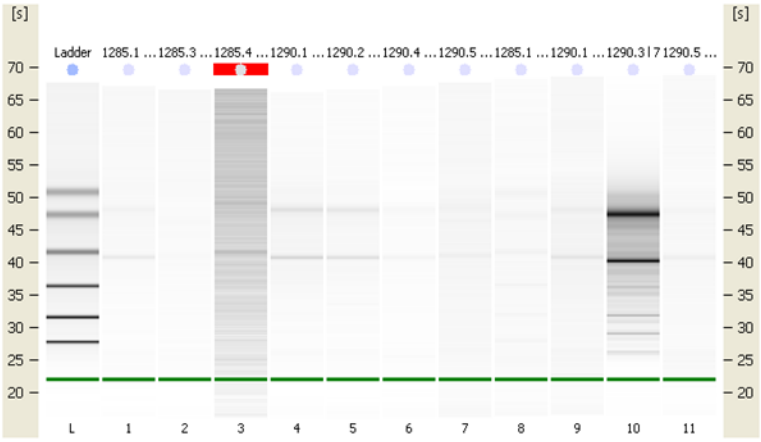

Figure A V-2. Bioanalyzer results for laser captured mammary tissues. Total RNA was extracted from mammary epithelial cells isolated with laser capture microdissection. RNA concentration and integrity were determined on RNA pico chips run on a bioanalyzer. This demonstrates that were able extract and isolate RNA from cells captured.

APPENDIX VI

Dual Fluorescent Whole Mammary Gland Staining

INTRODUCTION

In an effort to develop a broad and comprehensive evaluation for protein expression, we combined the principles of whole mounts and immunofluorescence. This technique would have allowed us to determine how prenatal PFOA exposure affects spatial expression of receptors and other proteins.

MATERIALS AND METHODS

The technique developed was modified from Landua *et al*., 2009 (*1*). Mammary glands removed during necropsy were mounted onto slides and fixed in paraformaldehyde (PFA) overnight at 4, then washed in phosphate buffered solution (PBS). Lipids were removed from gland by incubation in acetone with gentle agitation. Samples were blocked in goat serum, then incubated with primary antibodies for progesterone receptor (PGR, 1/1000 dilution) and/or estrogen receptor alpha (ERα, 1/200 dilution, (Abcam) for approximately 24 hours. The next day samples were incubated with fluorophore-conjugated secondary antibodies for another 24 hours, covered in foil to protect the solution from light. Glands were then counterstained with DAPI (Sigma D9542) for 4 hours, covered with Prolong Antifade Reagent (Invitrogen; Carlsbad, CA) to protect the fluorescent signal, then visualized with a Multiphoton Laser-scanning Microscope Zeiss 710 at the NIEHS Fluorescent Microscopy Core.

RESULTS

We were unable to develop a technique that accurately provided immunofluorescence of the entire gland. The mammary gland auto-fluoresces at wavelengths that overlap with some fluorophores. The secondary antibody for ERα was originally a 488 fluorophore but was changed to Alexa Fluor 555 due to the auto-fluorescence of the mammary gland at that wavelength. As observed in Figure Appendix VI-1, we were able to detect the epithelial ductal tree with Dapi staining (blue) and stromal ERα staining (green). However, we were unable to detect either receptor staining in the epithelial cells. It appeared as if there was non-specific staining of the secondary antibody for progesterone (red, Figure A VI-1). Due to these difficulties, we decided to abandon this technique.

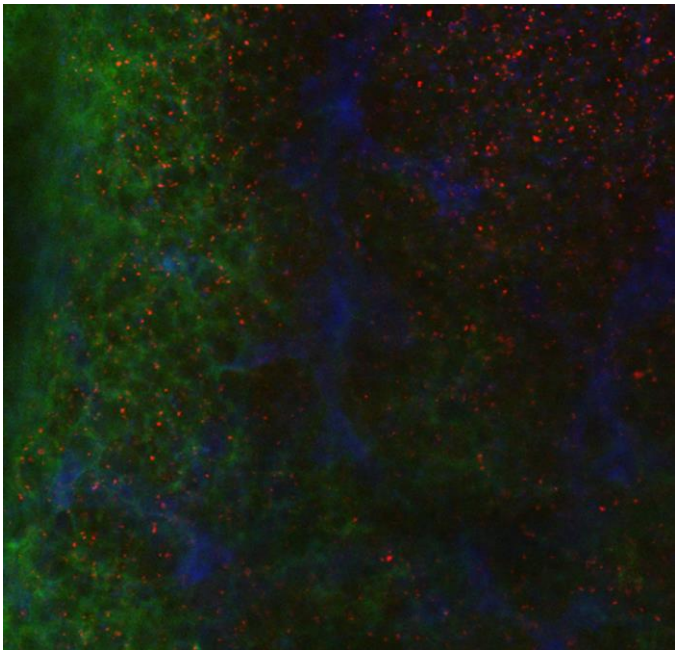

Figure A VI-1. Dual staining of a whole mount mammary gland. Gland was stained for estrogen receptor alpha (Green), progesterone receptor (red) and nuclei with DAPI (blue)

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