ELUCIDATING MECHANISMS THAT DRIVE INCREASED TUMORIGENESIS IN RATS EXPOSED TO ALCOHOL *IN UTERO*

By

HILLARY STIRES

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Dr. Wendie S. Cohick

and approved by

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ABSTRACT OF THE DISSERTATION

Elucidating Mechanisms That Drive Increased Tumorigenesis

in Rats Exposed to Alcohol *in Utero*

By HILLARY STIRES

Dissertation Director:

Dr. Wendie S. Cohick

Alcohol exposure during gestation increases breast cancer risk in offspring. While the mechanisms that underlie this effect are not fully understood, serum estradiol (E2) is increased in these animals during proestrus, suggesting a role for the estrogen axis. To test this hypothesis, it was necessary to develop a stress-free method of hormone administration that could be used in long-term studies of carcinogen exposure to ovariectomized (OVX) rats. Rats were OVX on post-natal day (PND) 40 then treated with daily peroral E2 with or without P4 by adding hormones to peanut butter. On PND 50 rats were injected with nitrosomethylurea (NMU) to induce mammary tumor development. After 26 weeks, there was no difference in tumor incidence suggesting that E2 alone at a normal physiological level can result in tumor development. These results indicate that this may be a useful method to examine the mechanisms of steroid action in mammary tumorigenesis. In addition to changes in circulating E2, local estrogen signaling may be altered in the mammary gland in response to alcohol *in utero*. To explore this possibility as well as obtain a global view of changes that occur in the mammary gland transcriptome, pregnant Sprague-Dawley rats were treated with alcohol or a control diet during pregnancy. Serum analysis demonstrated an increase in circulating E2 in alcohol-exposed dams during gestation suggesting that alcohol *in utero* may act as an endocrine disruptor during early development. Mammary glands from PND 2, 10, and 20 offspring were analyzed by RNASeq to look for changes in the estrogen axis. Initial analysis

with cummeRbund using a low read depth of 25 million reads demonstrated limited differences between alcohol-exposed rats and controls at PND 2 and PND 10. An analysis of additional samples at PND 20 using qRTPCR suggested that heterogeneity of the gland may prevent differences from being observed using the approach taken here. Further analyses of changes in the transcriptome from PND 2 to 10 suggested an increase in stromal cells over time that was corroborated by changes in mammary gland morphology. Comparisons were made with triple negative breast cancer (TNBC), a disease characterized by hormone independent growth similar to this period of mammary gland development. These results suggest that this may be a useful model to study TNBC. Future studies using greater read depth or a larger sample size may uncover differences between alcohol-exposed offspring and controls that were not seen with the present analysis.

Dedication

To my 7th grade science teacher, Mrs. Ambos: for inspiring my passion for science.

To my AP Biology teacher, Mr. Gunzelman: for inspiring my passion for biology.

To my research advisor at Lehigh, Carrie Garrippa: for inspiring my passion for research.

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List of Abbreviations

αERKO	Estrogen receptor alpha knockout
AF	Alcohol fed
AI	Aromatase inhibitor
AL	Ad libitum fed
BAL	Blood alcohol level
βERKO	Estrogen receptor beta knockout
BPA	Bisphenol A
CEE	Conjugated equine estrogens
CSF1	Colony stimulating factor 1
DAVID	Database for Annotation, Visualization, and Integrated Discover
DES	Diethylstilbestrol
DG	Dydrogesterone
DMBA	Dimethylbenzathracene
DOHAD	Developmental Origins of Health and Disease hypothesis
E1	Estrone
E2	17β-estradiol
EB	17β-estradiol benzoate
ECM	Extracellular matrix
EDC	Endocrine disrupting compounds
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen response element
FASD	Fetal alcohol spectrum disorder
FSH	Follicle stimulating hormone
GD	Gestational day

HPA	Hypothalamic – pituitary – adrenal axis
HPG	Hypothalamic – pituitary – gonadal axis
HRT	Hormone replacement therapy
IHC	Immunohistochemistry
IPA	Ingenuity Pathway Analysis
IUGR	Intrauterine growth restriction
LH	Luteinizing hormone
MMTV	Mouse mammary tumor virus
MPA	Medroxyprogesterone acetate
NBF	Neutral buffered formalin
NMU	Nitrosomethylurea
OVX	Ovariectomy
P4	Progesterone
PCA	Principal Components Analysis
PE	Paired end
PF	Pair fed
PND	Post-natal day
PR	Progesterone receptor
RANKL	Receptor activator of NF-κB ligand
RIN	RNA Integrity Number
RPKM	Reads per kilobase million
SERM	Selective estrogen receptor modulator
TDLU	Terminal duct lobular unit
TEB	Terminal end bud
TIL	Tumor infiltrating lymphocytes
TNBC	Triple negative breast cancer

Chapter 1

Review of the Literature

Introduction

Breast cancer is the second most diagnosed cancer and the second leading cause of cancer-related death in women in the United States (1). Research in this area has established a basic understanding of the biology of breast cancer allowing for the discovery of effective treatment options for certain breast cancers, specifically the use of tamoxifen and trastuzamab for breast cancers that overexpress estrogen receptor (ER) and HER2, respectively (2,3). Breast cancer is staged based on the progression of the disease and early detection leads to better prognosis (4). Programs that focus on early detection of breast cancer have been implemented to increase treatment success and to determine which women are at higher risk for developing the disease to implement prevention strategies.

Over-diagnosis and over-treatment complicate the efficacy of early detection. Use of mammography as an early detection program for all women has led to a decline in breast cancer death rates since 1989 but has also led to an overall increase in breast cancer incidence (5-7). Part of the increased diagnosis is attributed to false positives, which cause women to incur physiological, psychological, and financial stress from unnecessary treatment (8). There is currently debate about whether the risks of over-diagnosing breast cancer outweigh the benefits of detecting the disease in relation to the best age to begin testing (9,10) and the frequency at which mammograms should be performed (11).

Early detection is most successful when preneoplastic lesions are discovered and removed, preventing the disease from progressing (12). However, current standards of breast cancer surveillance may be too infrequent for fast growing, aggressive tumors such that the preneoplastic lesions are not detected until they have progressed to malignant disease (12,13). On the other hand, in cases where cancer is detected and verified, mammography is unable to distinguish between

disease that will go on to become malignant versus cancer that remains benign (12). A variety of genomic analyses have attempted to stratify breast cancer to determine who would benefit from aggressive cancer treatment (14,15). This method works well for groups of women, but is less effective and consistent on an individual basis (16,17). Therefore, a critical area of research is the development of biomarkers that would allow clinicians to distinguish between lesions that will go on to become malignant, necessitating treatment, and those that will remain benign.

In addition to population-wide breast cancer screening, information regarding family history can help identify women who have a higher risk for developing breast cancer. For example, women who carry hereditary mutations in the BRCA1/2 genes have 60-80% increased risk (18). As such, it is suggested that these women start getting mammograms at an earlier age (19). These women benefit from use of chemoprevention with selective ER modulators (SERMs) or prophylactic mastectomy to reduce risk of developing breast cancer (20,21). However, only approximately 10% of women with breast cancer have mutations in BRCA1/2 and there are no other genetic abnormalities that solely lead to breast cancer (22), indicating that genetics does not tell the full story of why breast cancer develops.

Exposure to a variety of environmental factors and dietary decisions can increase breast cancer risk (23). Specifically, exposures during critical periods of mammary gland development including embryogenesis and puberty may predispose women to breast cancer (24,25). In addition to direct-acting carcinogens, endocrine disrupting compounds (EDCs), which interfere with normal hormone action (26), are thought to lead to aberrant mammary gland development and ultimately breast cancer (25).

The Gail Model was developed to determine breast cancer risk and includes factors such as age of first menstruation and current age (27), but is only a beginning step to stratify women into high and low risk (28). It has been suggested that a large population of women would benefit from preventative treatment. Specifically, prophylactic use of SERMs has been shown to reduce ERα positive breast cancer development in high risk women (29,30). However, women who are at high

risk are unlikely to take advantage of neoadjuvant preventative care partially because of poor communication between physicians and at risk women but also due to fear of side effects (31). Women would be more likely to deal with side effects if they were convinced that chemoprevention is necessary. An extremely important aspect of preventative care is to determine which women would most benefit from the treatment (32).

Unfortunately, there is not one single tool that confers breast cancer risk assessment (33). To establish this tool, the biology of breast cancer needs to be more completely understood to help determine biomarkers that distinguish between high and low risk (12). Studying signaling pathways in the mammary gland is critical for understanding normal tissue as a basis for deciphering signaling in breast cancer. Emphasis has been placed on the role of estrogens in breast cancer development yet a complete understanding of their role in breast cancer is lacking. A more complete understanding of normal mammary gland biology would help to determine which pathways, when aberrant, can lead to breast cancer. Additionally, it is important to utilize multiple models to understand initiation and progression of a variety of breast cancers as well as to ensure there is consistency in methods across research groups.

Mammary Gland Development

Overview of Morphological Changes during Mammary Gland Development

In all mammals, mammary gland development begins during embryogenesis (34). First, the mammary lines appear on the ventral surface of the developing embryo. Shortly thereafter, mammary placodes form, which are thickened layers of pseudostratified epithelium. Mammary buds are established when the placodes invaginate into the surrounding primary mammary mesenchyme. Some of the epithelial cells elongate into the second mesenchymal layer, the mammary fat pad precursor, and begin to form rudimentary glandular trees. While each pair of glands typically develops asynchronously from the other pairs, there is no difference in proliferation rate between the different pairs during adulthood (35-37).

The mammary gland parenchyma grows isometrically with the body from birth until puberty, at which time allometric growth ensues in response to ovarian hormones. In contrast, the stromal tissue including the fat pad and connective tissue are already mature at puberty (38,39). Allometric growth of the parenchyma leads to a gland that is structurally prepared for pregnancy should it occur. Cell proliferation at the distal end of the mammary ducts leads to the formation of structures called terminal end buds (TEB), which are specialized sites of proliferation that lead the mammary ducts through the fat pad. The majority of the cells in the TEB are body cells that are tightly packed, while cap cells are found at the edge of the TEB (40). In response to a variety of growth factors (discussed below), the ducts elongate towards the edge of the fat pad with increasing complexity over each estrous (or menstrual) cycle. Peak proliferation occurs during metestrus (luteal phase of the menstrual cycle in humans) while the morphology is most expansive in estrus (41-45). The mammary ducts form through a process of controlled proliferation and apoptosis allowing for gland elongation and establishment of a ductal lumen, respectively. Body cells in the TEBs give rise to the mammary epithelial cells that have direct contact with the lumen and secrete milk while the cap cells differentiate into myoepithelial cells which form a layer around the epithelial cells and will eventually push the milk down the ducts (46). Within the luminal and myoepithelial compartments there are a variety of cell types such as stem cells and milk secreting cells leading to vast heterogeneity of the parenchyma (44). The mesenchymal compartment also encompasses a variety of cell types including adipocytes, fibroblasts, and immune cells. The different cell types of both the epithelium and the stroma interact to ensure proper growth and functionality of the mammary gland (47,48).

During pregnancy, the mammary gland undergoes extensive branching and alveoli form at the distal ends to produce and store milk during lactation for the developing offspring in response to progesterone (P4) and prolactin (48). At parturition, the gland is terminally differentiated and completely extends through the fat pad (48). Lactation includes two major phases: milk secretion from alveolar cells and milk removal via the nipple by the offspring. While these two phases are

required for proper milking, the timing for onset of lactation differs between species, as some begin producing milk during pregnancy while milk is not produced until after parturition in others (34). The offspring nurse from their mother until they are able to consume other sources of nutrients. Weaning signals the glands to return to their pre-pregnancy state through a process of controlled cell death called involution (34,47,48).

Species Comparisons in the Mammary Gland

The number and location of glands differs between species, for example mice have five pairs of nipples and glands, while rats have six, and humans have one (39). Microscopic mammary gland morphology differs between these species in several ways. The mouse has a much simpler ductal system with limited side branching during puberty and adulthood, while rats and humans develop lobules and ducts simultaneously. The terminal structure in the human, the terminal duct lobular unit (TDLU), is much more similar to the rat lobule than it is to the mouse (49). Humans have multiple segmented structures within their ducts while the ductal structures in the mouse and rat typically originate from the nipple and move outward as a single structure (38,50). Therefore, while mice offer the advantage of providing transgenic models to study specific effects of hormones, growth factors, etc. the rat mammary gland is structurally more similar to the human.

Ovarian Hormones in Mammary Gland Development

While many hormones and growth factors influence mammary gland development, studies during the first half of the twentieth century demonstrated the requirement of ovarian hormones in mammary gland development (51). Specifically, classical ablation experiments helped establish the roles of estrogens and progestogens (52,53). Ovariectomized (OVX) rodents have bare ductal structures with enlarged fat pads (51). Replacement of ovarian hormones demonstrated that estrogens stimulate ductal growth while progestogens act synergistically with estrogens to stimulate lobuloalveolar development (53). A more complete understanding of the roles of estrogens and progestogens in mammary gland development has suggested a role for paracrine signaling pathways. Estrogens and progestogens are primarily secreted by the ovaries and promote

development and function of female reproductive tissues as well as contribute to regulation of energy homeostasis. These steroid hormones are structurally related and interact with their specific receptors in a classical genomic pathway to induce transcription of genes. An important component of the genomic pathway is that the P4 receptor (PR) gene (*Pgr*) contains an estrogen response element (ERE) indicating that transcription of PR is at least partially regulated by estrogens (54,55). The steroid hormones can also act through a nonclassical mechanism to stimulate signaling cascades ultimately leading to growth and differentiation. Estradiol (E2) is the estrogen that has the highest affinity for ER while P4 is the strongest progestogen (56). The majority of circulating steroid hormones are found in the blood bound to proteins to prevent their enzymatic cleavage, which also renders them biologically inactive and allows steroid activity to occur only in target tissues (45).

Role of Ovarian Hormones in Prepubertal Mammary Gland Development

Mammary gland development proceeds in the absence of fetal ovarian hormones during embryogenesis, however maternal ovarian hormones may play a role in development (45,57). Injection of the pregnant dam with very high levels of E2 results in abnormal mammary development in offspring (45). Alpha fetoprotein binds estrogens in the developing fetus to prevent exposure to the high levels of ovarian hormones secreted by the mother in rodents. At birth, a few TEBs are present which have been attributed to residual maternal hormones (44).

Circulating E2 levels and ERα expression in the mammary gland increase during isometric growth, which raises questions about the role of E2 in mammary gland development during this time. In rats, total serum E2 is highest around postnatal day (PND) 10, which is about half way between birth and the onset of puberty (58). However, there is a lack of free E2 in circulation since much of the E2 is bound and sex hormone binding globulin levels are high (59,60). ERα levels are detectable in the mammary gland of mice starting at 3 days of age and increase between 1-2 weeks of age (61). However, E2 does not appear to play a role prepubertally in mammary gland development as PR levels and proliferation do not increase in response to exogenous E2 (62).

Additionally, if rats are OVX before puberty, mammary growth continues until puberty (44). Together these results suggest there are other signaling pathways besides ovarian hormones that influence mammary gland development prior to puberty.

Role of Ovarian Hormones in Pubertal Development of the Mammary Gland

The ductal structure of the mammary gland expands at the greatest rate around puberty with the onset of estrus due to increased circulating E2. The initial formation of the TEBs requires E2 (63-66). TEBs are observed around PND 20 in rats and mice, a time in development just before puberty, but they are still dependent on E2 as OVX leads to their regression (67). Without ERα, mammary glands appear rudimentary and lack any branching or TEBs (68,69). Initial studies with ERα knockout (αERKO) mice using transplantation techniques to define the roles of ERα in the stromal versus epithelial compartments indicated stromal ERα was necessary and sufficient for ductal development (70). However, this αERKO model was a hypomorphic mutant and thus had low levels of transactivation capability. A different αERKO model lacking all ERα transcripts demonstrated that ERα in the stroma was not essential while ERα in the epithelium was essential (69). The other ER, ERβ, does not appear to play a role in normal mammary gland development as ERβ knock out (bERKO) mice have normal mammary gland morphology and are able to reproduce and nurse normally (71,72) and double knockouts (αβERKO) develop similarly to αERKO mice (73).

P4 is also important for mammary gland development and plays a critical role during pregnancy. The PR in the epithelium, but not in the stroma, is required for normal mammary gland growth and development (74). There is decreased PR expression in the mammary gland following OVX (75).

The PR gene encodes two proteins, PRA and PRB, which are produced by alternative splicing from the same gene (76). Both PRA and PRB are expressed in the mammary gland in the luminal epithelial cells (77-79). In the murine, PRA is primarily seen in the virgin mammary gland while PRB is found in alveolar structures during pregnancy, accompanied by a decrease in PRA

(80). As such, PRA expression is important for side branching during mammary gland development while PRB is required for alveologenesis during pregnancy (81,82). The two PR isoforms work together to control normal cell fate decisions (83). In the mouse, the expression of PRA and PRB are typically spatially and temporally different whereas humans and rats can have cells that express both PRA and PRB, which may influence signaling (80,84,85).

Changes in Ovarian Hormones During the Adult Estrous Cycle

E2 and P4 are rarely present alone and their interplay maintains the integrity of the mammary gland in adulthood. Serum concentrations of E2 and P4 change over the estrous and menstrual cycles, which are divided into two main phases: the follicular phase to prepare for ovulation and the luteal phase that follows ovulation in an effort to prepare for pregnancy. The follicular phase is further separated into proestrus and estrus and the luteal phase into metestrus and diestrus. During proestrus, the corpus luteum degrades because of a lack of conception leading to a decrease in P4 secretion. P4 exerts negative feedback on luteinizing hormone (LH) and follicle stimulating hormone (FSH) such that decreasing P4 allows LH and FSH levels to increase. The release of these gonadotropin hormones from the pituitary causes antral follicles in the ovary to mature leading to increased E2 production. Serum E2 initially exerts negative feedback decreasing FSH and LH but rising E2 levels from the developing follicles cause the signal to switch to positive feedback leading to a spike in LH and ultimately ovulation during estrus. After ovulation, E2 levels drop and P4 levels start to rise as the ovulated follicle becomes the supportive corpus luteum, the main structure that secretes P4 during the estrous cycle. Metestrus is characterized by rising P4 levels that are maintained through diestrus and remain high if pregnancy is initiated. Without conception, the corpus luteum ceases to function leading to degradation. During the menstrual cycle, E2 levels also rise to prepare for pregnancy during the luteal phase (86,87).

Proliferation in the mammary gland increases during the luteal phase of the menstrual cycle and metestrus in rats indicating that P4 may drive proliferation (41-43). However, it is important to consider timing as E2 increases proliferation in the mammary glands of OVX mice after 24 hours

in stroma and after 48 hours in epithelium (88), indicating that the E2 peak during proestrus may be what drives proliferation in rodents. In humans, E2 is also present during the luteal phase and thus probably interacts with P4 to drive proliferation in the breast. Apoptosis is highest during estrus (43) correlating with the part of the cycle when the glands are most morphologically developed (44).

Similar to the biphasic effects of E2 on LH and FSH release, E2 has dose-dependent effects on mammary gland development. Increasing concentrations of E2 administered to OVX pubertal mice leads to increased ductal extension and number and size of TEBs up to a certain dose but then these parameters decrease as the concentration continues to increase (89,90). Increasing concentrations of P4 lead to increased lobuloalveolar development (91). E2 and P4 together increase proliferation of the mammary glands in mice more than E2 or P4 alone (90-92). In pubertal mice, TEBs proliferate in response to E2 while ductal epithelium responds to P4, E2, and E2 and P4, indicating that the various epithelial cell types within the mammary gland respond to hormones differently. Together, these results indicate a balance of E2 and P4 levels are important to maintain the mammary gland.

Paracrine Mediators of E2 and P4

Despite their important roles in mammary epithelial growth and differentiation, ERα and PR are only expressed in luminal epithelial cells and in a minority of cells within this population (93). The cells that express these receptors do not proliferate themselves. Instead, E2 and P4 bind to their receptors leading to increases in receptor activator of NF-κB ligand (RANKL), Wnt4, and amphiregulin (94,95). These paracrine signaling molecules stimulate surrounding cells to proliferate and differentiate.

RANKL is a member of the TNFα family that was originally discovered to be critically involved in osteoclast development, but is also required for normal mammary gland function during lactation (96). It is a direct target of PR and is required in the mammary gland epithelium for normal side branching and is sufficient to replace PR signaling for alveologenesis (97,98). Wnt proteins

are important for normal embryogenesis. Wnt4 is also a direct target of PR and is required for side branching (99). Recent evidence suggests Wnt4, but not RANKL, is essential for mammary epithelial stem cell function (100). In the rat, RANKL and Wnt4 increase in the mammary gland in response to both E2 and P4 following OVX but not to either hormone alone (101). RANKL is also found in the breast in women and levels correlate with pregnancy status and the luteal phase of the menstrual cycle when P4 is high (102-104).

Amphiregulin was first discovered as an epidermal growth factor receptor (EGFR) ligand in MCF7 cells (105). Without amphiregulin, there is no ductal growth in the mammary gland (106). P4 and E2 both signal through amphiregulin for TEB development during puberty in the mouse which suggests that there is an overlap in E2 and P4 regulation of amphiregulin (107). In the rat, amphiregulin mammary mRNA increases in response to E2, P4, and E2 + P4 but the protein only increases in response to E2 + P4 (101). Understanding the roles of E2 and P4 as well as their downstream paracrine signaling factors may lead to a better understanding of breast cancer development and result in better treatment strategies.

E2 and P4 in Breast Cancer

A defining characteristic of cancer is that it is able to sustain chronic growth, distinguishing it from normal tissue, which maintains a balance of growth signals ultimately leading to homeostasis. Understanding signals in normal tissues identifies potential mechanisms that cancer cells can hijack to enhance proliferation and evade growth-inhibitory signals. Cancer cells can alter signaling pathways by increasing concentrations of growth factors or by changing sensitivity to hormone levels by increasing receptor concentrations or downstream signaling molecules (108).

While the mechanisms by which ovarian hormones regulate normal mammary gland development are well established, the specific mechanisms by which they contribute to breast cancer initiation and progression are less defined. Work during the turn of the 20th century identified a role for ovarian hormones in breast cancer with oophorectomy resulting in regression of metastatic breast disease in three separate women (109). Subsequent work over the next century

demonstrated that for ovarian hormone ablation to succeed, the tumor must express ERα and that 75% of breast cancers overexpress the receptor (110-112). The successful discovery and use of anti-estrogens, either SERMs (such as tamoxifen) or aromatase inhibitors (AIs) in both preventing and treating ER-positive breast cancer, has further demonstrated a role for estrogens (113,114).

Evidence from Epidemiological Studies

Human studies indicate that exposure to estrogens for extended periods of time, as seen with early menarche and late menopause, is associated with increased risk of developing breast cancer (115). In premenopausal and postmenopausal women, higher levels of endogenous E2 are associated with increased breast cancer risk (116,117). Additionally, treatment with hormone replacement therapy (HRT) causes an increased risk for developing breast cancer (118,119). However, this is relatively complex as the duration of HRT use, the age when women begin using it, and the actual forms and combinations of hormones administered each may contribute to the increased risk (120,121). The use of oral contraceptives has shown varying effects on mammary epithelial proliferation and breast cancer risk (122,123). With fertility drugs that include P4, there is a 4-fold increased risk of breast cancer (124). These studies suggest that endogenous and exogenous ovarian hormone levels may influence breast cancer risk, both at a specific time point as well as with cumulative exposure over time.

Evidence from Animal Models

Animal models are critical for elucidating the role of ovarian hormones in breast cancer susceptibility. Tumors that develop spontaneously in mice are associated with the mouse mammary tumor virus (MMTV) or other virulent endogenous proviruses while spontaneous tumors in rats arise from endocrine organs or organs under endocrine control, most commonly mammary glands (38,125). Aryl hydrocarbons, including dimethylbenzathracene (DMBA) and nitrosomethylurea (NMU), are commonly used to initiate mammary tumors in rats to speed up the process of mammary tumor development. These tumors are ovarian hormone dependent as OVX rats have reduced susceptibility to tumorigenesis induced with either chemical carcinogen (126-128).

Additionally, pretreatment of intact animals with SERMs or AIs decreases mammary tumor formation in response to carcinogens, indicating a specific role for estrogens (129,130). Inducing tumors in rats with NMU during proestrus or estrus when E2 and P4 are high compared to metestrus when these hormones are lower results in increased tumor incidence and multiplicity and decreased tumor latency (131,132). Cumulatively, these studies corroborate findings in humans that E2 and P4 levels are important for breast cancer development.

Importance of Hormone Concentration

While the research described above indicates a general role for E2 and P4 in the development of breast cancer, the concentration of hormone may be important in determining if their actions are stimulatory or inhibitory. Free E2 and P4 levels increase with the onset of puberty then rise and fall over the menstrual (or estrous) cycle. During pregnancy, P4 and E2 levels are even higher allowing for maintenance of pregnancy and aiding in preparing the mammary gland for lactation. In menopause, E2 and P4 levels decrease as the ovaries atrophy leading to more local production of E2 in adipose tissue, including those of the breast (133).

As previously mentioned, OVX prevents tumor development in a chemical carcinogen rodent model. Interestingly, high levels of E2 and P4, similar to those seen in pregnancy, also protect against tumor development in rodent models (134). Each hormone is protective at high levels individually but the combination of E2 and P4 leads to even less tumor development following carcinogen administration (135-137). Parity-induced protection is a phenomenon seen in humans, mice, and rats (138,139). Several mechanisms have been proposed to explain this protective effect, including forcing cells to terminally differentiate (140), losing premalignant cells through involution (141), and causing persistent changes in circulating hormones (142). It is important to note that these hormone replacement studies, hormones were administered using silastic tubing, which theoretically allows for continuous release of hormone, although previous studies have indicated that there is an initial supraphysiological spike when the method is initiated (143). When 3-methylcholanthrene (an aromatic hydrocarbon) is administered daily for 50 days,

there is 60% tumor incidence in OVX animals compared to 100% incidence in sham. Treating OVX animals with 0.1 or 1.0 µg/day E2 leads to 100% tumor incidence following 3-methylcholanthrene administration while 20 µg/day E2 leads to a decreased tumor incidence (33%) indicating different levels of E2 can affect mammary tumor development in response to a chemical carcinogen (144). However, a separate study using oral gavage at 100, 300, or 900 µg/kg or I.V. at 0.4, 10 or 250 µg/kg E2 did not see a dose-dependent response to E2 (145). These studies demonstrate that different levels of E2 can affect mammary tumor development, but how the levels correlate with tumor development is not fully understood. The role of E2 is further complicated by studies that demonstrate E2 can cause apoptosis in cells (146). In humans, breast tumors respond to diethylstilbesterol (DES; a synthetic estrogen) treatment to the same extent as treatment with anti-estrogens such as tamoxifen, just with more side effects (147).

Mechanisms of How Estrogens Increase Breast Cancer Risk

Estrogens are thought to cause mammary tumorigenesis by increasing DNA damage either by increasing cell division or by directly targeting the DNA (148). Normal E2 signaling causes cells to divide and too much cell division allows for incorporation of DNA damage leading to aberrant growth (149,150). Alternatively, some of the free radical metabolites of E2 can directly damage DNA leading to cancer (151). Oxidation of estrogens ultimately leads to the formation of quinones that cause DNA adducts and these metabolites are higher in breast tissues of women with breast cancer (152,153). Increasing cell division requires both E2 and its receptor to be present while quinones leading to DNA adducts only requires the ligand.

Both of these hypotheses consider increased levels of hormone as a driver for tumorigenesis. Serum levels do not account for local levels of hormone, which could further increase exposure to estrogens and thus breast cancer risk (154). Estrogen synthesis is increased locally in breast tumors suggesting that breast tumors somehow circumvent normal signaling to increase E2 levels (155,156). Selecting women with high levels of serum E2 could be a method to

determine who would benefit from use of SERMs or AIs as a preventative tool before breast cancer develops (157,158).

Increasing ERα protein expression is another way that breast tumors can enhance actions of estrogens. ERα expression in epithelial cells in the normal mammary gland is relatively low at 5-15% while levels in tumors can be as high as 90% (94,159,160). Deregulation of ERα using a tetracycline-responsive transgenic mouse causes mammary glands to develop into hyperplastic glands ultimately leading to ductal carcinoma *in situ* (161). The tumors that develop from either NMU or DMBA express varying levels of ERα and PR (162-164).

As stated previously, ERα signals through paracrine mediators in the normal mammary gland so paracrine signaling may also play a role in breast cancer development. In breast cancer, ERα colocalizes with Ki67, a marker of proliferation, indicating that cells that express ERα proliferate, presumably in an autocrine manner (94,165). However, stimulating breast cancer cells with E2 also causes secretion of growth factors indicating paracrine signaling occurs (166). Amphiregulin is increased in tumor cells in response to E2 treatment and E2-dependent growth is inhibited without amphiregulin (167). Similarly, RANKL is upregulated in tumors that develop from E2 + P4 treatment compared with tumors that develop with E2 alone (168). An understanding of how ERα and PR act through paracrine signaling to affect breast cancer development is an active area of research (169).

Breast Cancer Heterogeneity

Estrogen signaling in breast cancer is a complex process and multiple subclones of cells probably integrate to confer survival for the tumor, indicating that intra-tumor heterogeneity plays a role in cancer survival. It was recently proposed that the forces of Darwinian evolution apply to breast cancer whereby subclones of tumor cells work together to increase fitness since the clones would most likely not survive alone (170). Patients with higher levels of intra-tumor heterogeneity have worse prognosis and are thought to have more advanced tumors (171). An understanding of

normal mammary gland biology has begun to elucidate mechanisms of intra-tumor heterogeneity but much work is still needed (172).

Inter-tumor heterogeneity is also important to study to understand the biology of breast cancer. Molecular expression profiling has been used to try to better stratify tumors to determine treatment options and patient outcomes leading to more accurate prognosis and diagnosis (14). These techniques are valuable for understanding breast cancer prognosis and treatment for groups of patients but are less successful as a prognostic tool for women on an individual basis (16). The original stratification of breast tumors by microarray placed breast cancers into four main categories: ER+/luminal like, basal-like, *ErbB2+* and normal breast (14,173). Since this classification over fifteen years ago, many other groups have tried to re-emulate the results and further classify breast cancers in an effort to determine better treatment options and survival rates.

Estrogen-driven breast cancer accounts for approximately 75% of breast cancers making it an important subgroup to study. This type of breast cancer has treatment options in the form of SERMs and AIs. ER+ luminal breast cancers have been further separated into luminal A and luminal B where there is lower expression of ER in the latter (173). Additional studies have indicated Ki67 and PR status may be more helpful to separate luminal tumors. Similarly, while *ErbB2+* breast cancers are typically more aggressive than luminal breast cancers (174), the tumors overexpress the Her2/neu protein, a tyrosine kinase receptor that can be targeted (175). A monoclonal antibody called trastuzamab was the first molecule shown to be efficacious in treating women with *ErbB2+* breast cancer (176). Her2 overexpression is found in 20-30% of cases and can be determined by immunohistocytochemistry (177).

The majority of basal-like breast cancers are also “triple-negative” breast cancer (TNBC) since they do not express ER, PR or Her2 (178). There is an adverse prognosis associated with this kind of cancer (178) and there is a bias towards younger women and African American women for developing it (174). While the percentage of women who have TNBC is low (10-15%), there are not currently any treatments available as a standard of care for women with this type of cancer.

Basal-like/TNBC have been analyzed by molecular subtyping to better understand pathology of the disease and to look for better markers for treatment (179). A recent study separated basal-like breast cancers into luminal androgen receptor, mesenchymal, basal-like immune suppressed, and basal-like immune activated (179). The benefit of this type of analysis is that there are treatment options for each of these four subtypes that could help cure the disease.

Unfortunately, these molecular subtypes do not necessarily shed light onto how these tumors initially develop. Molecular classifications may help determine the most efficacious treatment, however the terminology to describe the different subtypes is slightly misleading. Basal-like breast cancers do not necessarily arise from the basal compartment of the breast, but often times express luminal cytokeratins indicating a luminal origin (180). The general lack of understanding of how basal-like breast cancers develop is problematic for early detection and prevention. Part of the difficulty is the lack of models that emulate the heterogeneity of basal-like breast cancers. Recently, microarray of normal mammary stem cells compared to normal luminal or basal cells was analyzed then compared to a cohort of basal-like breast cancers. The markers pulled from the mammary stem cells that differed from more differentiated cells correlated with increased metastasis (181). Studying early mammary gland development may give insight into molecular pathways that can be affected in basal-like breast cancer.

Endocrine Disruptors and Breast Cancer

The Developmental Origins of Health and Disease hypothesis (DOHAD) suggests that exposure to suboptimal environments during distinct developmental time periods may lead to increased susceptibility to cancer, specifically hormone dependent cancers (182-184). A significant amount of research indicates that increased exposure to endogenous and exogenous estrogens throughout life increases breast cancer risk (185). Exposure specifically during gestation or puberty may increase risk more than at other time points. Rather than causing direct mutations, endocrine disruptors may reprogram the genome through epigenetic modifications leading to higher

susceptibility to disease later in life resulting in irreversible perturbations (182). Examples of two commonly studied endocrine disruptors include DES and bisphenol A (BPA) (24,186).

DES is a synthetic estrogen that was given to pregnant women to prevent miscarriages but instead led to the development of a variety of female cancers in daughters exposed prenatally (187,188). Rodent models have recapitulated the effects seen in humans. In ACI rats, exposure to DES *in utero* decreases mammary tumor latency and increases tumor multiplicity (189). Similarly, exposure to BPA perinatally leads to increased susceptibility to carcinogen-induced mammary tumors in rat offspring (190). BPA is a synthetic estrogen that is used as a plasticizer and is found in commonly used consumer products (25,191). Exposure to BPA *in utero* results in an increased incidence of atypical ductal hyperplasia and ductal carcinoma *in situ* in the mammary gland in adulthood in the absence of a carcinogen (192,193).

Direct E2 exposure *in utero* also increases mammary tumor incidence in adulthood (194). This effect is transgenerational as both the F1 and F3 generations show similar increases in tumorigenesis after the F1 generation is exposed to E2 *in utero* as measured by tumor incidence and multiplicity (195). Rodents exposed to E2 between PND 1 and 5 exhibit increased ductal junctions and mammary growth area after puberty, an effect which is observed prior to puberty (196).

Epigenetic reprogramming results from exposure to endocrine disruptors *in utero* and can be further altered with the onset of puberty and concurrent increases in ovarian hormones. For example, exposure to DES perinatally leads to altered methylation of an ERE gene (*Ltf*; lactoferrin) in the uterus of adult mice. OVX immediately prior to puberty ablates the changes in methylation in DES-exposed pups indicating that ovarian hormones drive the aberrant methylation seen in adulthood (197). The *Nsbp1* gene is hypomethylated in adult mice exposed to DES or genestein (a phytoestrogen) from PND 1-5 and OVX before puberty prevents the hypomethylation (198). Perinatal BPA exposure increases the sensitivity of the mammary gland to E2. When exposed animals are OVX then given E2, they exhibit enhanced mammary gland development compared to

unexposed animals (199). Together, these studies indicate that epigenetic modifications may begin postnatally but that the onset of hormones during puberty further alters the modifications and may thus alter the phenotype. A microarray analysis of uterine leiomyoma tissue exposed to DES *in utero* revealed a series of genes with EREs that were altered by the *in utero* exposure demonstrating an increased estrogenic sensitivity (200).

A comprehensive understanding of how common lifestyle choices during pregnancy influence cancer development in offspring would allow for warnings to pregnant mothers, which would ultimately help implement prevention strategies. An example of a modifiable lifestyle choice is consuming a high fat diet during gestation, which has been shown to increase breast cancer incidence in the offspring of rats (195). Our lab has focused on the effects of alcohol exposure *in utero* on mammary cancer development in the offspring using a rat model (201).

Despite warnings against consuming alcohol during pregnancy, the Centers for Disease Control estimates 1 in 13 women drink alcohol during pregnancy (202). This is supported by the high incidence of fetal alcohol spectrum disorder (FASD), which is reported to be as high as 2-7% of births in developed countries (203). More recently, women have been warned to not consume alcohol if they are not actively using birth control (204) since about half of pregnancies are unplanned (205). Much of the research on fetal alcohol exposure has focused on developmental and cognitive problems (206,207); however, the concept that offspring born with FASD may experience increased long-term susceptibility to disease has not been investigated as extensively.

Our work together with studies of Hilakivi-Clarke show that in rats, alcohol exposure *in utero* leads to increased susceptibility to mammary tumorigenesis in adult offspring (201,208). Subsequent studies from our lab examined mammary glands of rats exposed to alcohol *in utero* at different times in development and saw an increase in proliferation as measured by BrdU incorporation at PND 20 (before puberty). Mammary glands of alcohol-exposed rats had increased aromatase protein expression on PND 20 and PND 40 (a time during puberty) suggesting a role of local E2 production (209). These results suggest that the mammary gland exhibits changes earlier

in development that confer an increased susceptibility to cancer development in adulthood. After puberty, rats exposed to alcohol *in utero* have increased circulating E2 during proestrus (201), which may also contribute to increased tumorigenesis in adulthood since estrogens play such an important role in breast cancer development.

A better understanding of how alcohol *in utero* influences breast cancer development would guide better breast cancer prevention strategies for children born to women who drank during pregnancy. Specifically, elucidating the molecular signaling pathways involved in increased risk would expose genes of interest that could be used as biomarkers or targets for treatment. Women continue to drink during pregnancy despite warnings as evidenced by high numbers of children with FASD. There is an increased likelihood of women abstaining from alcohol consumption while they are pregnant if they know drinking puts their children at an increased risk for breast cancer development as well.

Objectives

The Developmental Origins of Health and Disease hypothesis (DOHAD) suggests that exposure to various environmental factors during critical periods of development can influence disease states later in life including breast cancer. Previous research has focused on the role of alcohol during pregnancy on impaired cognition in children but less of an emphasis has been placed on cancer development. Work from our lab and others using rodent models suggests that alcohol exposure *in utero* can enhance tumor development in offspring. Despite warnings against drinking during pregnancy, many women continue to do so as evidenced by high levels of fetal alcohol spectrum disorder in children (203). In an effort to find treatment options and increase warnings as part of a prevention strategy, it is important to elucidate mechanisms of how alcohol exposure during pregnancy can influence cancer development in the young.

Previous research demonstrated that E2 levels are higher in rats exposed to alcohol *in utero* during proestrus in adulthood. Since increased exposure to E2 is a risk factor for breast cancer, we wanted to test the hypothesis that this increase contributes to the increased susceptibility to carcinogenesis observed in these animals. We postulated that normalizing the increased circulating E2 levels seen in alcohol-exposed rats would eliminate the increase in NMU- induced tumorigenesis seen in these offspring. To investigate this, it was necessary to validate a method of hormone administration that could be used in long-term carcinogenesis studies. It was also necessary to determine if E2 alone could restore tumorigenesis in OVX animals or if progesterone was also required (Chapter 2)

An understanding of genes in the mammary gland that are altered in response to alcohol *in utero* would help to tease out driver pathways of enhanced proliferation seen in alcohol-exposed offspring just before puberty. Therefore, a second objective was to analyze the transcriptome of mammary glands from rats exposed to alcohol *in utero* in an attempt to elucidate pathways that may be perturbed in the mammary gland after birth but before puberty (Chapter 3). Our hypothesis was that genes involved in epigenetic modifications and genes with ERE would change, since

previous research suggests that these pathways are altered in response to a variety of environmental exposures *in utero*.

The study for Chapter 3 was designed to establish normal changes in gene expression that occur between PND 2 to 10 (a time before puberty) as a baseline for understanding changes that occur in response to environmental exposures *in utero*. The third objective focused on changes that occur prepubertally to understand how mammary gland development normally occurs during this time in an effort to see which pathways may be perturbed (Chapter 4). Parallels were made between this time of mammary gland development and TNBC.

Chapter 2

Peroral Estradiol is Sufficient to Induce Carcinogen-Induced Mammary Tumorigenesis in Ovariectomized Rats without Progesterone

Abstract

The interactions of estradiol (E2) and progesterone (P4) in breast cancer are complex. The rat chemical carcinogen model has been widely used to study the effects of E2 but conclusions on the additive effect of E2 and P4 are less clear. A newer method of hormone administration mixes hormones with nut butter for peroral consumption allowing for a less stressful method of administration with lower spikes in serum E2 levels. The present study was designed to determine if E2 alone can drive carcinogen-induced tumors in ovariectomized (OVX) rats or if P4 is also required using this method of hormone administration. Short-term studies were conducted to determine the dose of E2 that would lead to increased uterine weight following OVX. Subsequently, rats were OVX on postnatal day (PND) 40 then treated daily with E2 (600 µg/kg/day), P4 (15 mg/kg/day), or the combination. On PND 50, all rats were injected with nitrosomethylurea to induce mammary tumors. Uterine weights, body weights, and serum E2 levels were measured to demonstrate the efficacy of the method for increasing E2 levels during long term treatment. After 26 weeks, tumor incidence was similar in sham, E2, and E2 + P4 animals indicating that E2 was sufficient to induce tumorigenesis when hormone levels were normalized by this method. Immunohistochemistry indicated that tumors from these animals had similar proliferative rates and ER status, though PR status tended to be higher in the E2 + P4 group. This study demonstrates peroral administration can be used in long-term studies to elucidate relationships between different types and levels of steroid hormones.

Introduction

The role of estrogens in breast cancer has been studied for over 100 years and extensive work in this area has led to the well-accepted theory that increased exposure to estrogens over a woman's lifespan increases her risk of breast cancer (185). Progesterone (P4) is a progestogen that is also secreted from the ovary and similarly to estrogens, cycles over a woman's lifespan and decreases in menopause. Considerable data supports a proliferative role for P4 in the normal mammary gland (169,210), however, its role in breast cancer is controversial (210,211). The Women's Health Initiative showed that conjugated equine estrogen (CEE) and medroxyprogesterone acetate (MPA; a synthetic progestin) act synergistically to promote breast cancer compared with CEE alone (212,213), leading to the hypothesis that P4 increases breast cancer risk. However, subsequent studies focusing on the timing of administration and the effects of different types of progestogens (214,215) indicate that certain progestins may increase breast cancer risk but native P4 may not. These epidemiological studies have increased recognition of the complexities of P4's role in breast cancer and the need to further elucidate the interactions between estrogens and P4.

Since few mouse models develop luminal mammary cancer, rat models have been widely used to study mechanisms of estrogen-driven mammary tumorigenesis. Many studies using this model have shown that ovarian hormones are required for initiation and promotion of tumors (126,127) as well as sustained growth (216), yet few studies have focused on the specific roles of estrogens and P4. A specific role for estrogens is demonstrated by the finding that treatment of intact animals with tamoxifen or aromatase inhibitors (AIs) before nitrosomethylurea (NMU) or 7,12-dimethylbenz(a)anthracene (DMBA) administration delays carcinogen-induced tumor development (129,130). While the intact ACI rat spontaneously develops tumors in response to high doses of estrogens, high concentrations of both estrogen and P4 are required for the response in OVX animals (217), suggesting that P4 may also be important. Few studies have examined

whether P4 plays a role in carcinogen-induced tumorigenesis and results are unclear given the different doses, methods, and timing of hormone administration used (218,219).

A variety of methods are used to administer hormones following OVX including silastic tubing, hormone pellets, daily subcutaneous injections, and oral gavage. These methods initially result in supraphysiological levels of hormone or are stressful to the animal. Introducing hormones perorally in nut butter is a newer method of hormone delivery that is less stressful and leads to more normalized levels of ovarian hormones following OVX, without an initial supraphysiological spike in blood concentrations (143). The goal of the current study was to determine if estradiol (E2) alone will restore carcinogen-induced tumorigenesis when hormones are administered using peroral hormone administration or if P4 is also required with the hypothesis that with normalized levels of E2, tumors would not require P4 for growth. Short-term studies were conducted to determine the oral dose of E2 that was estrogenic, which was then used for a long-term study. Following OVX, daily E2 with or without P4 led to tumorigenesis in response to NMU that was similar to sham animals indicating that physiological levels of E2 can restore tumorigenesis without P4. There was a tendency for decreased tumor latency in E2 + P4-treated animals compared to E2-treated animals suggesting that additional studies are warranted to determine if P4 enhances latency or progression of carcinogen-induced tumors.

Materials and Methods

All animal procedures were approved by the Rutgers University Institutional Animal Care and Use Committee according to NIH guidelines. All surgery was performed under isoflurane gas and all efforts were made to minimize suffering.

Preparation and Administration of Hormones

E2, E2 benzoate (EB), and P4 (Sigma-Aldrich; St. Louis, MO) were independently dissolved in ethanol prior to dissolution in sesame oil (Sigma-Aldrich) at a concentration of 600 µg/ml (E2 or EB) and 15 mg/ml (P4). Equivalent volumes of ethanol without hormone were added to sesame oil to serve as the vehicle control for each hormone. Body weights were obtained prior to feeding each day and used to determine the appropriate volume of hormone to administer to each animal. Each day the appropriate volume of hormone or vehicle was mixed into approximately 4 g of peanut butter (Skippy Natural Peanut Butter) placed on 2x2 inch squares of parchment paper. Each animal was offered two allotments of peanut butter to achieve the appropriate treatment combination e.g. E2 and vehicle, P4 and vehicle, E2 plus P4, or vehicle and vehicle. The animals had access to the treatment until the next day when old papers were removed and new treatments were given. Hormone consumption was monitored and recorded daily.

Pilot Studies to Determine Estrogen Dose

Female Sprague Dawley rats (Charles River; Wilmington, MA) arrived on post-natal day (PND) 28 and were housed in a controlled environment with *ad libitum* access to food (Purina Mills Lab Diet; St Louis, MO) and water. To acclimate animals to the peanut butter, rats were offered peanut butter without treatment 2 hours after lights on each morning beginning on PND 35. Groups were normalized by body weight prior to surgeries, which were performed on PND 40 or 41. Bilateral ovariectomy (OVX) or sham surgery was carried out under isoflurane gas. Surgical procedures were performed using the aseptic no-touch technique. Animals were monitored for ten days post-operatively to ensure adequate recovery. Hormone treatments were started the day after surgery. Since the half-life of E2 in serum is only 2 to 8 hours (220,221), EB was chosen due to its

longer half-life. For the first pilot study, OVX rats were divided into three treatment groups (n=4): 1) EB (150 µg/kg/day); 2) EB + P4 (15 mg/kg/day); and 3) vehicle. A fourth group was given vehicle following sham surgery (n=4). In the second pilot study, OVX rats were divided into 3 treatment groups: 1) EB (300 µg/kg/day; n=5); 2) EB (600 µg/kg/day; n=5); and 3) vehicle (n=3) with a fourth group that received vehicle following sham surgery (n=3). After 10 days of treatment, rats were sacrificed by rapid decapitation. The uteri were excised and defatted then weighed. For sham animals, ovaries were removed from the uterine horns prior to weighing.

NMU Tumor Study

Rats arrived from Charles River on PND 28 and were housed and maintained as described above. Groups were normalized by body weight on the day of surgery performed on PND 40 or 41. OVX rats were subsequently treated with EB (600 µg/kg/day; n=8), P4 (15 mg/kg/day; n=7); EB + P4; (n=8); or vehicle alone (OVX; n=8) starting the day after surgery. A 5th group was given vehicle following sham surgery (Sham; n=8). After five weeks of peroral hormone administration, daily monitoring indicated that rats treated with EB were not consuming all of their treatment, thus animals were switched to E2 at the same dose for the rest of the study. On PND 50, rats were injected I.P. with 50 mg/kg NMU (Sigma-Aldrich). All injections were completed within one hour of dissolving NMU in sterile 0.9% saline (pH 4). Rats were palpated for tumors biweekly starting 4 weeks post-NMU injection. Tumor long length (L) and short length (S) were measured using a Vernier caliper and tumor volume was calculated ($S^2 * L/2$). Body weights were recorded biweekly. Rat chow was kept in hoppers for the duration of the study to monitor food consumption biweekly. Blood was taken from the lateral tail vein 4, 8, and 12 weeks after NMU injection three to five hours after hormones were offered. Rats were sacrificed if their tumor volume exceeded 10% body weight. As such, two rats from the group treated with EB/E2 (E) (sacrificed at 13 and 25 weeks post NMU), one rat from the E + P4 group (sacrificed 24 weeks post NMU) and one rat from the Sham group (sacrificed 16 weeks post NMU) were sacrificed prior to the end of the study. Remaining rats were sacrificed by rapid decapitation twenty-six weeks after NMU injection.

Tumors were excised and stored in 10% NBF. Pelts were removed then stretched, pinned to a wooden board, and submerged in 10% NBF. The uterus was excised, the fat removed, and weighed. For Sham animals, ovaries were removed from the uterine horns prior to weighing. Images of the uteri were taken prior to storage in 10% NBF.

Serum E2 Levels

Blood taken from the lateral tail vein was allowed to clot for 30 minutes at room temperature then spun at 1500 x g for 10 minutes at 4°C. Serum was collected and stored at -80°C until further analysis. E2 levels were determined using an ELISA following the manufacturer's directions (Calbiotech; Spring Valley, CA).

Mammary Gland Whole Mount Analysis

After fixing pelts in 10% NBF for at least 10 days, the left fourth inguinal mammary gland was dissected away from the skin, stretched on slides, and allowed to air-dry for 30 min. If this gland contained a tumor, the contralateral gland was used for analysis. Glands were then rehydrated in 70%, 50%, and 25% ethanol, placed in H_2O for 5 min, and stained in carmine alum for 1-2 nights (Sigma-Aldrich). After staining, slides were dehydrated in 70% and 95% ethanol followed by xylene. Glands were cleared in toluene for 1-6 weeks after dehydration to remove excess fat then air dried for 30 minutes before mounting SealPAK pouches (Kapak; Minneapolis, MN) with cedar wood oil (Acros; Geel, Belgium). To analyze mammary gland length, whole mounts were imaged using a Nikon DS-Fi1 camera (Nikon; Melville, NY) with NIS Elements software (Nikon). Mammary gland length was measured from images using the straight line function in FIJI from the far edge of the lymph node to the most distal point of the gland. To enhance magnification, glands were viewed and images taken using a Leica MDG41 stereomicroscope (Leica; Buffalo Grove, IL).

Tissue Histology

Fixed tumor and uterine tissues were dehydrated, cleared, and embedded in Paraplast using facilities located in the Histopathology Core of the Environmental Occupational Health Sciences Institute at Rutgers University. Samples were sectioned at 6 µm and placed on slides. For uteri,

cross sections were obtained. Slides were baked for 15 minutes at 60°C, followed by deparafinization in xylene and rehydration in decreasing concentrations of ethanol. Slides were stained with hematoxylin and eosin then mounted with Permount. A toxicological pathologist, who was blind to treatment, viewed tumor slides to determine whether the tumors were adenomas or adenocarcinomas. Uteri were scored to quantitate the grade (0-3) and stage (0-3) of metaplasia, which were added together to get a uterine score. Representative images of 0+0, 1+1, 2+2, and 3+3 were taken using an Olympus FSX100 microscope at 20X (Olympus; Waltham, MA).

Immunohistochemistry (IHC)

Fixed tumor tissue was dehydrated, cleared, and embedded as described above. Slides were baked at 55°C for 30 minutes, deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by boiling slides in 0.01 M sodium citrate buffer for 30 minutes then cooled at room temperature to 45°C before proceeding.

For Ki67, tissues were blocked in normal donkey serum (Santa Cruz Biotechnology; Dallas, TX) for 30 minutes at room temperature. Samples were incubated overnight at 4°C with either rabbit-Ki67 primary antibody (1:100, ab16667; Abcam; Cambridge, MA) or rabbit primary antibody isotype control (Life Technologies; Grand Island, NY), which served as a negative control on each slide. The following day the tissues were incubated with Alexafluor 488 labeled anti-rabbit secondary antibody (Life Technologies) for 60 minutes at room temperature. Slides were counterstained with DAPI (Life Technologies) then mounted with Prolong Gold Antifade (Life Technologies).

For estrogen receptor α (ERα) and P4 receptor (PR), endogenous peroxidase activity was blocked using 3% H_2O_2 for 10 minutes. ERα sections were blocked with normal goat serum (Vector Laboratories; Burlingame, CA) following the manufacturer's directions while PR was blocked with normal horse serum (Vector Laboratories) in a 1:1 dilution. Rabbit ERα primary antibody (MC20; Santa Cruz Biotechnology) was diluted in 1% BSA to 1:500 while Mouse PR primary antibody (MS-197-P0; ThermoScientific) was diluted in PBS + 0.1% triton to 1:300. Rabbit and mouse

primary antibody isotype control served as negative controls, respectively. Secondary antibodies were applied according to manufacturer's directions followed by development in 3,3'-diaminobenzidine (Sigma-Aldrich). Slides were counterstained with hematoxylin, dehydrated with increasing concentrations of ethanol followed by xylenes, then mounted using Permount.

For all IHC, tumor sections were viewed and five representative pictures were taken at random from one section per tumor using an Olympus FSX100 microscope at 20X (Olympus). Ki67 pictures were taken with the same exposure settings for all samples using green and blue fluorescence channels to visualize antibody staining and nuclei location, respectively. For ERα and PR, pictures were taken using bright field setting.

The amount of Ki67 staining per nuclei was calculated using previously described methods (222). ERα and PR were analyzed using the color deconvolution plug-in of FIJI Is Just ImageJ (FIJI) (223). Briefly, images were split into brown and purple images using the H DAB vector. Mean grey density was determined from the brown image then converted to optical density using the equation OD = log (255 / mean grey density), where 255 is the max intensity for 8-bit images. OD values from the 5 separate images per tumor were averaged to give a tumor OD. For animals with multiple tumors, average Ki67 staining or OD values were averaged for that animal before being included in the analysis.

Statistical Analysis

Body weights were analyzed using a repeated measures two-way ANOVA followed by a post hoc Tukey's multiple comparison tests through 13 weeks when the first rat was sacrificed. Body weights were also analyzed on the final day of the study by one-way ANOVA with Tukey's multiple comparisons post hoc analysis. Uterine weights, tumor burden, and mammary gland length were analyzed using a one-way ANOVA with Tukey's multiple comparisons post hoc analysis. Tumor incidence was analyzed using a Mantel-Cox Log-rank test. Uterine score, Ki67 expression, ERα expression, and PR expression were analyzed using a Kruskal-Wallis test with Dunn's

Multiple Comparisons posttest or a Mann Whitney test. GraphPad Prism version 5.0 (La Jolla, CA) was used to perform statistical analyses and $p \leq 0.05$ was considered significant.

Results

Short-term Studies

To determine the dose of peroral EB that would restore E2 to physiological levels in OVX animals, two short-term studies were conducted using uterine weights as a measure of estrogenicity. As expected, uterine weights in the OVX group were less than those in the sham group ten days after surgery in both studies. In the first study, 150 µg/kg/day EB ± 15 mg/kg/day P4 was unable to restore uterine weights over OVX (Figure 1A). In the second study, daily treatment with 300 µg/kg EB perorally was unable to rescue the decrease in uterine weight caused by OVX, while the 600 µg/kg dose increased uterine weight over OVX ($p < 0.05$; Figure 1B). Therefore, the 600 µg/kg daily dose was chosen for the long-term tumor study.

Long-term NMU Study

Hormone Consumption

For this study rats were OVX on PND 40 and treated daily with hormones until 26 weeks post NMU injection. Daily monitoring of hormone consumption was based on visualization of whether the paper was completely devoid of peanut butter (all), peanut butter was partially consumed (most), or appeared as it had when hormones were introduced (none). Hormone consumption over the first month of the study revealed that animals treated with EB consumed either most or none of both of their treatments (EB + P4 or EB + vehicle) while animals treated with vehicle or P4 consumed all of their treatments. After 4 weeks, animals treated with EB ± P4 consumed more food than Sham, OVX, or P4 animals as determined by measuring food hoppers biweekly, indicating that the reason EB ± P4 rats were not consuming all of the peanut butter was not related to a general decrease in overall food intake. Together, these results suggested that the animals had a taste aversion to the EB, either E2 or benzoate. Therefore, animals treated with EB were switched to E2 starting 4 weeks post NMU. While consumption increased when the treatment

was switched, the animals treated with E (EB or E2) never consumed as much as the P4 only, Sham, or OVX groups, all of whom consumed the entire treatment each day.

Uterine and Body Weights

Uteri from Sham animals and animals treated with E ± P4 appeared normal and were similar upon visual inspection. Uteri from OVX animals treated ± P4 were atrophied and visually indistinguishable from each other (Figure 2A). As expected, uterine weights were significantly decreased in OVX compared to Sham (Figure 2B). Treatment with E ± P4 increased uterine weights over OVX while treatment with P4 daily did not affect uterine weight ($p < 0.05$; Figure 2B). Body weights of OVX and P4 only were different from Sham beginning at 4 and 7 weeks post NMU injection, respectively ($p < 0.01$), while those of E only and E + P4 were not different from Sham ($p < 0.05$; Figure 2C).

Serum E2

To monitor serum E2 levels during the long-term study, blood was collected from rats via the lateral tail vein beginning 3 or 5 hours after hormones were administered at 4, 8, and 12 weeks post-NMU injection. Serum from Sham and OVX were assayed for comparison. E2 levels in serum of OVX and Sham ranged from 3.1-8.5 and 2.6-18.1 pg/ml, respectively. Serum E2 levels in E ± P4 ranged from 1.6 to 149.2 pg/ml across collection time points (Figure 3).

Uterine Histology

Since the serum E2 values were elevated over Sham, uterine morphology was analyzed to determine if there were any uterine abnormalities induced by E2 treatment. P4 only and OVX animals were not included in the analysis since their uteri were atrophied and did not display any abnormalities. Visual observation of uterine sections from E only, E + P4, and Sham animals revealed epithelial metaplasia that was extensive in some samples and less apparent in others. To quantify the changes, a toxicological pathologist determined how extensive the injury was (stage) and how disorganized the cells were (grade). Representative images of 0+0 (relatively normal), 1+1, 2+2, and 3+3 are presented in Figure 4. Stage and grade were added together to give an overall

uterine score and scores were compiled by group. There was more extensive uterine metaplasia in the E only group compared to Sham ($p<0.05$). The E + P4 tended to be less extensive than the E only group, but still more extensive than Sham.

Mammary Glands

Since E2 and P4 are important for normal mammary gland growth and maintenance, mammary gland whole mounts were prepared and imaged to analyze morphology. As expected, the ductal structures of the glands in the Sham group extended to the end of the fat pads with branching and alveolar buds appropriate for adult virgin animals (Figure 5A). The glands of animals treated with E2 ± P4 appeared similar (Figure 5B-C). The mammary glands from the OVX and P4 groups were indistinguishable from one another and exhibited very thin ducts and lacked alveolar buds (Figure 5D-E). To determine mammary gland length, distance between the lymph node and the most distal edge of the gland was measured as represented in Figure 5F. Mammary glands of OVX ± P4 animals extended significantly less distance into the fat pad relative to the Sham and E2 ± P4 glands (Figure 5G).

Tumor Development

To determine the influence of long-term peroral hormone treatment on mammary tumor development, rats were injected with NMU on PND 50. Tumors were palpated biweekly and tumor incidence was calculated (Figure 6A). The first tumors appeared 6.5 weeks post NMU injection in E only, E + P4, and Sham groups. There was no difference between tumor incidence in E only, E + P4, or Sham groups 26 weeks post NMU injection. There tended to be an increase in tumor latency in the E only group. One tumor developed in the OVX group 8.5 weeks post NMU injection. All tumors were adenomas or adenocarcinomas with no differences in tumor type between treatment groups (data not shown). Interestingly, autopsy revealed that the OVX animal that presented with a tumor was the only animal that had large metastases to the lungs which was confirmed by histological examination. Total tumor burden was calculated by adding the volumes

of individual tumors for each animal. There was no difference in total tumor burden although the E only group tended to have a greater burden than the E + P4 group or the Sham group (Figure 6B).

Tumor IHC

IHC was performed for Ki67 as a marker of proliferation, and ERα and PR to determine hormone receptor status. Representative images of low and high expression levels are presented for each marker in Figure 7. There were no significant differences in Ki67 staining between the Sham, E only, and E + P4 groups, although the E only group tended to be lower (Figure 7C). Similarly, there were no significant differences between groups for expression of ERα (Figure 7F). There was no significant difference in PR expression between the three treatment groups. However, comparing PR expression between E vs. E + P4 indicated expression was greater in animals treated with E + P4 compared to those treated with E only (Mann Whitney test, $p<0.05$; Figure 7I). Sham animals had PR expression that fell in the range of the E only and E + P4 groups. The OVX animal had one of the highest Ki67 expression levels and the lowest ERα and PR expression (data not shown).

Discussion

The goals of this study were two-fold: (1) to determine if estrogens alone can drive carcinogen-induced tumorigenesis in OVX rats or if P4 is also required and (2) to determine if tumors could be induced using a non-stressful method of hormone administration. Previous studies used methods that result in supraphysiological levels of hormone or induce stress (143), therefore peroral hormone administration is more suitable for long-term tumor studies. Body weights and uterine weights following six months of peroral hormone administration suggested that hormones were biologically active. The number of animals that developed tumors in response to NMU was similar between E-treated and Sham controls, indicating the lack of an absolute requirement for P4 for tumor development.

Pilot studies were needed to ensure that the doses chosen to restore physiological levels of hormones were sufficiently estrogenic (224). Previous short-term studies with a similar goal of increasing serum levels without supraphysiological spikes used a lower dose of E2 (28 µg/kg/day) that was unable to restore uterine weights (143). In the present work, only the 600 µg/kg dose increased uterine weights over the OVX control animals in a short-term ten-day study and was therefore used for the long-term study. Additionally, in the first short-term study with 150 µg/kg/day, no additive effect of EB + P4 on uterine weight was observed which agrees with previous research showing that treatment with E2 (10 µg/kg) and P4 by subcutaneous injection does not affect uterine weight relative to increases observed with E2 alone (90,225).

While peroral feeding of nut butter has been examined as a useful method of hormone administration, this is the first long-term study to examine its effectiveness in a carcinogenic-induced model of tumorigenesis. Therefore, several biological endpoints were examined to determine long-term estrogenicity of the chosen dose. While all animals did not consume the entire dose of E each day, serum E2 levels did not correspond with the pattern of feeding that was observed, nor did they indicate lower circulating E2 in the animals that did not consume the treatments as well. E2 levels in serum of Shams ranged from 2.6 to 18.1 pg/ml while those of OVX

animals ranged from 3.1 to 7.7 pg/ml. The ELISA used in the present work has been reported to be the most accurate and sensitive compared to other commercially available assays for rodent serum (226). Interestingly, similar to the present results, it could not distinguish between Sham and OVX animals based on E2 serum levels in animals that were not specifically determined to be proestrus (226). Serum levels in E-treated animals ranged between 1.6 to 149.2 pg/ml over 12 weeks of the study. Previous studies have shown that intact animals sacrificed during estrus or proestrus have serum E2 levels ranging from 2.4 to 145.4 pg/ml (143,227). Therefore, while the values reported here for E-treated animals were higher than Shams in this study, the levels were generally within a physiological range.

Since weekly E2 values represented a single point in time for each animal, other endpoints were evaluated to better assess the physiological relevance of the dose used in this study. OVX causes rats to become hyperphagic and increases positive energy balance leading to increased weight gain compared with sham operated animals (228). Treatment with E2 reduces body weight gain (229-231) while P4 alone has no effect on body weight following OVX (232). Similar results were found in the present study, indicating long-term efficacy of the E treatment. Uterine weights in the long-term study followed those of the short term study where OVX decreased uterine weight and E ± P4 rescued it. Uterine histology was evaluated as a more sensitive marker of estrogenicity. Uteri were sectioned and stained with H&E to analyze cellular endpoints including luminal epithelial height and gland number (233). This analysis indicated that uteri did not have a normal histology and exhibited differing levels of metaplasia. While uterine metaplasia in response to NMU has been described (234), the metaplastic response was greater in OVX animals treated with E relative to Shams in the present study. As expected, treatment with P4 tended to decrease the metaplasia induced by E alone. These results suggest that a lower dose of E might have been sufficiently estrogenic in a long-term study without inducing uterine metaplasia.

In the present study, mammary gland morphology in OVX animals was maintained with either E or E + P4 treatment. Interestingly, there was not an obvious difference between the two

treatments. Mammary gland morphology was not maintained with P4 alone, indicating that there is an absolute requirement for estrogens. Similar results were reported in a recent study that examined E2 ± dydrogesterone (DG) using a similar method of hormone administration. Histological analysis of mammary glands revealed that DG did not reverse the atrophic morphology observed in OVX rats however treatment with E2 ± DG resulted in mammary glands similar to those of sham controls (235). While the measurement included here was a qualitative overview of mammary gland morphology, these results are interesting as previous research has indicated that estrogens are required for pubertal mammary gland development but P4 is also required for mammary gland maintenance during adulthood in mice (236).

The main goal of the present study was to determine if E2 alone could restore mammary tumorigenesis in OVX rats treated with hormones or if P4 was also required. Overall tumor burden, tumor incidence, and tumor multiplicity were similar in Sham, E only, and E + P4 groups. These results indicate that contrary to previous research using a chemical carcinogen in an OVX rat model (218,219), estrogens alone can fully restore tumorigenesis. The differences might be attributed to the level of circulating hormones achieved by treatment, which were not monitored in previous studies. Alternatively, the timing of hormone administration could influence tumor development. Specifically, silastic tubing provides a constant slow release of hormone while peroral treatment gives a daily pulse of hormone, which better mimic the cyclical fluctuations in steroids. In future studies, it would be worth comparing multiple types of hormone administration to better understand hormone kinetics and their effects on long-term physiology.

While gross tumor parameters did not differ between E, E + P4 and Sham groups, E-treated animals tended to have a delay in tumor onset (i.e. latency) indicating that P4 may play a role in tumor initiation or enhancing tumor promotion. The small number of animals in the present study may have precluded detecting a significant change in these parameters. Interestingly, tumors from the E + P4 group had increased PR expression compared to the E only group. In the normal mammary gland, treatment with P4 downregulates expression of PR (237). However, tumors do

not always respond to hormones in a similar manner as the normal gland. For example, cells that express ERα and PR are limited and do not typically divide, but act in a paracrine manner to stimulate neighboring cells to divide (94,95). Luminal breast tumors have increased levels of ERα and PR and the cells that express these receptors also divide. The switch from paracrine signaling to autocrine signaling is an active area of research.

A clear role for estrogens is indicated by studies showing that treatment with tamoxifen or AIs leads to tumor regression (130,238). However, this experimental paradigm does not determine whether P4 is also required for tumorigenesis. Interestingly, few studies have used ovarian ablation and hormone replacement to answer this question and those that have been performed have shown varying results. Tumors failed to develop in response to NMU when 26-30 mg of E2 was administered to OVX rats using silastic tubing. Interestingly, tumors did develop in animals treated with estrone (E1) and tumor incidence nearly doubled when P4 was also included (219). The authors attributed the lack of an effect of E2 to the high dosage administered (219) based on reports that high levels of E2 and P4 (similar to levels seen in pregnancy) around the time of NMU administration prevent mammary tumor development in intact rats (136,239). The reason for the difference in tumor burden with E2 versus E1 was unresolved. In contrast to findings of Bigsby (219), tumors did form in response to DMBA following ovarian ablation and replacement with daily gavage feeding of 100, 300, or 900 µg/kg/day E2 or daily intravenous administration with 0.4, 10 or 250 µg/kg/day E2; however, total tumor burden was not restored to that of sham animals (145). The combination of E2 and P4 was not examined in this study and there was no relationship between the dose and tumor incidence (145). In another study, OVX rats treated by subcutaneous injection with 0, 1, 10, 100 or 1000 µg/kg every other day did not develop palpable tumors in response to DMBA, however adding 4 mg P4 beginning 85 days after DMBA administration increased tumor susceptibility in rats treated with 1 and 10 µg/kg E2. No sham operated animals were included in this study (218). While interpreting these findings is complicated by the use of inconsistent hormone concentrations and methods of administration, they suggest that estrogens

alone can promote carcinogen-induced tumorigenesis but that P4 may be required for a full response. Treating with silastic tubing can lead to high levels of hormone, which is important to consider as very high levels of estrogens can prevent mammary growth and tumor development.

In the current study, E treatment alone was found to be sufficient for restoration of carcinogen-induced tumorigenesis when OVX rats were given replacement hormone perorally. This study demonstrates the usefulness of this method in terms of providing a low-stress method of hormone administration that can be used to achieve circulating concentrations of physiological levels of hormones. This model will be useful in future studies designed to understand the effects of combinations of progestogens and estrogens on mammary tumor development.

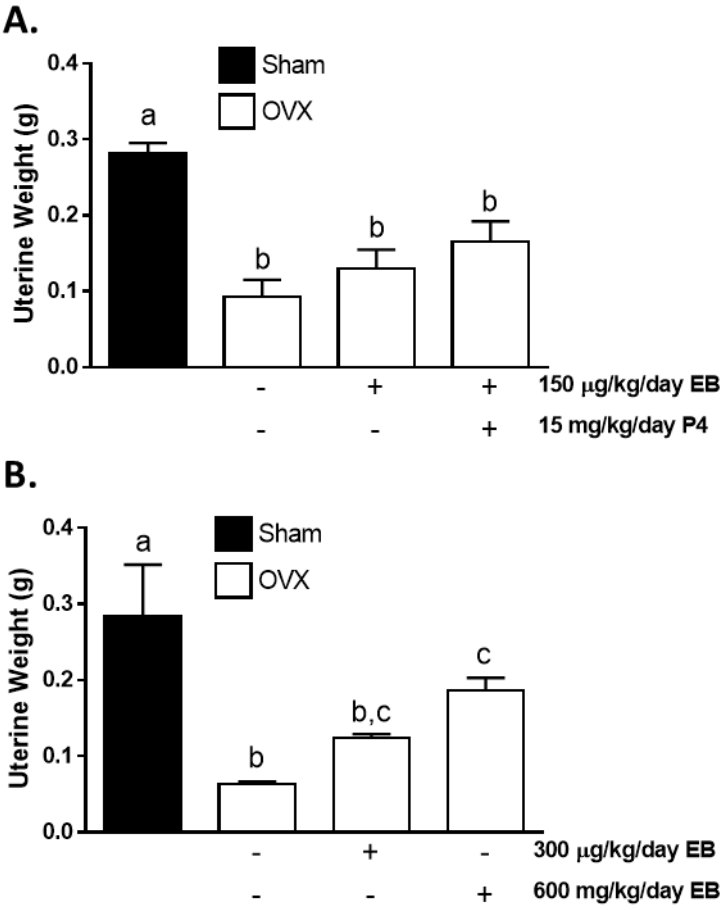

Figure 1. The most effective dose of EB for peroral administration following OVX is 600 µg/kg/day. Uteri were excised and uterine wet weights were determined at sacrifice (for Sham animals, ovaries were removed prior to weighing). Uterine weights are expressed as mean ± SEM, $p<0.05$, one-way ANOVA; Tukey's multiple comparisons posttest; different letters denote significant difference. (A) In the first short term study, uterine weights of hormone-treated rats were not different from OVX (n=4). (B) In the second study, 600 µg/kg/day EB increased uterine weight over OVX (n = 5, 3, and 3 for EB treated, Sham, and OVX animals respectively).

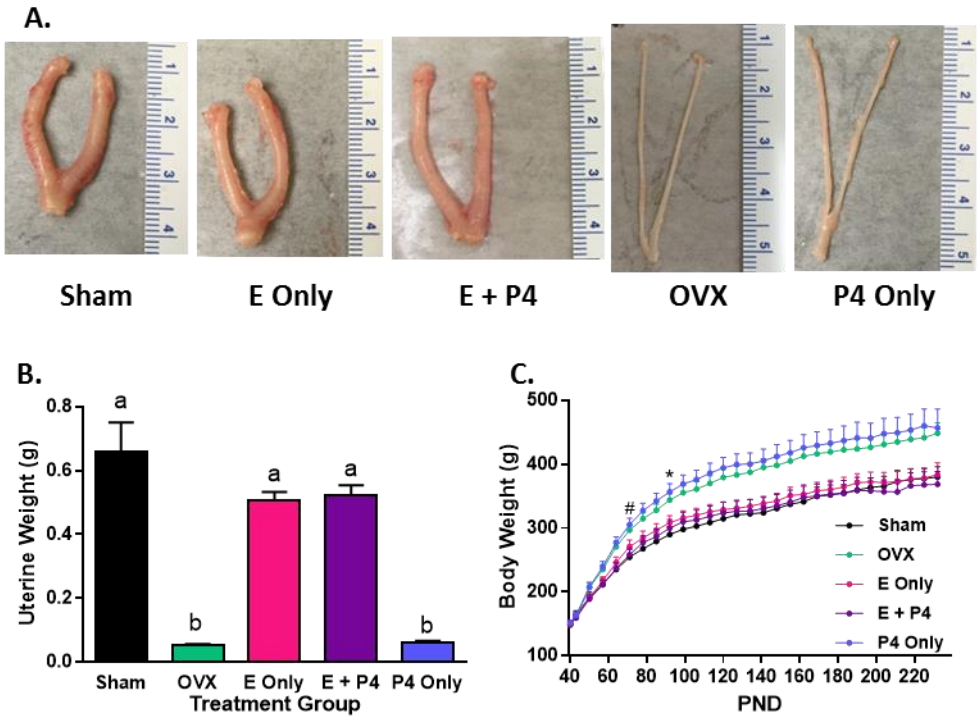

Figure 2. Long-term, daily, peroral E treatment was physiologically active for the duration of the study. At sacrifice, uteri were excised and weighed. Images of uteri were taken before storage in 10% NBF. (A) Representative images of uteri from each treatment group. (B) Uterine weights were reduced in OVX rats treated with vehicle but increased with E or E + P4 treatment. P4 alone was unable to restore uterine weights. Uterine weights are expressed as mean ± SEM; n=8, 7 for P4 only; $p<0.05$, one-way ANOVA; Tukey's multiple comparisons posttest; different letters denote significant difference. (C) Rats were weighed biweekly for the duration of the study. Long term exposure to E ± P4 resulted in body weights that were similar to Sham. Daily treatment with P4 did not prevent weight gain following OVX. Body weights are expressed as mean ± SEM, n=8, 7 for P4 only; repeated measures two-way ANOVA through the first animal sacrifice at 13 weeks post NMU (PND 148) (treatment: $p < 0.01$) with Tukey's multiple comparisons posttest (* start of $p<0.05$ for OVX, # start of $p<0.05$ for P4 only; compared to Sham). The pattern persisted in remaining animals at the end of the study ($p<0.05$, one-way ANOVA, Tukey's multiple comparisons posttest).

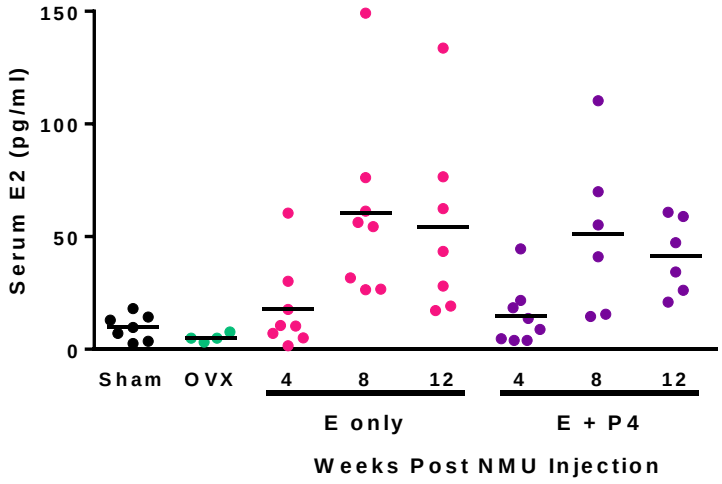

Figure 3. Serum E2 levels were elevated with peroral hormone treatment. Blood collection from the lateral tail vein was initiated 5, 3, and 5 hours after treatment was presented 4, 8, and 12 weeks after NMU injection respectively. It took two hours to collect blood from all animals on any given day. Serum from Sham animals, pools of OVX untreated animals, E only, or E + P4 was analyzed using an E2 ELISA (Calbiotech).

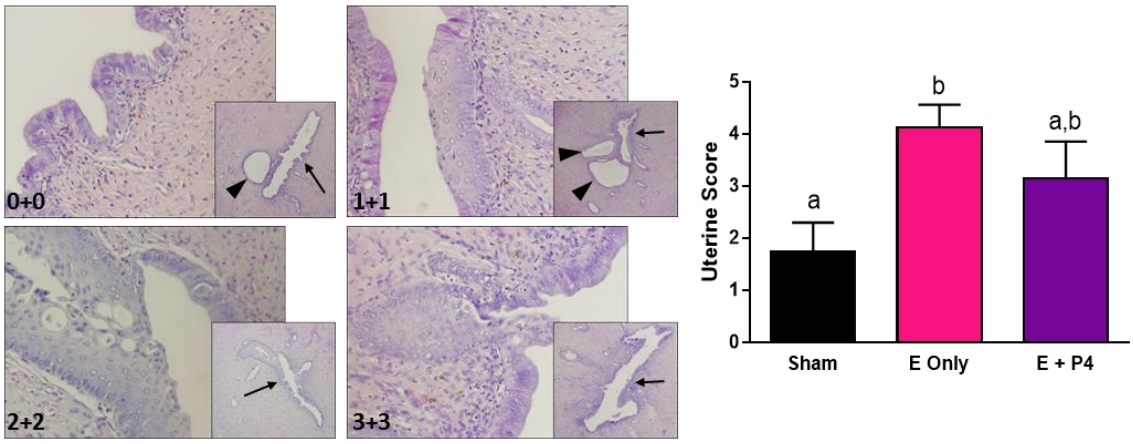

Figure 4. Treatment with E led to increased uterine metaplasia in treated animals. Cross sections of uteri were stained with hematoxylin and eosin. A toxicological pathologist staged and graded the slides from 0-3 for each category leading to scores of stage + grade, specifically focusing on the uterine lumen (arrows) and not dilated ducts (arrow heads). Images are representative of 0+0, 1+1, 2+2, and 3+3 respectively. The larger images were taken at 20X and the inset at 4X using an Olympus FSX100 microscope. Scores were derived by adding stage and grade to get a final score of 0-6. P4 only and OVX animals were not included in analysis as all uteri were atrophied. Uterine scores are expressed as mean ± SEM; n=8; $p<0.05$, Kruskal-Wallis test with Dunn's Multiple Comparison's posttest; different letters denote significant difference.

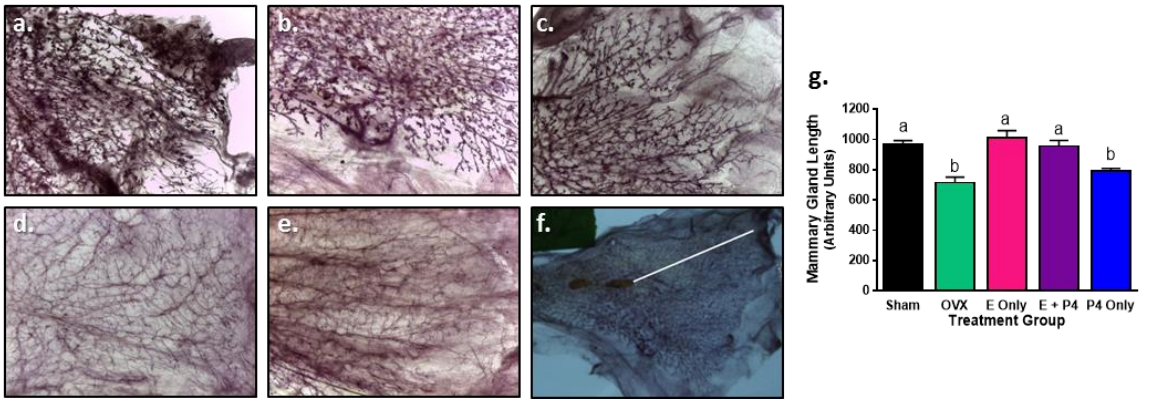

Figure 5. Mammary gland development of E ± P4 treated animals was similar to Sham. Representative images of whole mounts (taken at 4x using a Leica MDG41 stereomicroscope) demonstrate ductal thickness was similar in Sham (A), E only (B), and E + P4 (C) while glands were similar between OVX (D) and P4 only (E) with thinner ducts relative to the other groups. Mammary gland length was measured from the lymph node to the most distal point using FIJI as shown in F. Mammary glands from Sham, E only, and E + P4 exhibited similar ductal lengths while OVX and P4 only were shorter (G). Mammary gland length is expressed as mean ± SEM; n= 8, 7 for P4 only; $p<0.05$, one-way ANOVA; Tukey's multiple comparisons posttest.

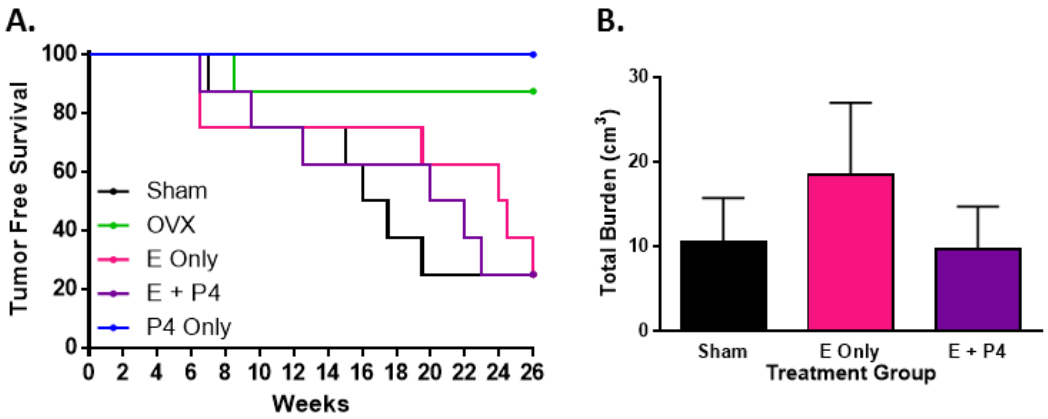

Figure 6. Peroral treatment with E ± P4 after OVX results in tumor development following NMU. (A) Rats were injected with NMU on PND 50 then palpated biweekly for the appearance of tumors. There was no difference in tumor incidence between E, E + P4 and Sham but all three groups were different from P only and OVX, which were not different from one another. n= 8, 7 for P4 only; $p<0.05$, Mantel-Cox Log-rank test. (B) Tumors were measured with a Vernier caliper the morning of sacrifice. To calculate tumor burden, the volume of each tumor was added together per animal. There were no differences in tumor burden among the treatment groups; n=6.

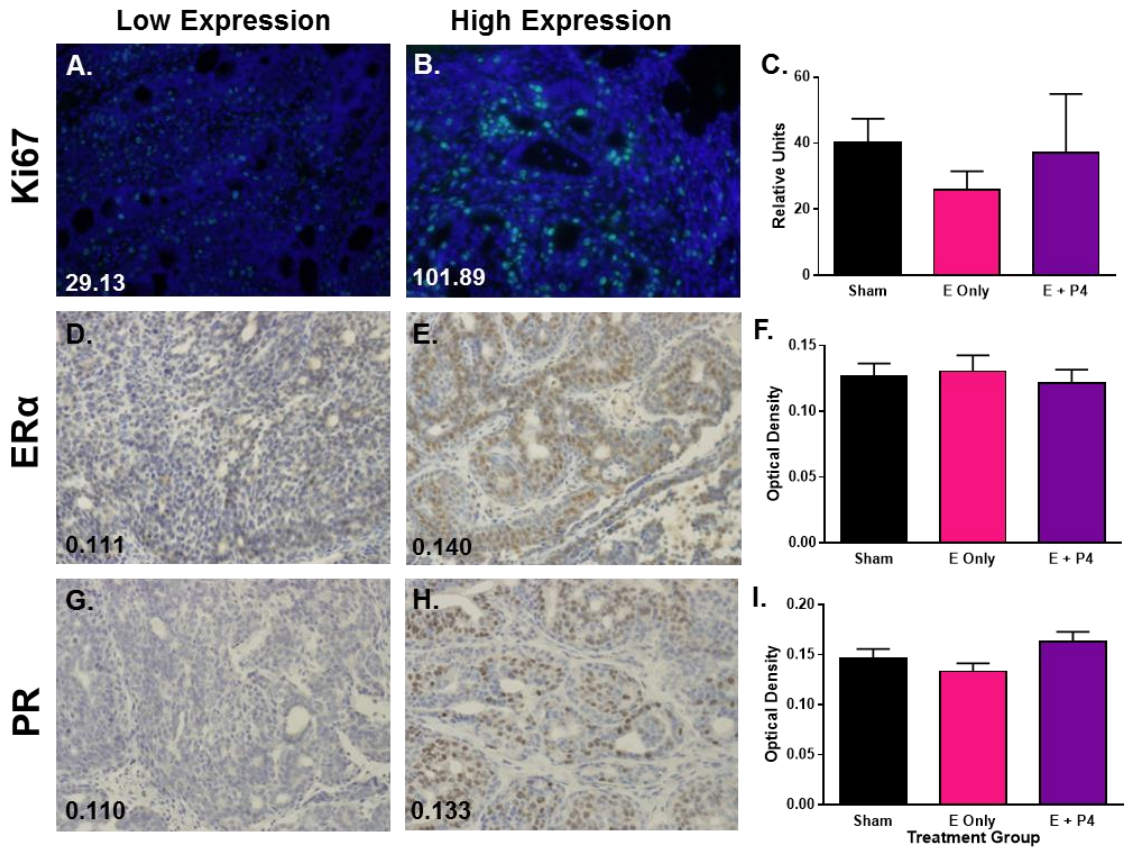

Figure 7. The types of tumors that formed did not differ with treatment with E±P4. Ki67, ERα, and PR IHC was performed and measured as described. Images depicting low (A, D, G) and high (B, E, H) expression of Ki67, ERα, and PR are presented with corresponding scores in the bottom left corner of each image. There was no difference in Ki67 or ERα expression between treatment groups; n=6. Animals treated with E + P4 had more PR expression than animals treated with E alone (Mann-Whitney test comparing E only to E + P4, n=6; $p<0.05$).

Chapter 3

Effect of Alcohol Exposure *in Utero* on the Mammary Transcriptome in Early Development

Abstract

Environmental exposures during development influence the mammary glands of offspring leading to increased susceptibility for cancer development in adulthood. Following endocrine disruptor exposure *in utero*, mammary gland gene expression is altered in genes involved in epigenetic modifications and estrogen signaling in offspring during development and mammary cancer susceptibly is increased. Alcohol *in utero* increases mammary tumor development but how mammary gene expression is altered is largely unknown. To determine if alcohol *in utero* leads to changes in genes related to epigenetics and the estrogen axis, mammary glands of rat pups exposed to alcohol *in utero* were harvested at PND 2, 10, and 20. For PND 2 and 10 glands, whole gland transcriptomes were analyzed by RNASeq with a read depth of 25 million reads. This analysis indicated limited changes in gene expression between alcohol-exposed offspring and controls at each time point. RNASeq analysis of a portion of the mammary gland at PND 20 led to further analysis by qRTPCR, suggesting the heterogeneity of the gland represented a problem with analyzing a portion of the gland. Additional reads for the PND 2 and 10 samples already analyzed and the addition of more animals to remove biases in biological variability are necessary to complete this transcriptomic data set.

Introduction

The Developmental Origins of Health and Disease hypothesis (DOHAD) suggests that influences early in development can result in permanent changes in physiology and metabolism, leading to diseases in adulthood including diabetes, obesity, and cancer (240). For example, daughters of women who took diethylstilbesterol (DES) during pregnancy have an increased risk of developing various hormone-dependent cancers including breast cancer (241). Animal studies support findings in humans that DES exposure *in utero* leads to increased mammary tumorigenesis (189), and other environmental exposures during gestation, such as the plasticizer bisphenol A (BPA) or a high fat diet, also lead to increased risk of breast cancer in offspring (190,194). A common theme with these exposures is that they disrupt the estrogen axis by either directly binding to the estrogen receptor (191) or by increasing circulating estradiol (E2) (185,194). Alcohol consumption increases E2 levels in rodents (242) and in women (243). While drinking during pregnancy is often associated with developing the neurological abnormalities involved in fetal alcohol spectrum disorder (FASD) (244), there may also be an increased risk for cancer development in offspring (245,246) including breast cancer (201,208,247).

Mammary gland development begins during the fetal period, but the most expansive development is under the influence of hormones during puberty and then pregnancy (48). These three phases of development are thought to be periods of vulnerability to perturbations imposed by endocrine disrupting chemicals (248). During fetal and embryonic life, the animal is susceptible to small changes in hormones that could affect later development, suggesting that slight changes in hormone levels could have a lasting impact (249). Environmental exposure during gestation can alter subsequent mammary morphology as evidenced by an increase in the number of terminal end buds (TEB), the proliferative structure guiding elongation in the gland, in response to neonatal DES or BPA (199,250). Gene expression profiles can also change. Mammary glands of mice exposed to BPA or DES *in utero* express increased levels of EZH2, an enzyme overexpressed in cancer that has methyltransferase activity important for DNA methylation leading to epigenetic modifications

(251). Interestingly, gene expression of differentiation markers in mammary epithelial cells exposed to DES *in utero* were greater than controls at postnatal day (PND) 35 and less than controls at PND 49, indicating aberrant regulation of genes at an earlier time point was overcorrected (252). In the adult uterus, genes with estrogen response elements (ERE) increase following *in utero* DES exposure, suggesting a reprogramming of estrogen responsive genes because they are "hyper responsive" to estrogens in adulthood as a direct result of their *in utero* exposure (183,200). We have previously shown that alcohol *in utero* leads to a hyperproliferative gland at PND 20 as well as increased aromatase protein and IGF-1 mRNA expression in the mammary gland (209). While there were no differences in TEB number (209), these results suggest that there are differences in gene expression in mammary glands in response to alcohol during gestation, specifically genes with ERE and genes involved in epigenetic modifications such as EZH2.

One of the greatest technological advances for biological science in the 21st century is improved genomic sequencing. RNASeq utilizes next generation sequencing to analyze the entire transcriptome giving an overview of all RNA transcripts present at a certain time, which allows for analyses including expression levels of genes, alternative splicing and other post-transcriptional modifications, and discovery of new transcripts (253). For sequencing, nucleic acid starting material is sheared and made into single stranded template, which is then immobilized and amplified on a glass slide or nano-bead (254). The use of high resolution imaging to detect dNTP incorporation increases accuracy and precision, and continues to improve as technologies advance (255). Following sequencing, reads are mapped to an annotated genome and expression can be analyzed with a variety of software packages, one of the most common is the Tuxedo Suite (256,257). Once expression profiles are established, gene networks and signaling pathways can be annotated to determine biological relevance.

An understanding of genes that change in the mammary gland following exposure to alcohol *in utero* may help to elucidate biological pathways involved in the increased mammary tumorigenesis seen in response to a chemical carcinogen during adulthood in rats. Gene expression

patterns are expected to be established close to the period of *in utero* exposure, therefore we examined early time points in development i.e. PND 2, 10, and 20 with the hypothesis that genes involved in epigenetic regulation and genes with EREs would be altered at these time points. Initial RNASeq analysis with a low read depth revealed limited differences in gene expression between mammary glands of alcohol and pair fed controls at PND 2 and 10. Analysis of PND 20 samples for which only a portion of the gland was analyzed indicated that variability in gene expression patterns may be related to the heterogeneity of the gland.

Materials and Methods

Animals and Treatment

Timed pregnant Sprague Dawley rats (Charles River; Wilmington, MA) arrived on gestational day (GD) 5 (GD 0 is the day of vaginal plug) and were housed in a controlled environment with a 12-hour light/dark cycle (6:00 AM/6:00 PM). Upon arrival, dams were separated into three groups, which were normalized by body weight. Rats were acclimated to the environment for two days prior to initiation of treatments (n=6): Alcohol Fed (AF), Pair Fed (PF), and Ad Libitum Fed (AL). The AF dams acclimated to a liquid alcohol diet (Bio-Serv; Frenchtown, NJ) starting on GD 7 and GD 8 with 2.2% (v/v) ethanol, then 4.4% (v/v) ethanol for GD 9 and 10, and finally 6.7% ethanol (v/v; 35% of total calories) for GD11 through parturition. PF dams had access to an isocaloric liquid diet (Bio-Serv; Frenchtown, NJ) at a volume equal to 120% of the average amount of diet consumed by the AF dams from the previous day. The additional 20% was included to compensate for the design of the feeder bottle. The AL group had *ad libitum* access to a solid pellet diet (Purina Mills Lab Diet, St Louis, MO). Rations were measured and replaced just before lights out every day. All dams had *ad libitum* access to water throughout the study. Dam weights were recorded every other day. Blood was taken from the lateral tail vein of pregnant dams on GD 19, beginning at 8:00 PM with lights off under a red light. At birth, all female pups were weighed then cross-fostered to AL dams to ensure alcohol exposure was confined to gestation. Male pups were decapitated after sexing at birth. One of the PF dams was ultimately not pregnant therefore n=5 for that treatment group. Litters were normalized to 9 female pups so that each dam nursed 3 sisters from an AF dam, a PF dam, and a different AL dam. On each day of sacrifice one of the sisters from each of treatment group were euthanized by rapid decapitation. On PND 2 the left and right inguinal mammary glands were removed and each gland was stored separately in RNAlater (Qiagen; Valencia, CA). On PND 10, the left and right glands were cut into pieces, combined, and divided between three tubes of RNAlater. On PND 20, the left gland was cut in half and the first half was stored in 10% neutral buffered formalin (NBF) while the second half was cut

into pieces and combined with the right gland that was also cut into pieces and stored in 4 tubes of RNAlater. All samples were stored at -80°C until further analysis

Dam Blood Alcohol Levels and Serum E2

Tail vein blood was allowed to clot for 30 minutes at room temperature then spun at 1500xg for 10 minutes at 4°C. Serum was collected and stored at -20°C. Blood alcohol levels (BAL) of all dams were measured using the Analox Alcohol Analyzer (Analox Instruments; Lunenburg, MA) according to the manufacturer's directions. The calibrators were made using a pool of blood from AL dams and 100% ethanol.

Serum taken from the tail vein was also analyzed for serum E2 levels using an E2 ELISA following the manufacturer's directions (Calbiotech; Spring Valley, CA).

RNA Isolation

For PND 2, RNA was isolated from the entire left gland, for PND 10, RNA was isolated from both glands, and for PND 20, RNA was isolated from a 100 mg piece of the gland. RNA was isolated using the RNeasy lipid tissue mini kit according to the manufacturer's directions (Qiagen). RNA quantity was assessed using the Nanodrop ND-100 (ThermoScientific; Waltham, MA) and RNA quality was verified using the Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano kit (Agilent Technologies; Santa Clara, CA).

RNASeq

Pups from the same dams were selected from PND 2, 10, and 20 such that mammary glands from sisters were analyzed across time while at each time point each sample represented offspring from a different dam (n=3 per time point). RNASeq library preparation and sequencing was carried out at the Genomic Services Laboratory, HudsonAlpha Institute for Biotechnology (Huntsville, AL). RNA integrity was rechecked by HudsonAlpha prior to sequencing. PolyA libraries of RNA were made from total RNA using NEBNext magnetic oligo d(T)25 beads (New England Biolabs Inc.; Ipswich, MA). RNA was fragmented then converted into cDNA for library preparation using the NEBNext mRNA Library Prep Reagent Set for Illumina (New England Biolabs Inc.). PolyA

RNASeq libraries were prepared with uniquely indexed primers using the TruSeq RNA Library Preparation Kit (Illumina; San Diego, CA). cDNA libraries were amplified through six PCR cycles using KAPA HiFi HotStart Ready Mix (Kapa Biosystems Inc.; Woburn, MA). The cDNA library quality and quantity was assessed using the Qubit Fluorometer, Agilent Bioanalyzer, and KAPA Biosystems Library Quantification. After KAPA quantitation and dilution, the libraries were clustered four per lane and sequenced on an Illumina HiSeq 4000 instrument with 50 bp paired end (PE) reads. Reads were converted to .fastq files using the default settings on bcl2fastq conversion software v1.8.3 and quality control on raw data was performed using FastQC (Bahraham Bioinformatics; London, UK).

Bioinformatics

After sequencing, reads were mapped to the reference rat genome (Rn6; NCBI; Bethesda, MD) using TopHat v2.0 (258). CuffDiff v2.2 was used to determine differential gene and transcript expression (257). To analyze differential expression, cummeRbund was used to look at overall changes in gene expression as well as differences in individual genes (259). Data were subjected to principle components analysis (PCA) to determine clustering patterns.

qRTPCR

The High Capacity cDNA Reverse Transcription Kit (Life Technologies; Grand Island, NY) was used to reverse transcribe 1 µg RNA using the GeneAmp® PCR System 9700 (Life Technologies). Each primer was validated using standard curves (see Table 1 for primer sequences). A pool of RNA from PND 20 mammary glands was used for primer validation. To validate primers, serial dilutions of pool RNA were created (1:2 to 1:20,000) following reverse transcription. qRT-PCR was performed using Power SYBR Green Master Mix (Life Technologies) on 96 well plates using a StepOnePlus™ Real-Time PCR System (Thermo Scientific; Pittsburgh, PA).

For all runs, β-actin (*Actb*) was used as the housekeeping gene. Data were analyzed using the StepOne™/StepOnePlus™Software v2.3. Standard curve Ct values were plotted using

Microsoft Excel and amplification efficiencies were calculated using the slope to ensure there were an average of 3.3 cycles between each 10-fold dilution for each gene. To ensure the gene of interest was amplifying at the same rate as the housekeeping gene, the change in Ct values from the genes were plotted to ensure the absolute value of the slope was < 0.1. Primer validation was performed at least three times for each gene and primers were only used when these parameters were accepted and there was a single peak in the melt curve. Melt curves were analyzed to ensure a single gene product was amplified before completing analysis. All primers in Table 1 were considered valid as each melt curve had a single peak, the serial dilutions of a pool of mammary gland RNA had an average of -3.3 slope, and the primer of interest amplified at the same rate as *Actb*, the reference gene.

Sample cDNA was diluted to 1:20 or 1:200 based on the standard curve for qRTPCR analysis. As above, *Actb* was used as the housekeeping gene and the pool was used as a calibrator for $2^{-\Delta\Delta Ct}$ analysis.

Statistical Analysis

Dam body weights were analyzed using a repeated measures two-way ANOVA followed by a post hoc Tukey's multiple comparison test. Date of parturition was analyzed by a Chi Square analysis. Pup weights and dam serum E2 levels were analyzed using a one-way ANOVA with Tukey's multiple comparisons post hoc analysis. GraphPad Prism version 6.0 (La Jolla, CA) was used to perform statistical analyses and $p \leq 0.05$ was considered significant.

Results

Dam and Pup Weights

Dam weights were recorded every other day throughout gestation. Starting on GD 17 and continuing through parturition, AL dams weighed more than AF and PF dams, which did not differ from one another (Figure 1A). AF dams tended to give birth later than AL dams (Figure 1B). At birth, female pups were weighed and averaged per litter to remove bias from different numbers of female pups per dam. Average female pup weight from dams exposed to a liquid diet *in utero* was less than female pups from dams fed solid chow *ad libitum* (Figure 1C). There were no significant differences in total litter size between the treatment groups (data not shown).

Dam Serum

To determine the BAL achieved with this study paradigm, blood was collected beginning 2 hours and finishing by 6 hours after diets were administered (Table 2). For AF dams, the average serum ethanol level was 147 mg/dl and ranged from 86-179 mg/dl. BAL were lower at the earlier time points compared with serum taken 4 or more hours after treatment.

Serum E2 was also measured to determine if alcohol treatment increased circulating concentrations. Serum E2 levels of AF dams were higher than those of PF animals while serum E2 levels of AL fed dams were not different from either group (Figure 2).

RNASeq

Analysis of RNA quality using the Agilent Bioanalyzer at Rutgers prior to shipment and at HudsonAlpha just before sequencing indicated that all samples were of good quality (RNA integrity number; RIN > 8.0; Table 3). Initial sequencing resulted in reads that came in under yield (5-6 million reads) so sequencing was performed again to get a total of 25 million reads. Individual .fastq files were aligned to the rat genome (Rn6) using TopHat v2.0 leading to an overall alignment rate of 92-95%. After mapping, the two .bam files for each sample corresponding to the two sequencing runs were merged using samtools merge.

PCA plots and scatter matrices were created for PND 2, 10 and 20 samples to assess the variance of the samples (Figure 3). For the PCA plot, samples that cluster closer together have less variability than samples that cluster further apart meaning distance correlates with variability. Therefore, if treatment effects exist, the PCA plot would show that samples from the same treatment group cluster together and independently of samples from other treatment group. The scatter matrix plots the log(RPKM) values of all of the genes of one sample against all of the genes of another sample for all samples. If the two samples have identical expression, their scatter matrix would look like a straight line. Ideally, samples from different treatment groups should have more scatter and look like less of a straight line than samples from the same treatment group. Initial comparisons of AF vs. PF glands revealed that there was little difference between the samples at each time point (Figure 3 upper panels). For PND 10 samples, it looked like the PF samples were separating from the AF samples. However, the scatter matrix comparing each sample to the others showed the expression profiles were similar for all of the samples as the plots all appeared to be very close to straight lines. While cummeRbund analysis revealed 47 and 67 genes changed for PND 2 and PND 10, respectively, further analysis of these individual genes combined with analysis of the PCA plot prevented a definitive conclusion about a treatment effect with such a low read depth.

For PND 20 samples, the PCA plot and scatter matrix indicated two of the three samples clustered together for each treatment group (Figure 4). The other sample appears to be switched between AF and PF. To rule out the possibility that the two samples that clustered with opposite groups were switched, several analyses utilizing qRTPCR were considered.

Remaining RNA from each the sample that had been sent for sequencing was analyzed by qRTPCR for select genes to ensure results matched RNASeq. A recent report suggests that modulating the immune system in fetal alcohol-exposed rats may combat an increase in tumorigenesis (247). Many of the genes exhibiting a pattern that suggested the samples had been switch were immune related genes, therefore three genes associated with the immune system as

well as one other gene of interest (*Pgr*) were selected (Table 4). These genes had log2fold changes in RPKM values that were greater than |2|.

RPKM values were compared to changes obtained from qRTPCR. As indicated in Figure 5, expression patterns were similar between qRTPCR and RNASeq for each gene suggesting that the two outlier samples were not switched during preparing them for shipping or at the facility at HudsonAlpha.

To determine if the two samples in question were outliers, the samples not sent for sequencing were also analyzed by the four genes of interest (Figure 6). As expected, the outlier samples (squares) did not follow the pattern of the rest of the samples from their treatment group. Interestingly, there was one sample that clustered with the AF outlier for Cr2, Runx3, and Lef1 suggesting the samples had not been switched but that the piece of the gland that the RNA was isolated from was not representative of the whole gland.

The mammary gland is a heterogeneous tissue so isolation of a portion of the tissue may be representative of only a few cell types or effects on specific cell types may be masked if all cell types do not respond similarly. Since the original gland had been cut into pieces before storage in RNAlater, RNA from an additional piece of each gland was isolated from all samples to see if there was consistency in expression across different pieces of the gland. RIN were determined on these samples as well and two samples had RINs <7.0 and were thus not included in the second isolation analysis.

RNA that was isolated from a second piece of the gland rarely matched the $2^{-\Delta\Delta ct}$ value of the originally isolated RNA for each gene. Linear regression analysis was completed for all samples that had two different isolations of RNA and resulted in R^2 values less than 0.55 suggesting a lack of correlation between the first and second isolation from the same gland (Figure 7). These results indicate that the samples may not have been switched, but that the heterogeneity of the tissue lead to differences in expression. To further explore this possibility, genes representing various cell types within the mammary gland were selected to look at RPKM values. Table 5 shows

inconsistency in RPKM values for each gene representing different cell types suggesting an uneven cell type distribution for each sample.

Discussion

The goal of this study was to determine whether alcohol exposure *in utero* affects mammary gland gene expression during prepubertal development. Exposure to alcohol during gestation has been shown to negatively affect cognitive function in children (207) and animal models (260) but the effects on tumor development, specifically on mammary cancer, have not been studied as extensively. Previous work in our lab (201) and the Hilakivi-Clarke lab (208) demonstrates that alcohol *in utero* increases mammary tumorigenesis in offspring using a rodent model. An increase in proliferation in the mammary gland at PND 20, but not 40 or 80 (209), suggests that perturbations occur earlier in development. Therefore, the current study focused on earlier time points in development including PND 2, 10, and 20. Pups from PND 20 were included to follow up on previous analyses of the mammary gland, while PND 2 represents a time just after birth allowing for analysis close to when pups were exposed to the treatment. The PND 10 samples were included as an intermediate time point between the PND 2 and PND 20.

AF dams tended to give birth later than the control animals which is contrast to findings in human studies that show babies born to mothers who drink alcohol tend to be premature (261). It was previously suggested that premature birth exacerbates the effects of alcohol *in utero* (262) but our results suggest that perturbations caused by alcohol are not caused by premature birth. It is possible that increased circulating E2 in alcohol consuming dams drives tumorigenesis in their offspring, acting as an endocrine disruptor. In this study, similar to previous studies using lower levels of alcohol (208), serum E2 levels were increased in AF dams.

The liquid diet method of administering alcohol, also known as the Lieber-DeCarli diet paradigm, was originally proposed during the mid-1960s to study the effects of alcohol consumption (263). Prior to this, ethanol was added to drinking water but since rats have an aversion to alcohol in drinking water, ethanol levels did not increase to clinically relevant levels that lead to liver damage (264,265). Subsequent formulations of the liquid diet were created to ensure proper nutrient, mineral, and vitamin content (266). The ethanol liquid diet contains 35.5%

ethanol leading to a BAL that was previously reported as 100-150 mg/dl (265), similar to findings presented in this study that range from 86-179 mg/dl. One study analyzed serum two hours after feeding the Lieber-DeCarli diet and saw BAL of 104.3 mg/dl (267), similar to earlier time points presented here. The control liquid diet is the same as the ethanol liquid diet but contains carbohydrate in the form of maltose dextrin to replace the calories from ethanol (268). Animals are pair fed to normalize calorie consumption since rats consume less of the alcohol diet than the control liquid diet when given *ad libitum* access (265). The diet contains high levels of fat (35% of calories) to emulate the western diet and to show that fatty liver disease is caused by alcohol consumption in conjunction with a high fat diet, but not with a high fat diet alone (266).

The solid chow diet was also included in the original Lieber-DeCarli paradigm to demonstrate that the high fat liquid diet did not shift baselines from those of an animal fed a typical chow diet. However, the nutrient composition of the solid diet is not matched to that of the liquid diet. Additionally, the form of the solid diet differs and it is presented *ad libitum* suggesting the AL animals consume more than their liquid diet consuming counterparts. As a result of all of these differences, the AL group is not an appropriate control for the study.

It is important to note that the PF and AF dams gained less weight than the AL dams leading to smaller females at birth. The original DOHAD was put forth by Barker and colleagues following observations of increased heart disease in children born to mothers who were pregnant during the Dutch famine (269,270). Subsequent research demonstrated that intrauterine growth restriction (IUGR) can lead to increased morbidity and mortality of the neonate (271). While research on the effects of IUGR on cancer development in offspring is limited, it is possible that the growth restriction seen in the AF and PF dams and offspring may represent a second insult in addition to alcohol exposure and confound the interpretation of the results. Alcohol exposure during gestation is a risk factor for IUGR (272) suggesting the PF diet is a good control to tease out the specific effects of alcohol. Despite this, alternative approaches for administering the diet should be considered, such as an *ad libitum* liquid diet containing similar nutrients and composition, that

would prevent growth restriction. In the current study, the PF diet provides the best control since it controls for nutrient composition, lack of solid chow, and overall caloric intake, therefore subsequent RNASeq analysis used the PF pups as a control for the AF pups.

Initial RNASeq analysis of pup mammary glands revealed limited differences in gene expression between pups exposed to alcohol or a control liquid diet *in utero* at PND 2 or 10. These results are surprising since we have previously reported differences in mammary gland aromatase protein and IGF-1 mRNA at PND 20 but not in adulthood (209) suggesting that changes should also be present earlier. While 25 million reads should be enough to see large changes in gene expression, increased read depth which increases sequence coverage (273) could tease out smaller differences in gene expression. There are intrinsic differences among animals that contribute to biological variability (274), especially in rat studies since Sprague Dawley rats are an outbred strain. Increasing the sample size would help reduce biological variability and may lead to differences that are not seen on a smaller sample set. Sequencing the epithelial cells could decrease biological variation and should be considered for PND 2 and 10 samples in the future.

At PND 20, RNASeq results suggested a difference between AF and PF samples if two of the samples were switched. However, subsequent qRTPCR analysis revealed that the samples were not switched but that isolating pieces of tissue from the same gland can result in different gene expression patterns. Genes representing different cell types within the mammary gland should not differ in RPKM values if the cell types have a homogeneous distribution, however, results from the PND 20 samples suggested that expression differences were related to different expression patterns by cell type (Table 4). This is to be expected as the mammary gland is a very heterogeneous tissue containing many different cell types (48). The PND 20 mammary gland does not fill the fat pad as much as a mature adult gland (209) indicating adult glands may lead to a more homogenous expression profile across different pieces of the gland. Future studies should select specific cell types using laser capture microdissection at PND 20 to get a more comprehensive analysis of differences between AF and PF pups.

An understanding of genes that change in the mammary gland following exposure to alcohol *in utero* may help to elucidate biological pathways involved in the increased tumorigenesis seen in response to a chemical carcinogen during adulthood in rats. Since gene expression patterns should be established closer to the period of *in utero* exposure, the present work focused on earlier time points in development including PND 2, 10, and 20. While there were no differences detected between AF and PF samples at any time point, future studies with increased reads or animals may be needed to detect significant differences in gene expression. Generally, it is important to consider the origin of the tissue isolated for RNASeq to ensure proper comparison.

Gene	Accession Number	Primer Sequence	Product Length
Cr2	NM_001105989.2	F: GCCAGTCTGTGAGAGTGATTT R: GTCCTGTGTGGTGTCCATTAT	74
Lef1	NM_130429.1	F: TGGTCAGCGAGAGACAATTATG R: TCATTCTGGGACCTGTACCT	88
Runx3	NM_130425.1	F: CAATCCAAGGCTCCTCAGAC R: GTAGGGAAGGAGCGATCAAAC	67
Pgr	NM_022847.1	F: TTCTACTGCTGTGCCTTAC R: GGCCTTCCAAAGGAATTGTG	124
Actb	NM_031144	F: CCATTGAACACGGCATTGTC R: GCCACACGCAGCTCATTGTA	82

Table 1. Accession numbers and primer sequences for genes analyzed by qRTPCR.

Alcohol Samples	Time Serum Taken	BAL (mg/dl)
AF-1	8:56 PM	116.06
AF-2	9:31 PM	86.02
AF-3	10:30 PM	171.52
AF-4	11:15 PM	179.14
AF-5	11:45 PM	160.26
AF-6	12:13 AM	171.16
Average Alcohol		**147.36**

Table 2. Alcohol levels are elevated for at least 6 hours following lights off. Serum was collected from the lateral tail vein on GD 19 2-6 hours after lights out then analyzed for ethanol levels using the Analox Analyzer.

Sac Day	Sample	HudsonAlpha Analysis		Cohick Lab Analysis	
		Qubit (ng/µl)	RIN	Nanodrop (ng/µl)	RIN
PND 2	AF_02_0	408	8.7	424	9.4
	AF_02_1	420	9.6	409	9.2
	AF_02_2	330	8.0	337	8.7
	PF_02_0	320	9.1	363	9.4
	PF_02_1	476	9.1	566	9.4
	PF_02_2	312	9.1	345	9.3
PND 10	AF_10_0	494	8.8	575	9.2
	AF_10_1	526	8.7	596	8.9
	AF_10_2	384	8.5	426	8.9
	PF_10_0	464	8.4	529	9.1
	PF_10_1	444	8.2	448	8.8
	PF_10_2	334	8.4	382	8.9
PND 20	AF_20_0	390	9.0	404	9.3
	AF_20_1	979	9.3	814	9.7
	AF_20_2	455	9.5	476	9.8
	PF_20_0	445	9.0	474	9.5
	PF_20_1	340	9.5	370	9.9
	PF_20_2	370	9.3	410	9.7

Table 3. Concentration and RNA integrity values for samples analyzed by RNASeq prior to and after shipping. Total RNA from each sample was measured for concentration (Nanodrop or Quibit) and integrity (Bioanalyzer) in house and at HudsonAlpha prior to RNAseq analysis indicating that samples were not compromised during shipping.

Gene	AF0	AF1	AF2	PF0	PF1	PF2	Fold Change
Cr2	**5862**	4	6	**5807**	**4037**	3	**-8.52**
Lef1	**2063**	35	43	**1558**	**2333**	53	**-4.01**
Runx3	**1119**	45	73	**685**	**1283**	62	**-2.45**
Pgr	**112**	370	155	**103**	**20**	173	**2.63**

Table 4. Four genes were selected to complete qRTPCR troubleshooting. RPKM values were determined for each gene and a log2fold change was calculated comparing AF to PF samples. The three samples with the greatest RPKM values are bolded for each gene.

Tissue	Gene	AF0	AF1	AF2	PF0	PF1	PF2
Non vs. myo vs. luminal	Cd24	1613	**4517**	**3281**	1796	449	**4042**
Epithelial	Krt8	1772	**7163**	**4486**	2510	487	**5105**
Epithelial	Krt14	646	**2566**	**1204**	686	83	**1692**
Epithelial	Epcam	3336	**9896**	**6212**	3808	748	**7445**
T Cells	Cd3e	**2297**	16	6	**1817**	**2594**	9
T Cells	Cd3g	**1927**	5	7	**1424**	**1814**	8
B Cells	CD19	**3553**	17	23	**4052**	**3342**	20
Dendritic Cells	Itgax (Cd11c)	**386**	37	53	**122**	**267**	91

Table 5. Expression of genes representing different compartments of the mammary gland shows an uneven pattern among samples. RPKM values from genes demonstrating different cell types in the mammary gland from samples sent for RNASeq are shown. Bolded values are the three greatest RPKM values for each gene.

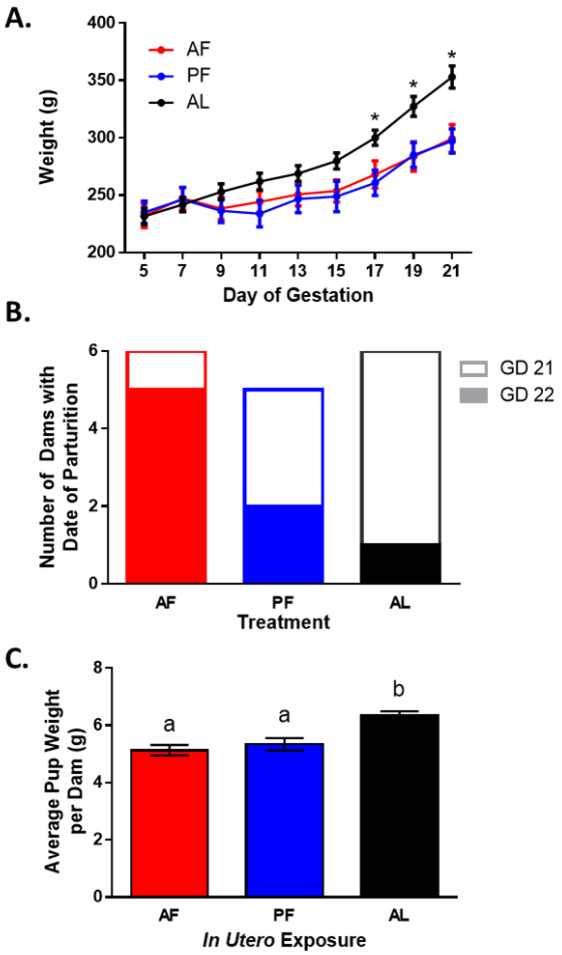

Figure 1. Liquid diet during gestation decreases growth in dams and offspring. (A) Dam weight was recorded every other day. Body weights are expressed as mean ± SEM, n=6 for AL and AF, 5 for PF; repeated measures two-way ANOVA (treatment: $p < 0.01$) with Bonferroni-Dunn multiple comparisons test (* PF and AF of $p<0.05$ compared with AL). (B) Gestational day that dams gave birth. Chi-square analysis; $p = 0.06$. (C) Average weight of female pups at birth. Weights of pups born to each dam were averaged. Bars represent mean ± SEM of average number of pups per dam, n= 6 for AF, 5 for PF, and 6 for AL; $p<0.05$; one-way ANOVA; Tukey's multiple comparisons posttest; different letters denote significance.

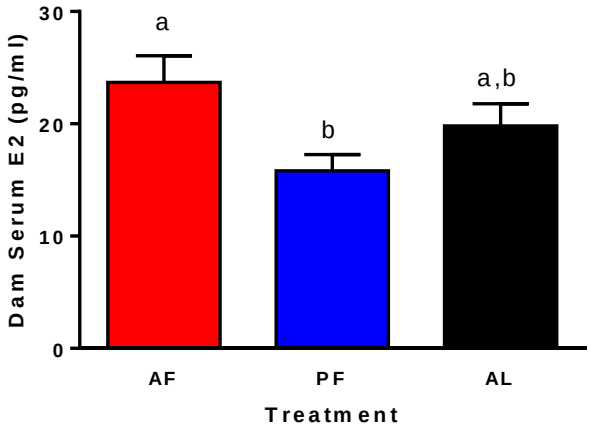

Figure 2. Alcohol treatment during pregnancy elevates serum E2. Serum was collected from the lateral tail vein on GD 19 then analyzed for E2 levels by ELISA (CalBiotech). Bars represent mean ± SEM; $p<0.05$; one-way ANOVA; Tukey's multiple comparisons post-test; different letters denote significant difference.

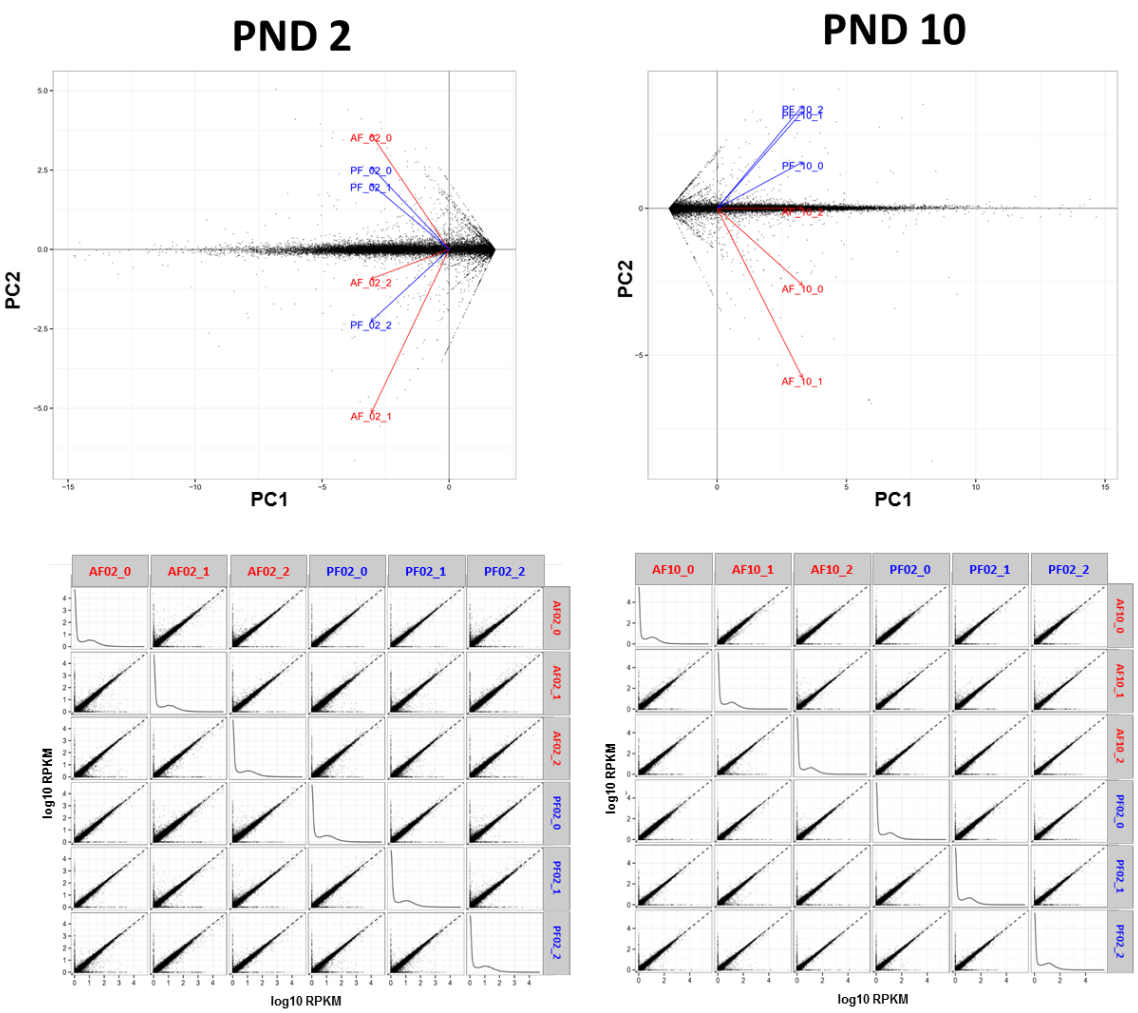

Figure 3. PCA plots and scatter matrices indicate overlapping variances for the samples at each time point. PCA plots and scatter matrices were created using cummeRbund for samples at PND 2 and 10. There do not appear to be differences in PF and AF mammary gland mRNA at PND 2 and 10 using 25 million reads.

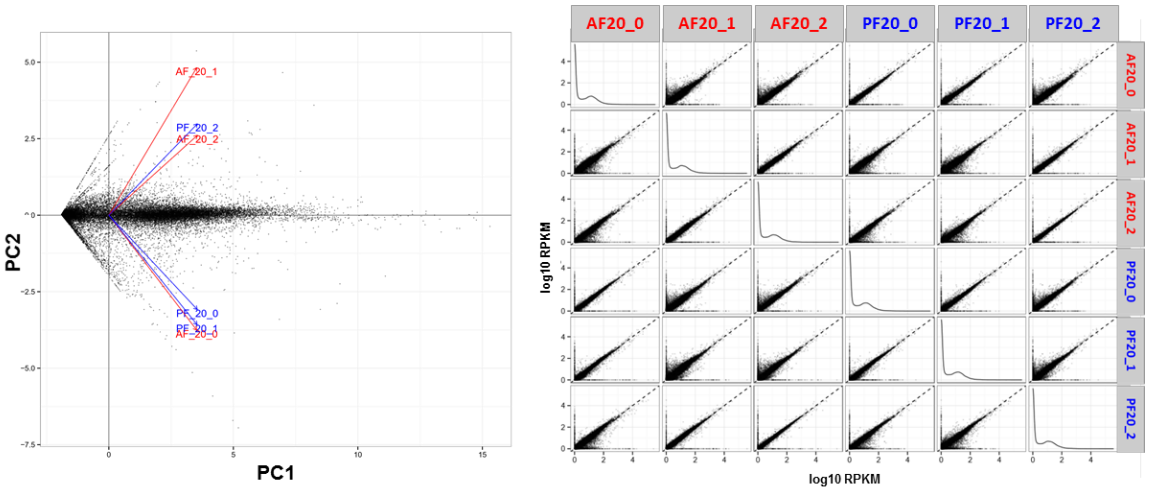

Figure 4. PCA plot and scatter matrix for RNA samples from PND 20. The PCA plot and scatter matrix were produced using CummeRbund. The plots suggest the possibility that two samples were switched as one PF sample clustered with the AF samples while one AF sample clustered with the PF samples.

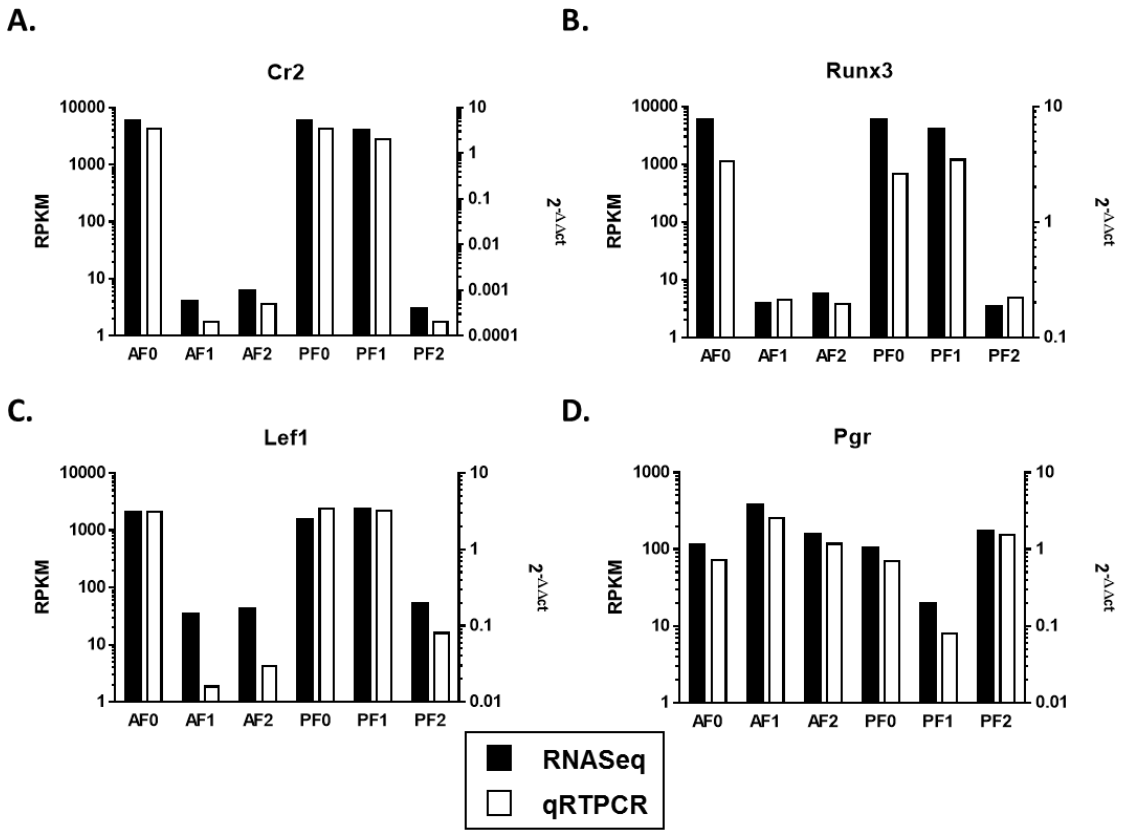

Figure 5. qRTPCR analysis validates RNASeq results. qRTPCR analysis was performed for (A) Cr2, (B) Runx3, (C) Lef1, and (D) Pgr using a second aliquot of the RNA that had been sent to Hudson Alpha. Results are expressed as $2^{-\Delta\Delta ct}$ using the pool as a calibrator. RPKM values from the RNASeq analysis were included to compare qRTPCR patterns with RNASeq patterns.

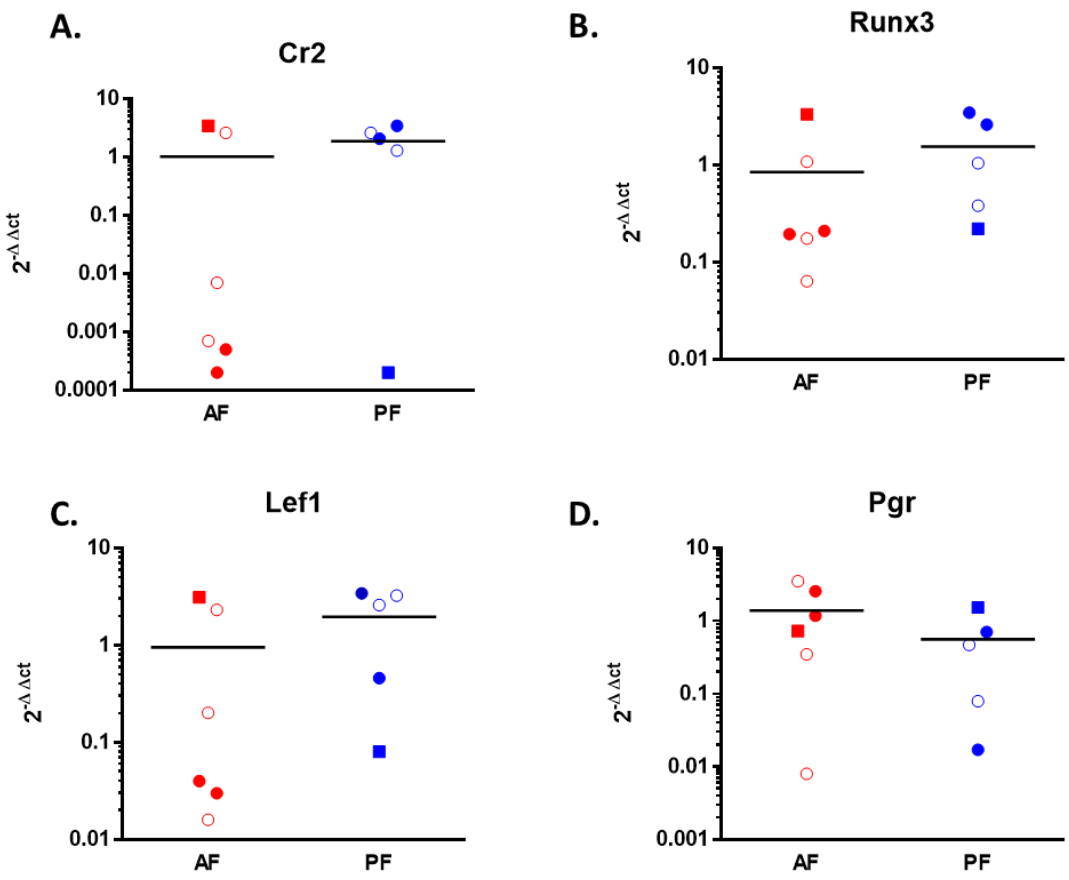

Figure 6. qRTPCR analysis of additional RNA samples suggests no treatment effect of alcohol exposure *in utero* at PND 20. qRTPCR analysis was performed for (A) Cr2, (B) Runx3, (C) Lef1, and (D) Pgr on all samples and is expressed as $2^{-\Delta\Delta ct}$ using the pool as a calibrator. Closed symbols represent samples sent for sequencing while open circles represent samples not sent for sequencing. The outlier samples from the samples sent for sequencing (AF0 and PF2) are displayed as a (■).

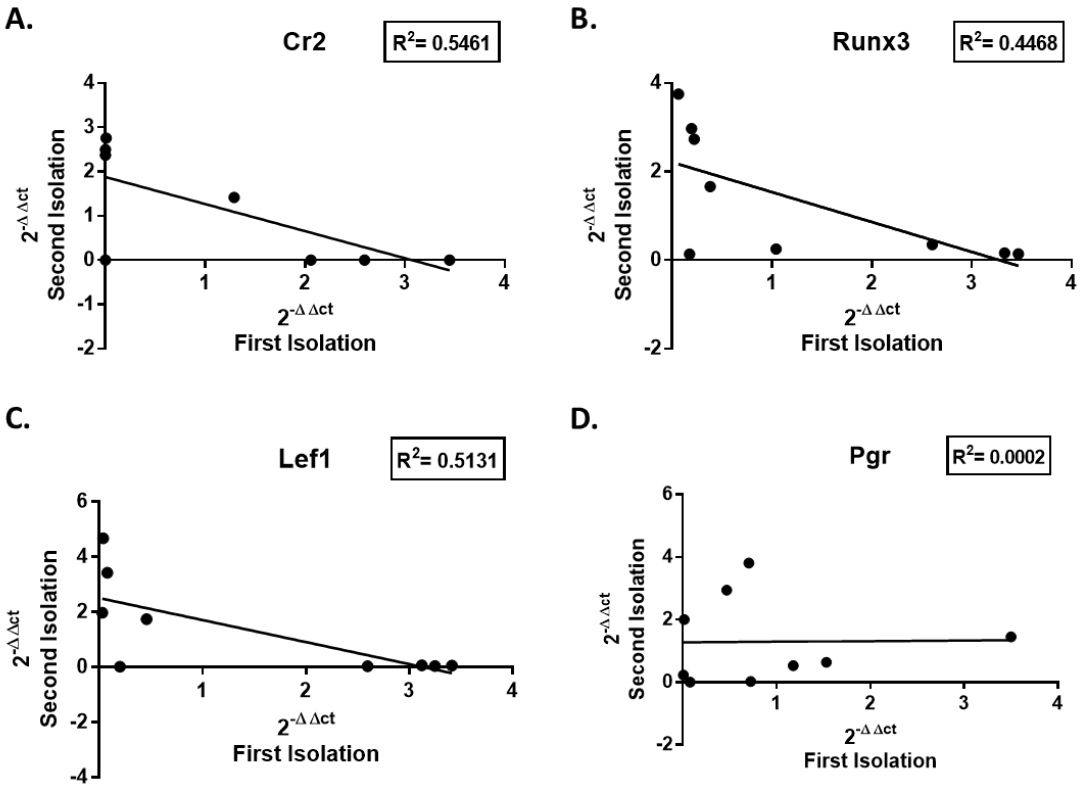

Figure 7. Gene expression by qRTPCR from two different pieces of the same mammary gland were compared for similarities in expression. qRTPCR analysis was performed for (A) Cr2, (B) Runx3, (C) Lef1, and (D) Pgr on all samples and is expressed as $2^{-\Delta\Delta ct}$ using the pool as a calibrator. qRTPCR results from the first isolation of a piece of tissue were compared to RNA from a second piece isolated from the same gland and R^2 values were calculated. RNA isolated from two pieces of the same gland did not show consistent expression.

Chapter 4

Prepubertal Mammary Gland Development as a Model for Hormone Independent Breast Cancer

Abstract

An understanding of molecular signaling pathways that drive mammary gland development has helped understand how breast cancer develops leading to better treatment strategies. Unfortunately, signaling in triple negative breast cancer (TNBC) is not completely understood and targeted therapy has been largely unsuccessful. Prepubertal mammary gland development has not been studied as extensively as other periods of development because growth is isometric meaning the gland grows at the same rate as the rest of the body. Despite this, the gland develops from a rudimentary tree at birth to a gland ready for expansion during puberty suggesting changes in cellular morphology and gene expression exist during this time period. To understand prepubertal mammary development, glands of rats from post-natal day (PND) 2 and PND 10 were compared for transcriptome analysis and morphological characteristics. Since growth during this time period is ovarian hormone independent, expression patterns of development were compared with a cohort of TNBC tumors. Overall, there were changes in the stromal tissue from PND 2 to 10, which is interesting since recent reports subdivide TNBC into immune-related and mesenchymal-like breast cancers based off of gene expression profiles. These results suggest that prepubertal development could be used to study mechanisms of TNBC development.

Introduction

In 2015 over 200,000 new cases of invasive breast cancer were reported and over 40,000 women died from the disease in the United States (1). Molecular signatures were originally established over 15 years ago to better classify breast cancer patients for treatment success (14). Many years of breast cancer research has led to the development of treatment options for women with specific types of tumors including those tumors that express estrogen receptor (ERα) or Her2 (2,3). Unfortunately, a subset of women has breast cancer that does not express either of these receptors and have been left with limited treatment options (275). Triple-negative breast cancers (TNBC) as determined by immunohistochemistry (IHC) largely overlap with the subset of cancers that are defined by gene expression studies as basal-like breast cancer (14,275). These cancers have a worse prognosis than other breast cancers and are more often seen in younger women and African American and Hispanic women (276). TNBC are typically very heterogeneous but subclassifications of basal-like breast cancers suggest roles for signaling stromal cell types in progression of the disease (277). A better understanding of the biology of how TNBC develops including signaling networks between difference cell types would lead to better prevention and treatment strategies.

Studying normal mammary gland biology helps elucidate molecular signaling pathways that lead to breast cancer when they become aberrant (108). Mammary gland development is broken into three main phases: embryogenesis, puberty, and pregnancy including lactation and involution (278). Previous research has suggested similarities between pathways that guide normal mammary gland development during embryogenesis and adulthood and pathways that are deregulated in breast cancer (279-280), but other phases of development have been studied less extensively. Specifically, prepubertal mammary gland development represents a time of development that is thought to arrest from birth until puberty (281,282) allowing the gland to simply grow isometrically (283). However, there are sequential changes in differentiation of epithelial cells that occur from birth to 2 years of age in humans (284). Preovulatory macaques exhibit differences in mammary

epithelial cell morphology from columnar epithelium surrounded by rounded myoepithelial cells to cuboidal luminal epithelial cells with flattened myoepithelium that is not associated with changes in proliferation leading up to puberty (285). Recent reports in the bovine suggest that allometric growth of mammary glands may begin before puberty (286), which may only be partially attributed to signaling through ERα (287). While ERα levels in the mammary gland increase from birth until puberty (62), growth is ovarian hormone independent and free serum estradiol levels are low in rats (44,59). Development at this time is not confounded by the influence of circulating ovarian hormones and thus signaling pathways during this period of normal growth and differentiation may give insight into pathways affected in TNBC, a disease that is also ovarian hormone independent (275).

Previous transcriptome studies analyzing mammary gland development include mammary glands from mice at multiple time points. One study completed mRNA and miRNA profiling on whole glands as early as 12, 15, 19, and 25 days of age but emphasized changes in miRNA expression between virgin, lactating, and involuting glands. Within this analysis, it was evident that large changes exist across mammary gland development but an in depth understanding of changes at earlier time points was lacking (288). Other studies focus on different developmental time points including examining terminal end buds at different ages (289,290) and analyzing expression patterns over pregnancy (291,292). One study analyzed mammary gland development by DNA array and focused on genes that represented various cellular compartments of the mammary gland as evidenced by changes in numbers of cell types, rather than changes in expression within individual cells. Using this method, they discovered a role for brown fat and adaptive thermogenesis in prepubertal development (293). This result suggests that there is an increase in cells that support the developing epithelium and interact in a coordinated fashion during this time.

The mammary gland is comprised of many cell types that communicate differently at time points across development. The mesenchyme plays a critical role in the development of the mammary parenchyma (294). The mesenchymal tissue of the mammary gland gives rise to the

connective tissue (295) including fibroblasts, adipocytes, immune cells and vascular cells (282). Fibroblasts reside immediately adjacent to the myoepithelium and play a critical role in epithelial cell proliferation (296). Similarly, adipocytes communicate directly with the epithelial cells (297). The fat pad develops from condensed mesenchymal tissue during late embryogenesis that ultimately leads to monocular adipocyte formation 2-3 days after birth (298,299). Lymphatic and blood vessels are intricately interwoven within the mammary gland and expand over pregnancy and lactation as growth of the mammary tree progresses (300,301). As for immune cells, both innate (302) and adaptive immune systems (303) have been shown to be critically important for mammary gland development.

To gain a better understanding of development during prepubertal mammary gland development, transcriptomic analysis was conducted on whole mammary gland RNA isolated from post-natal day (PND) 2 and 10, which represent periods of hormone independent, isometric growth. The adjacent glands were analyzed histologically to visualize morphological changes in cell types. Initial analysis suggested an increase in immune-related genes, which was further validated by IHC and other morphological analyses. Finally, expression patterns of genes that significantly changed from PND 2 to 10 were compared to a TNBC cohort to determine if this early period of mammary gland development could serve as a model for studying this disease. Overall, changes in stromal genes suggest a more in depth analysis of the communication between the stroma and parenchyma during early development is warranted.

Materials and Methods

RNASeq

RNASeq was completed as described in Chapter 3. Briefly, after *in utero* exposure to AF or PF diets, female offspring were euthanized by rapid decapitation on PND 2 and 10. Whole gland RNA was isolated then sent for sequencing at HudsonAlpha using 50 bp PE reads to obtain 25 million reads.

Bioinformatics

After sequencing, reads were mapped to the reference rat genome (Rn6; NCBI; Bethesda, MD) using TopHat v2.0 (258). To analyze differential expression between PND 2 and 10, cummeRbund was used to look at overall changes in gene expression as well as differences in individual genes by combining samples from AF and PF pups at each time point (259). Data were subjected to principle components analysis (PCA) to determine clustering patterns. Genes with greater than |1.5| log2fold change and RPKM values > 1 were selected for gene enrichment analyses performed using Database for Annotation, Visualization, and Integrated Discover (DAVID; https://david.ncifcrf.gov/tools.jsp) and Ingenuity Pathway Analysis (IPA; (www.qiagen.com/ingenuity; Qiagen; Redwood City, CA) to integrate genes and biological pathways. To see if differences in mammary gland RNA expression were similar to TNBC, expression values of genes that changed in the prepubertal RNASeq data set between PND 2 and PND 10 were selected from a set of 579 TNBC that had been analyzed by Affymetrix (GEO accession number: GSE31519) (304).

qRTPCR

RNA from PND 2 and PND 10 mammary glands was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies) with 1 µg RNA. Primer sets were designed using the PrimerQuest Tool (Integrated DNA Technologies; Coralville, IA) ensuring the amplicon spanned an exon junction. Primer sequences for all genes analyzed are listed in Table 1. Primers were validated using a pool of RNA from PND 10 mammary gland RNA. qRTPCR was

performed using Power SYBR Green PCR master Mix (Life Technologies) using an Applied Biosystems Thermocycler. *Sdha* and *Atp5b* were used as housekeeping genes because they were previously described to be appropriate for mammary tissue (305). For each gene of interest, the $2^{-\Delta\Delta ct}$ method described by Applied Biosystems was calculated using the geomean of the two housekeeping gene Ct values for each sample and the pool as a calibrator.

qRTPCR results were analyzed using a linear regression to compare results with RNASeq results and assessing the goodness of fit by R^2. A Student's T-test was used to compare qRTPCR results at the two ages using GraphPad Prism version 6.0 (La Jolla, CA) with $p \leq 0.05$ considered significant.

Mammary Gland Morphology

To assess if changes in genes determined by RNASeq analysis were caused by changes in morphology of the tissue over time, a second animal study was performed following the study design described in Chapter 3 with the following exceptions. Four dams per treatment group were treated with AF, PF or AL diets during gestation. At birth, pups were cross-fostered to AL dams then sacrificed on PND 2 and 10. The left mammary gland was stored in RNAlater for possible RNA analysis in the future. The right gland was stretched on a slide and stored in 10% neutral buffered formalin (NBF) until further analysis.

Mammary Gland Whole Mounts

After fixing glands in 10% NBF overnight, the gland was allowed to air-dry for 10 minutes. Glands were then rehydrated in 70%, 50%, 30% and 10% ethanol, placed in water for 5 min, and stained in carmine alum overnight (Sigma-Aldrich). After staining, slides were dehydrated in 70% and 95% ethanol followed by xylene. Glands were cleared in toluene for 30 minutes after dehydration to remove excess fat then mounted in SealPAK pouches (Kapak; Minneapolis, MN) with cedar wood oil (Acros; Geel, Belgium). To analyze mammary gland morphology, glands were viewed and images taken using a Leica MDG41 stereomicroscope (Leica; Buffalo Grove, IL) and a Nikon DS-Fi1 camera (Nikon; Melville, NY) with NIS Elements software (Nikon).

Tissue Histology

Mammary glands were fixed in 10% NBF overnight. Glands were removed from slides then dehydrated, cleared, and embedded in Paraplast using facilities located in the Histopathology Core of the Environmental Occupational Health Sciences Institute at Rutgers University. Glands were sectioned at 6 µm and placed on slides.

Hematoxylin and Eosin

Slides were baked for 15 minutes at 60°C, followed by deparafinization in xylene and rehydration in decreasing concentrations of ethanol. Slides were stained with hematoxylin and eosin, mounted with Permount then visualized and imaged using an Olympus FSX100 microscope at 20X (Olympus; Waltham, MA).

Immunohistochemistry (IHC)

Slides were baked at 55°C for 30 minutes, deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by boiling slides in 0.01 M sodium citrate buffer for 30 minutes then cooled at room temperature to 45°C before proceeding. Endogenous peroxidase activity was blocked using 3% H_2O_2 for 10 minutes then sections were blocked with normal goat serum (Vector Laboratories; Burlingame, CA) following the manufacturer's directions. Rabbit CD3 primary antibody (ab5690; Abcam; Cambridge, MA) was diluted in 1% BSA to 1:100. Rabbit primary antibody isotype control served as a negative control. Secondary antibody was applied according to manufacturer's directions (Vector Laboratories) followed by development in 3,3'-diaminobenzidine (Sigma-Aldrich). Slides were counterstained with hematoxylin, dehydrated with increasing concentrations of ethanol followed by xylenes, then mounted using Permount. Representative images of mammary glands were taken using an Olympus FSX100 microscope using the bright field setting (Olympus; Waltham, MA).

Results

RNASeq

Since there were not major differences in gene expression between mammary glands of AF and PF pups at individual time points using 25 million paired end reads (Chapter 3), comparisons between PND 2 and 10 were analyzed by combining the treatment groups at each time point. The PCA plot indicated that samples clustered together and independently of one another at each time point (Fig 1 A). Cuffdiff analysis indicated that 5625 genes significantly changed between PND 2 and 10. A volcano plot (Fig 1 B) was used to show the distribution of genes that change (in red) based on fold-change and p-value. The lack of distribution on the x-axis suggests that the observed gene changes did not have a large fold-change. As previously described, *Esr1*, the gene that encodes ERα, increased from PND 2 to 10.

Mammary Gland Morphology

Mammary gland whole mounts were prepared from PND 2 and 10 pups. Whole mounts representing the tissue that was isolated for RNASeq analysis demonstrated that the parenchyma of the gland was a small portion of the overall tissue at both PND 2 and 10 (Fig 2; arrows). As expected, the gland was larger at PND 10 than at PND 2. In both cases, two mammary epithelial trees were present in the whole mount preparation indicating that two glands were actually isolated: the 4^{th} and 5^{th} inguinal mammary glands. A prominent lymph node was evident at both time points (Fig 2; arrow heads) and was more clearly seen at a higher magnification (Fig 3). At PND 10, there was more branching of the ductal structure of the gland than there was at PND 2 (Fig 3; arrows). In both cases, the 4^{th} mammary gland was growing towards the lymph node as expected.

A microscopic histological examination using hematoxylin and eosin showed further differences between PND 2 and 10 glands. At PND 2, the fat cells were smaller and the extracellular matrix (ECM) and fibroblasts surrounding the ducts was not evident, but was clearly distinguishable at PND 10 (Fig 4; arrows). These results suggest that there may be changes in genes corresponding to the stromal tissue in the mammary gland.

RNASeq Pathway Analysis

Pathway analysis using DAVID and IPA using the 5625 genes that changed between PND 2 and PND 10. Of those genes, 347 had >|1.5| log2fold difference in RPKM expression and were subsequently included in pathway analysis. DAVID and IPA analyses showed that pathways involved in immune regulation were the top pathways that changed from PND 2 to 10 (Table 2 and 3). Specifically, genes involved in the innate immune system associated with T and B cells changed including "T Cell Receptor Signaling" and "B Cell Development". These results suggested that there were changes in immune cells in the mammary gland over time.

qRTPCR Validation

Genes representing T cells (*Cd3e* and *Cd3g*) and B cells (*Cd19* and *Rt1doa*) were selected for qRTPCR validation. Additionally, one gene that was highly expressed in PND 2 glands (*Dlk1*) and one gene that was highly expressed in PND 10 glands (*Sncg*) were selected. The *Dlk1* gene encodes for protein delta homolog 1 and has previously been shown to be upregulated in mammary mesenchyme during fetal development in mice (306) and is involved in inhibiting adipogenesis (307). The protein γ synuclein, encoded by the *Sncg* gene, is also known as breast cancer specific gene-1. It plays a role in the heat shock protein chaperone complex (308) and levels of *Sncg* correlate with increased breast cancer stage (309).

For the 6 selected genes, qRTPCR was performed on the same RNA that was used to generate cDNA libraries for RNASeq as described. For each gene, $2^{-\Delta\Delta Ct}$ from qRTPCR was plotted against RPKM values from RNASeq analysis (Fig 5) and linear regression was analyzed. For all genes, R^2 values were greater than 0.85.

In addition to the samples that had been sent for sequencing, qRTPCR was performed on PND 2 and 10 samples that had not been sent for sequencing, including mammary glands from AL pups. Including AL samples did not lead to differences between AF, AL, and PF at either time point (data not shown). To determine how a larger sample size (n=11 for PND 2 and n=16 for PND 10) affected the changes in gene expression, qRTPCR results were compiled by time point. All genes

analyzed showed significant differences that were in the same direction as the RNASeq results (Fig 6).

IHC

There are three possible explanations for a change in immune markers over time: 1. the lymph node was establishing itself in this tissue and B and T cells were infiltrating, 2. B and T cells were accumulating along the edge of the mammary gland at PND 10 guiding the gland to grow, or 3. the less likely possibility that the mammary epithelial cells were themselves expressing markers of T and B cells leading to changes of cytokine and chemokine signaling. To see which of these options was correct, mammary glands were sectioned and stained by IHC for CD3, a marker of T cells (310). We expected CD3 protein expression to increase from PND 2 to 10 since transcription of two of the subunits for this protein (*Cd3e* and *Cd3g*) increased in both the RNASeq data set and the qRTPCR validation.

IHC analysis of mammary glands stained with CD3 indicated that there was little CD3 expression in the gland except for in the lymph node (Fig 7). There did not appear to be an increase in intensity of staining in the gland.

Comparison to TNBC

In order to determine if the genes that change during prepubertal mammary gland development are also perturbed in TNBC, Affymetrix gene expression data for 579 TNBCs were downloaded from Gene Expression Omnibus (accession number GSE31519; http://www.ncbi.nlm.nih.gov/geo/). Karn et al. previously characterized these 579 tumors as TNBC based on their low expression of *Esr1*, *Pgr*, and *Erbb2* mRNA. The tumors were analyzed using the Human Genome U133 Affymetrix array (304).

The genes that were up- or down-regulated by at least |1.5| log2fold change between PND 2 and 10 were compiled. Of the 347 genes that changed, 63 were not on the human Affymetrix gene array and 40 did not have orthologues to human genes. Of the remaining 242 genes, 183 were up-regulated from PND 2 to 10 and 59 genes were down-regulated (see Appendix). Expression

levels of these 242 genes were examined in all tumors in the TNBC data set. Heat maps were generated of the expression patterns of the TNBC tumors using the dendogram function in cummeRbund for the genes that were up and down regulated from PND 2 to 10 separately (Fig. 8). The dendogram allowed genes that changed similarly across samples to cluster. Genes boxed in blue indicating genes that were downregulated in both the prepubertal mammary gland and TNBC and genes boxed in orange indicating genes that were upregulated in both data sets were further analyzed. IPA analysis of genes in the orange box implicated changes in pathways of the immune system including “B Cell Development” and “iCos-iCOSL Signaling in T Helper Cells”.

Discussion

The goal of this work was to determine which factors drive prepubertal mammary gland development. Since mammary gland development in this time period is ovarian hormone independent, we thought it would be interesting to compare changes across this time period with those observed with TNBC, since this cancer is also driven by hormone-independent mechanisms. Initial RNAseq analysis suggested changes in immune function; therefore, we were interested in determining if changes in gene expression corresponded with changes in morphology. TNBC subtypes also suggest differences in immune profiles as described below.

After birth but before the onset of ovarian hormone release during puberty, the mammary gland grows isometrically with the body. Despite isometric growth, changes occur in tissue and cell morphology during this developmental window. Identification of these changes may provide insight into how mammary gland remodeling occurs in the absence of ovarian hormones and may suggest pathways that become aberrant during the development of TNBC. Whole mount analysis revealed an increase in branching of the epithelium from PND 2 to 10 suggesting that the mammary epithelium is continuing to develop despite previous suggestions that the gland remains quiescent (282). The PND 2 gland has a less developed stroma compared to the PND 10 gland. While the epithelium does not appear to be vastly different between PND 2 and 10, there is an increase in ECM and fibroblasts surrounding the parenchyma over time. Together, these results suggest that there are signals in the mammary gland during prepubertal development that lead to changes in overall structure of the gland.

Initial RNASeq analysis comparing PND 2 to 10 mammary glands suggested an increase in genes involved in the adaptive immune system. Signaling cytokines such as *Ccl5* and *Ccr7* that are associated with T cells and B cells respectively, were increased from PND 2 to 10 suggesting increased cytokine signaling as well. qRTPCR analysis using genes that are markers of T and B cells corroborated findings in the RNASeq data set both on an individual basis as well as when the sample size was increased to include additional samples that had not been sent for sequencing. The

immune system has been studied as a well-adapted system that allows for destruction of harmful pathogens while distinguishing invading cells from normal tissue. The innate immune system provides a quick but nonspecific response and includes phagocytic cells such as neutrophils, macrophages, and dendritic cells. By contrast, the adaptive immune system consists largely of B and T cells that become involved in the immune response only after an antigenic challenge is recognized, allowing for a tailored response to pathogens with help from memory cells. Phagocytic macrophages and dendritic cells can be involved in the adaptive response when they act together with B cells as antigen presenting cells in a process that involves the major histocompatibility complex (MHC; class II) (311). Cells of the immune system reside in normal tissue and inflammatory responses involved in wound healing are also present during involution of the gland following pregnancy suggesting an overlap in classical immune response and tissue remodeling during development (312,313).

The role of the immune system in involution has been studied extensively (314) and the role of the innate immune system in nulliparous glands has been an active area of research for the past 15 years (315). Macrophages are present in the stroma adjacent to the mammary epithelium as early as 2 weeks of age (302). Csf1 is an important regulator of mononuclear cell lineage (316) and an inactivating mutant of Csf1 leads to a reduced number of macrophages in mice (317). Using this model, macrophages have been shown to play a large role in mammary gland development as evidenced by the finding that a depletion of macrophages by irradiation leads to a decrease in mammary development (302). Specifically, macrophages are involved in branching morphogenesis (318) and organizing the structure of the terminal end bud (319). Mast cells are also required for normal proliferation during puberty (320).

The role of the adaptive immune system in mammary gland development is mostly unknown. A recent report demonstrated a relationship between T cells and mammary epithelial cells acting as antigen presenting cells (303). In the current study, the pathways that changed the most between PND 2 and 10 mammary glands were all involved in the adaptive immune system.

In an effort to determine the distribution of T cells in the mammary gland, IHC was performed using CD3, a marker specific to T cells. The IHC results suggest that there was not a difference in the presence of T cells near the developing epithelial tree, but rather that there were numerous T cells in the lymph node near the 4th inguinal mammary gland. It is possible that the orientation of the sections for IHC did not allow for detection of the interaction between the ductal tree and the lymph node. The whole mounts appear to show a direct interaction of the epithelium with the lymph node, therefore future studies should investigate the role of the lymph node in mammary gland development.

Immune system evasion has been implicated as important for all cancer development (108) and specifically for breast cancer (321). Tumor infiltrating lymphocytes (TIL) are sometimes found in breast cancer and predict patient survival and response to chemotherapy (322). A recent report suggests that spatial relationships of cell types, specifically immune cells near tumor cells, can influence prognosis (323). However, many times levels of TIL are modest, partially because the immune system is simultaneously attempting to dampen a response to self-antigens (324). Interestingly, a recent transcriptome analysis subdivided basal-like breast cancer into four subtypes: basal-like immune suppressed, basal-like immune activated, mesenchymal, and luminal androgen receptor (179). While a role for the immune system in TNBC has been proposed, this area warrants further exploration as a better understanding of the role of the immune system in breast cancer may lead to improved treatment options for women with TNBC.

A decrease in *Dlk1* from PND 2 to 10 suggests a decrease in adipogenesis, a process expected to occur over this time period (62). However, an increase in the size of the adipocytes suggests accumulation of fat during this period which may be signaling to the growing epithelium. Retinoid signaling has been suggested to be important in mammary gland development during embryogenesis based on the presence of retinoid receptor expression in the fat pad precursor (325). While not among the top ten pathways that were affected from PND 2 to 10 using IPA analysis, TR/ RXR Activation, VDR/ RXR Activation, and LXR/ RXR Activation were all pathways that

changed from PND 2 to 10, suggesting retinoid signaling continues to change as the embryo develops during prepubertal development and that the fat pad may be guiding development (see Appendix for list of top 100 pathways that changed).

While markers for fibroblasts including vimentin (*Vim*) and smooth muscle actin (*Acta2*) did not change from PND 2 to 10 by RNASeq, there was an increase in fibroblasts and ECM surrounding the ducts of the mammary gland. In breast cancer, cancer associated fibroblasts (CAF) exhibit distinct gene expression profiles compared with normal fibroblasts (326). CAF enhance growth of mammary tumors by secreting a variety of paracrine factors (327). Similarly, in breast cancer there are changes in the ECM. Normally, the ECM is required to maintain the structure and integrity of the tissue, but the normal structure is aberrant in cancer leading to resistance to certain treatment regimens (328). That the ECM is established during the prepubertal developmental time period suggests further research to understand the development of these networks is warranted.

Communication between the epithelium and surrounding stroma during mammary gland development is well-defined in embryonic and pubertal development. Signaling pathways involved in this communication have subsequently become targets in breast cancer (172,277). However, a better understanding of the development of these processes may lie in the study of prepubertal mammary gland development, as this is the time when the stromal cells are developing. This time of hormone independent mammary gland development could be used as a model to describe pathways that become aberrant in TNBC. One caveat of this comparison is that mammary glands ultimately become responsive to hormones during puberty as ERα expression increases, a phenomenon not seen in TNBC. However, the little that is known about TNBC suggests an important role of the stroma (179) including adipocytes, fibroblasts, and immune cells, all of which increase during prepubertal development. Further investigations are warranted to explore relationships between these various cell types in an effort to guide better strategies to prevent and treat TNBC.

Gene	Accession #	Primer Sequence	Product Length
Cd3e	NM_001108140.1	F: CTGCTTCTGGTGCCTTCTT R: CGTTCCACTGCATCCTCTTAG	93
Cd3g	NM_001077646.2	F: GCCGAGGAGCAAAGAAGAA R: CGAAGATAAAGCCGGACACA	102
Rt1doa	NM_183051.2	F: CATCTGGACGTCTTGGTAGAAC R: CACCATGCAGATGAGGACAT	123
Cd19	NM_001013237.3	F: AGGACTGGTGGATGGATAGT R: AGCCGCCATAGAAACCATAC	72
Dlk1	NM_053744.1	F: CCTGTGTGAGAAGTGCGTAA R: AGGTGCAAGCCCGAATATC	125
Sncg	NM_031688.1	F: GTACGCAAGGAGGACTTGG R: CCTCTGTGGTTGACTGGAAG	114
Sdha	NM_130428.1	F: AGACGTTTGACAGGGGAATG R: TCATCAATCCGCACCTTGTA	120
Atp5b	NM_134364.1	F: GGCACCAATCAAAATTCCT R: AGCCTCAGCATGAATAGGAG	124

Table 1. Accession numbers and primer sequences for genes analyzed by qRTPCR.

Table 2. DAVID Functional Annotation	Count	P-value	Benjamani
Positive regulation of immune system process	32	2.8 E-13	2.7 E-10
Positive regulation of immune response	23	4.2 E-11	1.3 E-8
Positive regulation of response to stimulus	27	1.3 E-9	3.1 E-7
Antigen receptor-mediated signaling pathway	12	1.4 E-9	3.1 E-7
Immune response-regulating cell surface receptor signaling pathway	13	2.8 E-9	4.5 E-7

Table 3. Ingenuity Canonical Pathways	Count	P-value	Ratio
iCOS-iCOSL Signaling in T Helper Cells	24	1.5 E1	2.2 E-1
T Cell Receptor Signaling	19	1.1 E1	2.0 E-1
B Cell Development	12	1.1 E1	3.6 E-1
CD28 Signaling in T Helper Cells	21	1.0 E1	1.7 E-1
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	17	9.8 E0	1.9 E-1

Table 2 and Table 3. Pathway analysis using DAVID (2) and IPA (3) indicates that genes involved in the immune system change in the mammary gland from PND 2 to 10.

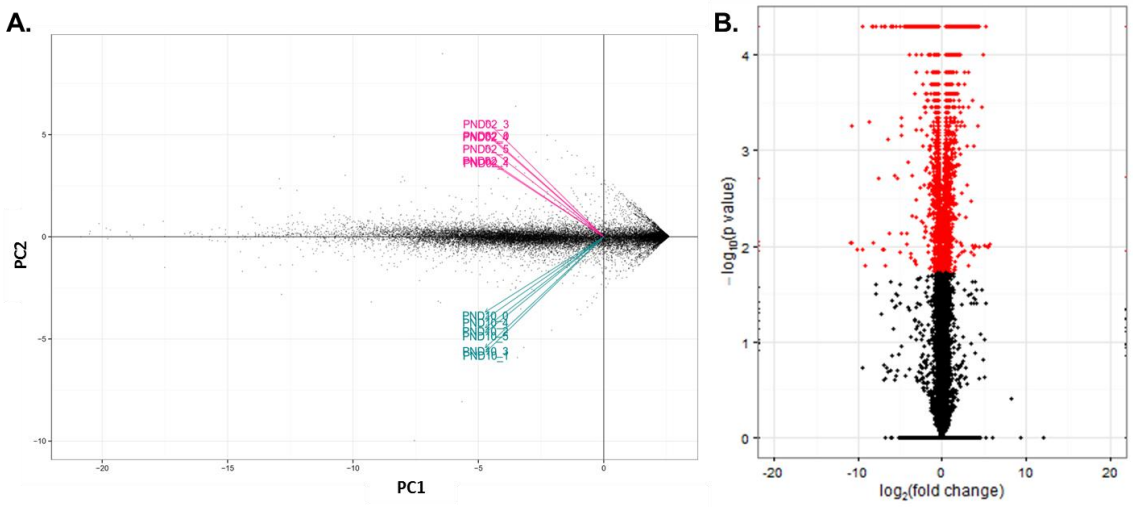

Figure 1. PCA and volcano plots of RNASeq analysis comparing PND 2 vs 10 samples. Plots were created using cummeRbund. (A) The PCA plot demonstrates the relationship between data from PND 2 (pink) and PND 10 (teal) samples indicating separation between the time points. (B) The volcano plot demonstrates that a substantial number of genes changed significantly between PND 2 and 10 ($p<0.05$; 5625 genes; red dots).

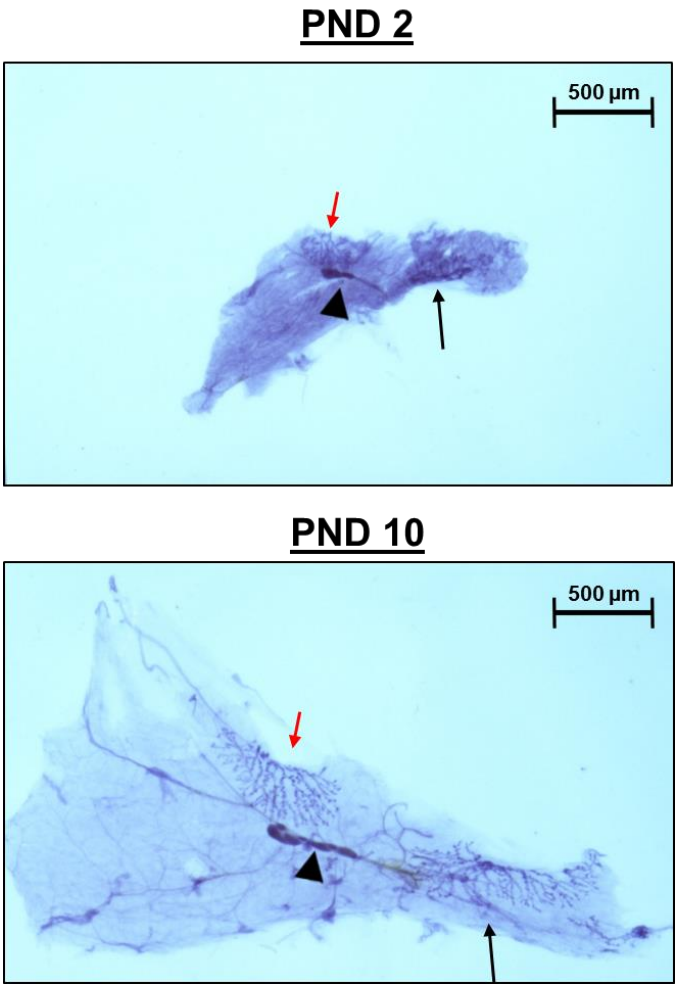

Figure 2. Mammary gland whole mounts from early developmental time points show limited parenchymal structure relative to the fat pad. Representative images of mammary glands taken at PND 2 and 10. Arrows indicate two mammary epithelial trees within the gland while arrow heads indicate the lymph node, the red arrow indicates the 4th inguinal mammary gland.

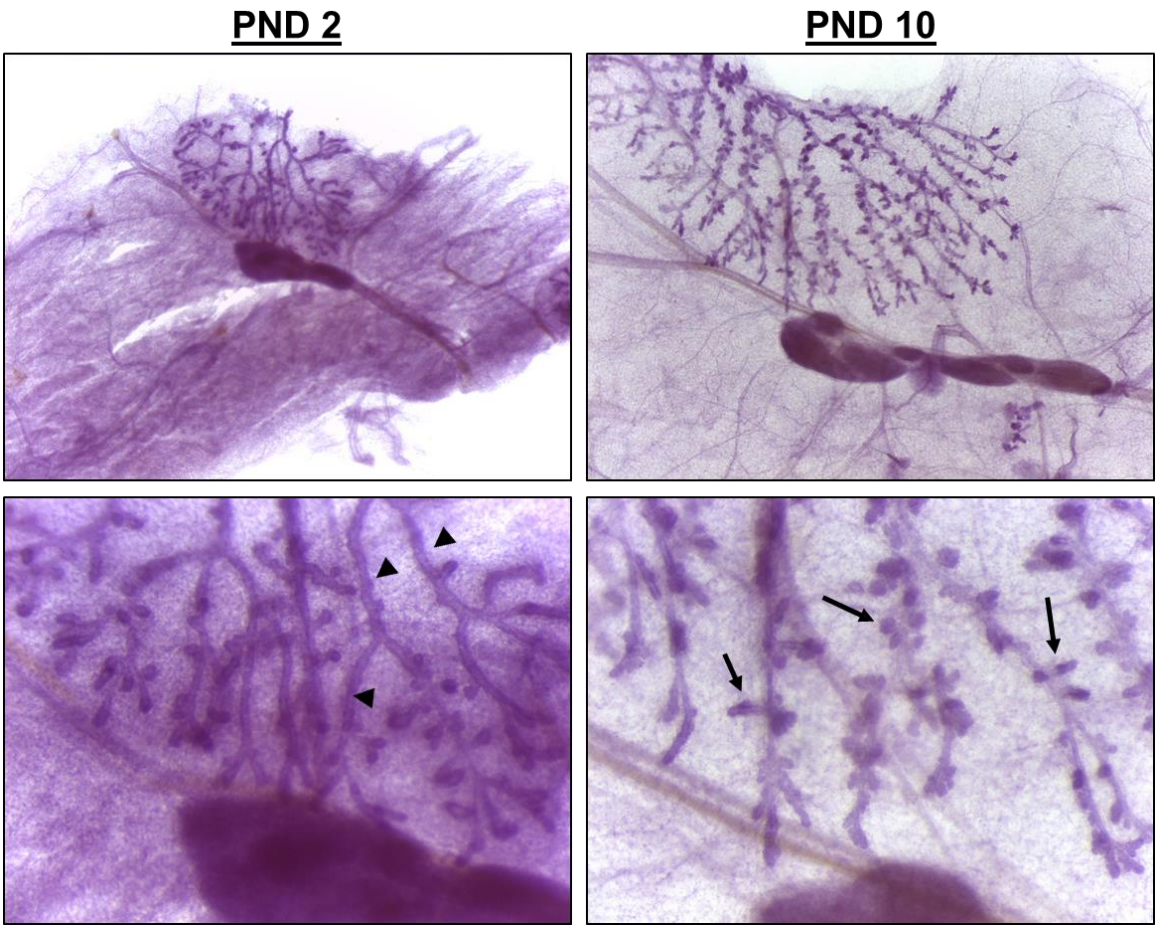

Figure 3. The fourth inguinal mammary gland grows toward the lymph node. Mammary gland whole mounts from PND 2 and 10 were imaged using a Leica dissecting microscope (upper panel 1.6X, lower panel 4.2X). At both time points the mammary gland parenchyma is growing towards the lymph node. At PND 10 there is more branching (indicated by arrows) than at PND 2 (arrow heads demonstrate lack of branching).

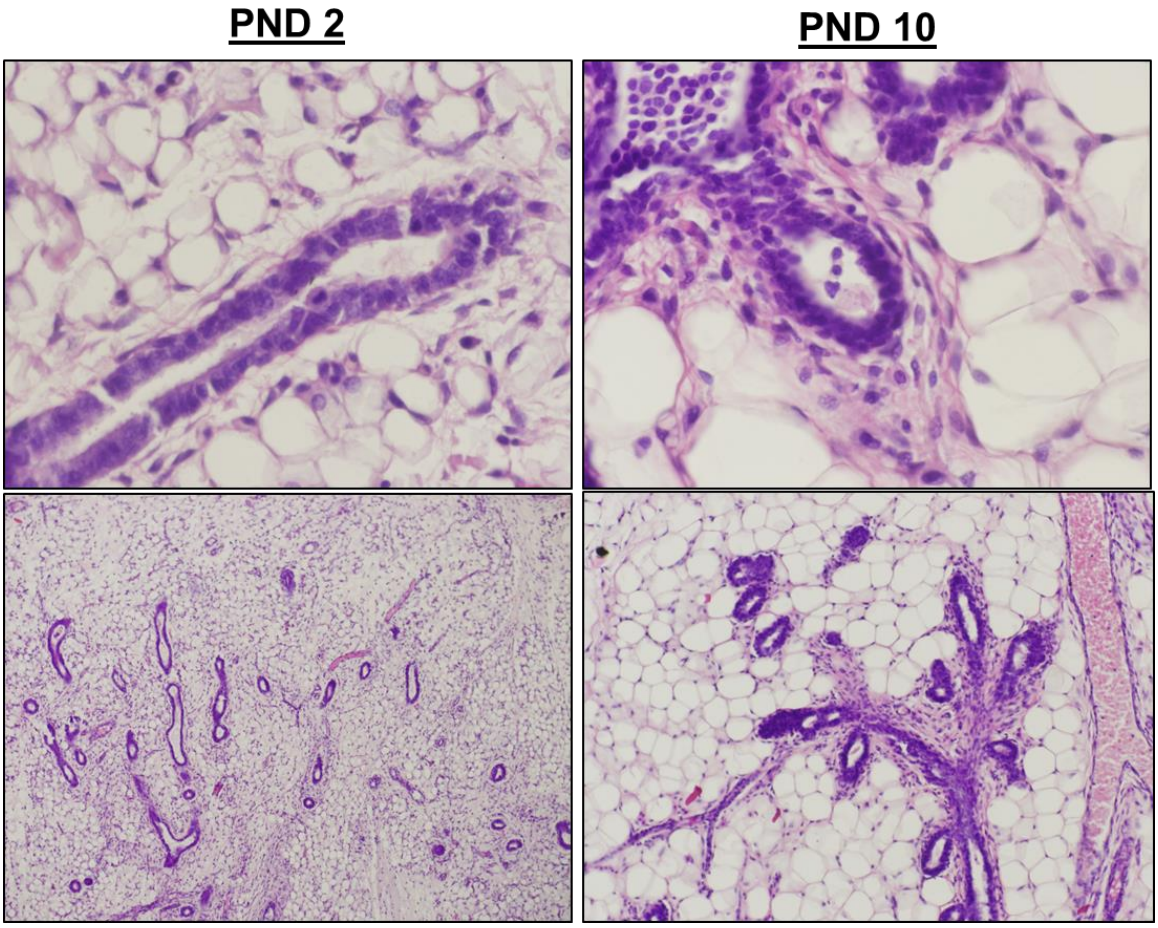

Figure 4. Histological analysis indicates differences in the mammary mesenchymal tissue surrounding the ducts between PND 2 and PND 10 mammary glands. Transverse sections of mammary glands at PND 2 and 10 were sectioned and stained using hematoxylin and eosin. Representative images of mammary glands at each time point were taken at 10x (top) and 40x (bottom). At both time points, mammary gland epithelial cells organized into ductal structures were present. At PND 10, there was increased collagen and other supporting stromal tissue surrounding the epithelium compared to PND 2. Additionally, the overall size of the fat cells was smaller at PND 2 than PND 10.

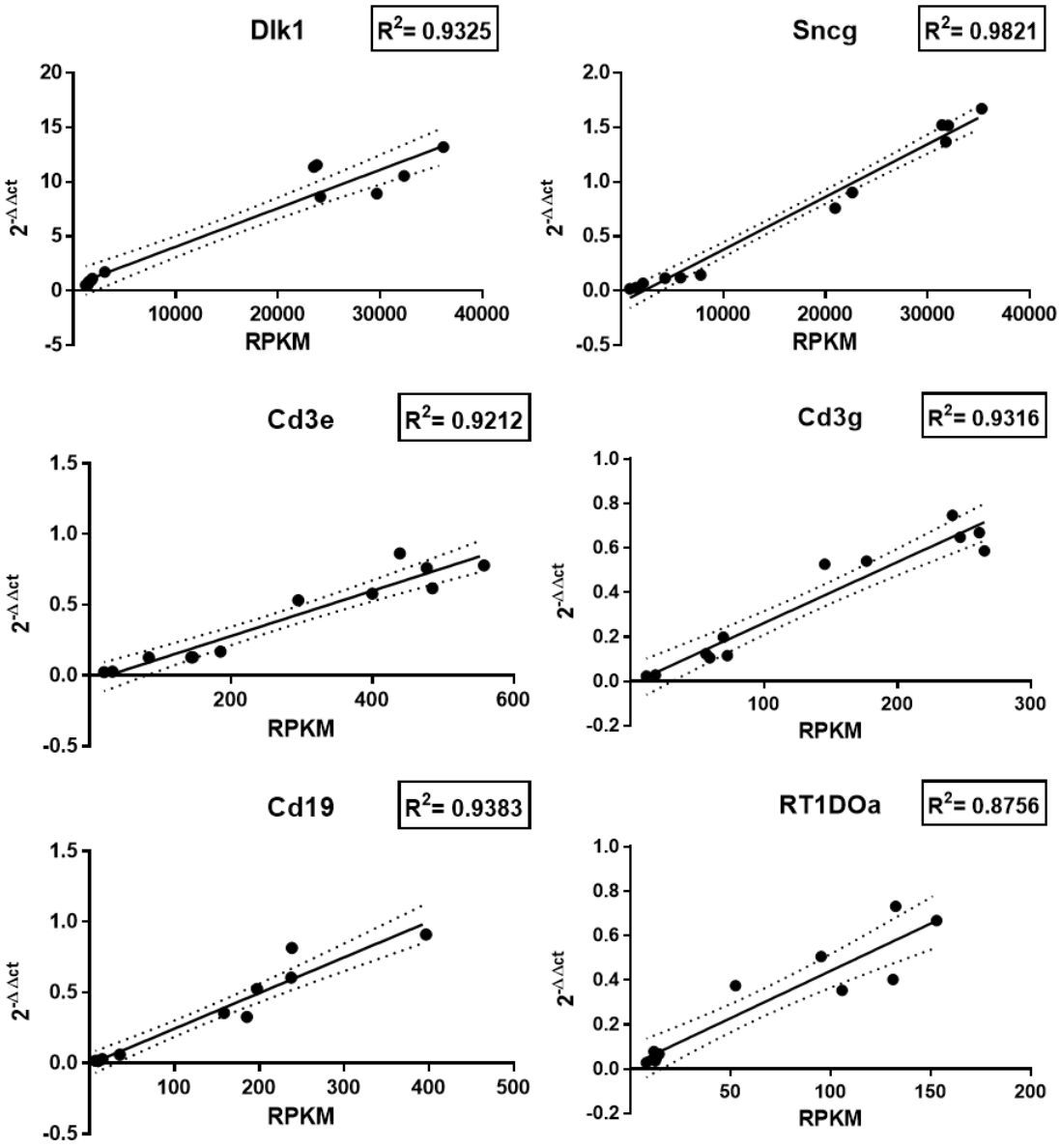

Figure 5. qRTPCR results correlate with RNASeq results. For each gene, qRTPCR was performed on RNA that had been analyzed by RNASeq. qRTPCR $2^{-\Delta\Delta Ct}$ values were plotted against RNASeq RPKM values and an R^2 was calculated for each gene.

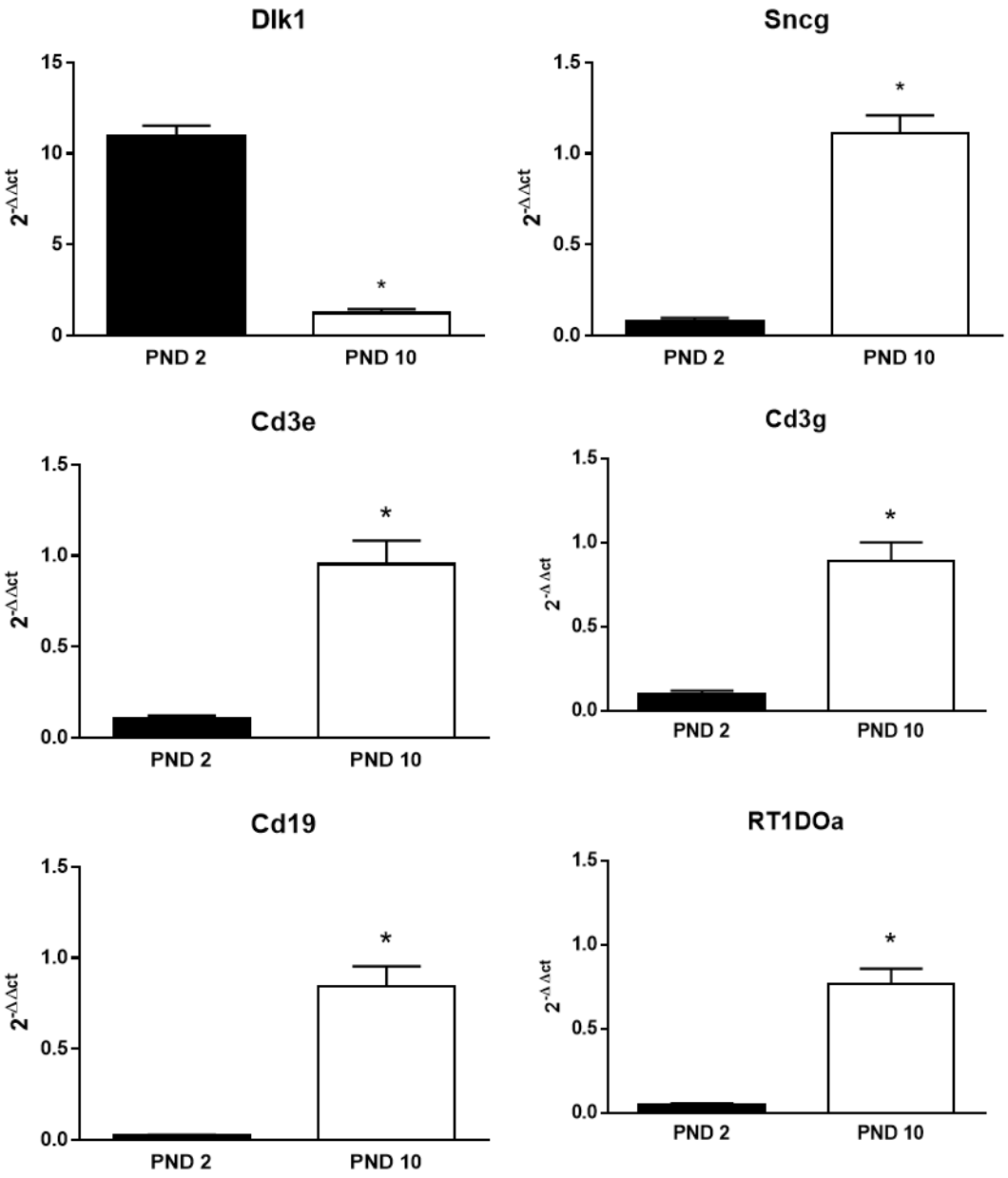

Figure 6. qRTPCR analysis expanding the sample size showed similar patterns to the RNASeq results. qRTPCR was performed for each gene on a larger sample size including ad lib (AL) pups (n=11 for PND 2 and 16 for PND 10). * $p<0.0001$, unpaired T-test.

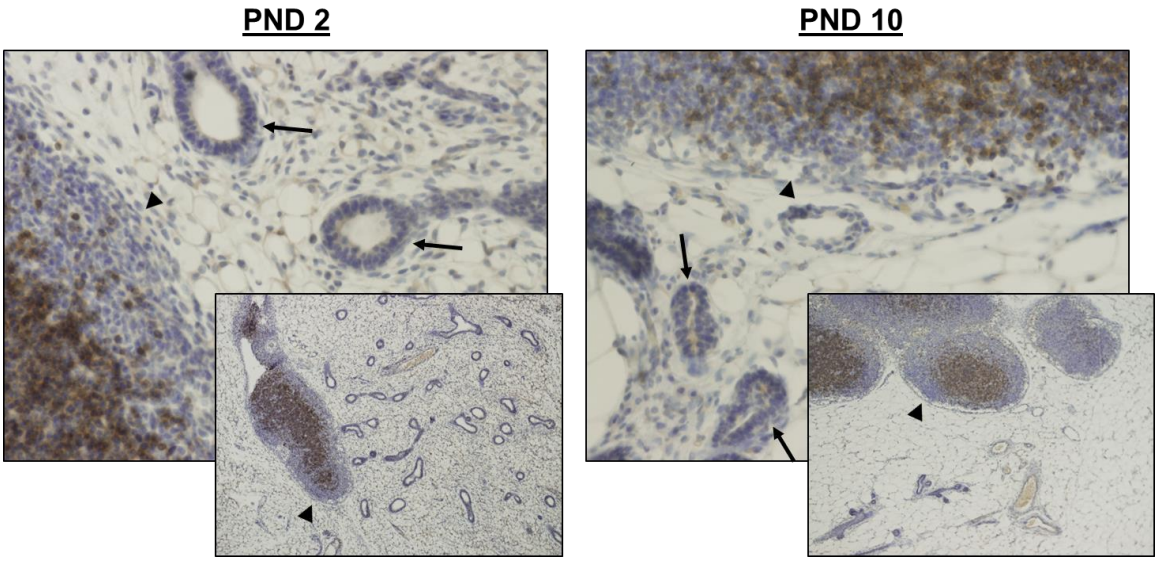

Figure 7. IHC analysis of CD3 protein expression at PND 2 and 10 indicates that T cells are mostly in the lymph node and not near the edge of the epithelium. IHC for CD3 (brown) was performed and tissues were counterstained with hematoxylin (purple). Representative images were taken using an Olympus microscope at magnifications of 4x (inset) and 20x (larger image). The lymph node (arrow head) was near the mammary epithelial ducts (arrow) in both cases, but physical interaction was not detected.

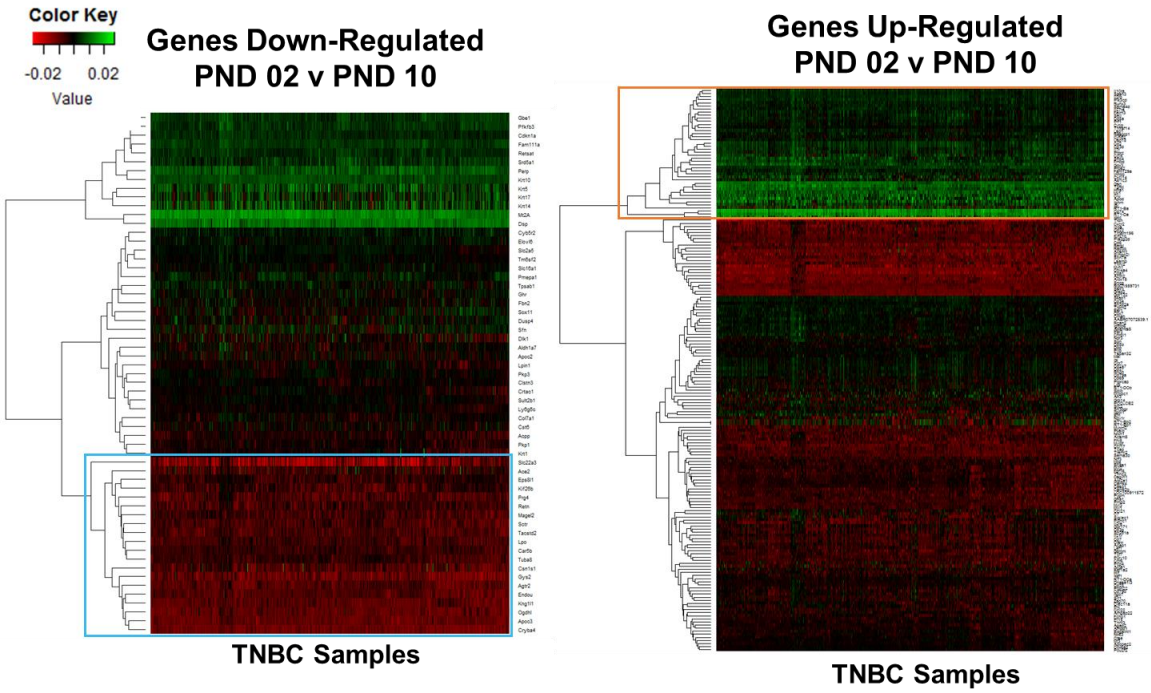

Figure 8. Genes that change in the mammary gland by at least |1.5| log2fold from PND 2 to 10 also change in a triple negative breast cancer (TNBC) cohort. Genes that were significantly up or down regulated by at least 1.5-log2fold from PND 2 to 10 in the mammary gland were selected then compared with the same genes in a TNBC cohort. Breast cancer samples were segregated along the X axis and genes displayed on the Y axis. The blue box represents genes that were down-regulated in both TNBC and PND 2 vs 10 mammary glands, while the orange box represents genes that were up-regulated in both data sets.

Conclusions and Future Directions

Breast cancer cannot be fully explained by inherited genetic mutations. The Developmental Origins of Health and Disease hypothesis (DOHAD) offers an explanation as to how environmental exposures during critical periods of growth may influence tissue development and ultimately lead to cancer (183). Research in our lab supports this hypothesis by demonstrating that alcohol exposure during gestation leads to increased susceptibility to tumorigenesis in a rodent model (201). We have also shown that there is an increase in circulating serum estradiol (E2) during proestrus in adulthood in response to alcohol *in utero* (201). Estrogens are thought to be critically important for breast cancer development (185), therefore, the goal of the studies presented here was to elucidate mechanisms of mammary tumorigenesis in rats exposed to alcohol *in utero* focusing on the role of the estrogen axis. There were two main approaches used to decipher a role for E2: the first approach considered changes in systemic E2 while the second examined changes in local gene expression.

Since serum E2 is higher in alcohol-exposed offspring, we hypothesized that this might contribute to an increase in mammary tumorigenesis. This hypothesis is supported by the observation that increased exposure over a woman's lifetime to high E2, due to early menarche and late menopause, correlating with an increased circulating E2 at cancer diagnosis, increases breast cancer risk (115,329). To test this hypothesis, we considered the most logical means of decreasing circulating E2 levels so that levels were the same between alcohol-exposed animals and controls. Ovariectomy (OVX) removes the main source of E2, which would cause circulating E2 to significantly decrease, however, OVX also prevents mammary tumor formation following treatment with a chemical carcinogen (127). An alternative approach would be to use an aromatase inhibitor (AI) to decrease E2 levels. However, AIs also prevent tumor development when animals are exposed to a chemical carcinogen (129). Therefore, to normalize E2 levels and allow for

comparisons in tumor development following NMU administration, OVX was followed by E2 administration to normalize levels.

A review of the literature to determine the most effective method and dose for E2 administration revealed that there were limited studies that OVX then gave hormones back to restore tumor development following administration of a chemical carcinogen. An effort to determine the most effective dose was confounded by literature suggesting that increasing E2 levels to the high levels seen in pregnancy also prevents tumor development (134). These studies suggested that a certain range of serum E2 levels leads to tumor formation. Of the few studies that did give hormones back following OVX, several suggested that progesterone (P4) was also required to promote tumor development (218,219). We felt this paradox relating to the role of P4 was also interesting to study in the context of hormone replacement therapy (HRT) and breast cancer. A large epidemiological study to understand the effects of HRT on a variety of outcomes of health and disease demonstrated that women who took E2 and P4 had a greater risk of developing breast cancer compared with women who took E2 alone (213). Contradicting reports following that study pointed to timing of hormone exposure or type of progestogen as confounding variables (214,215), suggesting an animal model to study different times and kinds of hormone exposure may lead to a better understanding of this conundrum.

The goal of the study presented in Chapter 2 was to see if tumors would develop in OVX rats treated with normalized levels of serum E2 and if P4 was also required. There are many different methods to administer hormones following OVX including silastic tubing placed at the time of surgery and daily injections. However, previous work has suggested these methods lead to supraphysiological levels or cause stress (143), which are confounding factors negatively impacting tumor development (330). Peroral administration provides a less stressful method of administration without spikes in hormone levels (143). Using this method, treatment with E2 with or without P4 led to tumor development similar to that observed in Sham animals with no differences between the two hormone-treated groups. This result led us to conclude that E2 at the appropriate dose could

lead to tumor development without P4. There was a tendency for an increase in tumor latency in the group treated with only E2. The lack of significance could be a result of a type II error due to a small sample size. Therefore, determining if P4 has effects on tumor latency deserves further study.

While extremely high and extremely low levels of E2 inhibit tumor development, slight variations within a normal range of E2 do not seem to influence tumor development. There was considerable variability in E2 levels as determined by ELISA and uterine histoarchitecture, which did not correlate with tumor development, multiplicity, or onset. One other study reported a lack of correlation between E2 at different doses and tumor incidence (145). A larger study using multiple doses of E2 and a larger sample size would help determine if E2 at different doses drives tumorigenesis differently. Additionally, it would be interesting to analyze different patterns of administration of E2 and P4 on tumor development. This study used a daily hormone administration, which does not emulate the estrous cycle of the rat, which has about 6 hours of high E2 during proestrus over the course of 4 to 5-day estrous cycle.

The variation observed within a normal range of serum E2 raises the question of whether this approach adequately controls circulating E2 levels enough to test our hypothesis. Furthermore, proliferation of the mammary gland is maximal 48 hours after E2 administration (106), therefore, it is plausible that differences in cyclicity of hormones could influence tumor development differently whereby higher levels of E2 across the cycle seen in intact animals would lead to increased tumor development but daily high levels would not. An alternative approach would be to treat control animals with low levels of E2 during proestrus to increase their levels to those of the alcohol-exposed animals.

Current research focuses on understanding the role of P4 in mammary tumorigenesis (169) and suggests that the additive effect of E2 and P4 is more important than either hormone alone. The combination of E2 and P4 may also be important to drive tumorigenesis in alcohol-exposed animals. Serum P4 levels following exposure to alcohol *in utero* are lower in alcohol fed (AF) compared to pair fed (PF) during proestrus, however there is no difference between AF and ad lib

(AL) rats (267). Animals in this study were not in full proestrus as they were sacrificed earlier in the day suggesting a more stringent study may help to further understand the role of P4 following exposure to alcohol *in utero*. Since E2 and P4 largely signal through paracrine mediators to induce proliferation in the mammary gland, studies focusing on these intermediate factors would strengthen an argument that E2 and P4 drive increased tumorigenesis in alcohol-exposed animals.

The ovary is the main source of estrogens in females and would thus be the most likely driver of increased E2 and P4 production. It may be interesting to study the ovary in alcohol-exposed rats including whole transcriptome analysis by RNASeq to see if genes associated with estrogens and P4 synthesis are affected by the *in utero* exposure. It would also be important to conduct enzyme activity assays to ensure changes in enzyme function correspond with changes in mRNA. Alternatively, the liver is a site of E2 metabolism that could be altered by alcohol *in utero.* Previous reports have demonstrated altered enzyme metabolism in fetuses following alcohol exposure (331) but an understanding of changes to pathways involved in ovarian steroid metabolism are lacking. Again, a whole genome approach with the liver may give insights into signaling pathways affected.

Unfortunately, we were only able to detect a small number of changes in gene expression in mammary glands of rats exposed to alcohol *in utero* compared with control animals on PND 2 and 10 and problems with variability within animals led to difficulty in interpreting results from PND 20 (Chapter 3). A concern with the RNASeq analyses presented here is that we may be experiencing type II error since we have previously reported differences in cell division and gene expression in the mammary glands of alcohol-exposed pups at PND 20 (209). Read depth is the number of reads that are sequenced and increasing read depth leads to improved quantification (332). We used 25 million reads for analyses in Chapter 3 and 4 which is a 500 times greater read depth than was able to distinguish differences in highly expressed genes between single cells (333). Using a greater read depth in our studies may distinguish subtler differences. It is also important to note that the analysis with 20,000 reads compared single cells (333), which removes all noise from

other cell types. Biological variability plays a role in increasing error (332,334) and is especially problematic when multiple cell types are considered. The mammary glands used in our analyses contained multiple cell types as evidenced from troubleshooting analyses completed in Chapter 3 suggesting biological variability negatively influenced our ability to detect differences. Another way to increase the possibility of detecting differences between AF and PF pup mammary glands besides increasing read depth is to increase the sample size. While three replicates is the lowest number for inferential analysis (332), an increase in sample size would also account for biological variability, which is especially important in outbred rodent strains such as the Sprague Dawley rat.

An alternative analysis is to repeat the study but to remove the lymph node or select a single cell type using laser capture microscopy. Results from troubleshooting in Chapter 3 should be used as a warning that all RNA analyses should contain homogenous tissue. In a study analyzing gene expression over pregnancy, there were changes in inflammatory mediators and cytokines suggesting a role for the immune system in modulating these responses in the absence of a lymph node (292).

While the immune system does not directly influence E2 and P4 production, there is a relationship between ovarian hormones and the immune system that requires a delicate balance for normal mammary gland development (335). Colony stimulating factor 1 (Csf1) is regulated by estrogens (302) and macrophages exposed to estrogens promote stromal development through paracrine signaling (336). The paracrine signaling molecule RANKL increases in response to P4 stimulation in the mammary gland but was originally discovered to be critically important in osteoclast differentiation (337). RANKL also plays a role in the adaptive immune system through T cell activation and has been shown to be involved in breast cancer metastasis to bone (338). Together, these results suggest an interplay between the immune and estrogen axes in controlling the regulation of mammary gland development and breast cancer development which complicates understanding signaling pathways in either independently. The data presented in Chapter 4 suggest an alternative approach to studying mammary gland development at a stage where the influence of

hormones is not as prevalent, but where the microenvironment is developing and thus likely plays a critical role in growth.

It is possible that the increase in tumorigenesis in rats following alcohol exposure *in utero* is a result of altered communication between the estrogen axis and the immune system. Work in the Weinberg lab focuses on how alcohol *in utero* influences brain development including the hypothalamic – pituitary – adrenal (HPA) and hypothalamic – pituitary – gonadal (HPG) axes and a recent report from their lab analyzed rat brains following exposure to the Lieber-DeCarli diet (262). Specifically, the prefrontal cortex and hippocampus were subjected to whole genome mRNA expression arrays resulting in differences in neuroimmune function (339). This result, along with the multitude of studies that point to deregulation in the immune system in response to alcohol *in utero*, suggests the immune system may play a role in increased mammary tumorigenesis.

It is interesting that the changes in gene expression between mammary glands at PND 2 to 10 as described in Chapter 4 demonstrated an increase in immune-related genes, specifically genes of the adaptive immune system. The most likely explanation for this increase is B and T cell infiltration into the lymph node, so an understanding of how the lymph node influences mammary gland development is warranted. If the lymph node plays a role, there may be differences in how the lymph node establishes itself in the mammary gland between alcohol-exposed offspring and a control.

The results presented in this thesis suggest further studies should be completed to elucidate mechanisms of how alcohol *in utero* increases mammary tumorigenesis. Alternative methods of studying the roles of E2 *in utero* would concretely determine if increased E2 is involved. It may be worth completing transcriptome analyses on other tissues of rats exposed to alcohol *in utero* including ovaries, liver, and cells of the immune system. Additionally, increasing sequencing reads or samples for RNASeq analysis of mammary glands may give way to changes that were not detected with 3 samples per treatment group and 25 million reads. An understanding of the

mechanisms involved in increased mammary tumorigenesis in alcohol-exposed animals would ultimately lead to better prevention and treatment strategies for breast cancer.

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Appendix

Table 1. Genes that change by |1.5| log2fold from PND 2 to 10.

Rat Gene ID	PND 02 v 10 log2fold	Affymetrix ID	Rat Ensembl
Krt1	-8.65691	205900_at	ENSRNOG00000028996
Krt10	-7.18688	207023_x_at	ENSRNOG00000030170
Prg4	-4.15061	206007_at	ENSRNOG00000002385
Dlk1	-3.97249	209560_s_at	ENSRNOG00000019584
Krt14	-3.88322	209351_at	ENSRNOG00000003899
Csn1s1	-3.77156	208350_at	ENSRNOG00000055596
Pkp1	-3.54498	205724_at	ENSRNOG00000010076
Sult2b1	-3.23123	205759_s_at	ENSRNOG00000021046
Retn	-2.9179	220570_at	ENSRNOG00000001001
Krt17	-2.81212	205157_s_at	ENSRNOG00000026371
Lpo	-2.74769	210682_at	ENSRNOG00000008422
Agtr2	-2.6773	207293_s_at	ENSRNOG00000050006
Tacstd2	-2.62314	202285_s_at	ENSRNOG00000007740
Endou	-2.44947	206605_at	ENSRNOG00000056446
Dusp4	-2.3413	204014_at	ENSRNOG00000011921
Tuba8	-2.34101	220069_at	ENSRNOG00000048169
Krt5	-2.3264	201820_at	ENSRNOG00000050420
Dsp	-2.29943	200606_at	ENSRNOG00000013928
Ace2	-2.28541	219962_at	ENSRNOG00000031665
Car5b	-2.22278	207129_at	ENSRNOG00000029330
Kng1l1	-2.21872	206054_at	ENSRNOG00000030387
Slc2a5	-2.17174	204429_s_at	ENSRNOG00000017693
Magel2	-2.13423	219894_at	ENSRNOG00000010158
Cst6	-2.07989	206595_at	ENSRNOG00000020455
Gys2	-2.03864	214621_at	ENSRNOG00000059753
Perp	-1.97184	217744_s_at	ENSRNOG00000011994
Eps8l1	-1.95814	218778_x_at	ENSRNOG00000027942
Ly6g6c	-1.92443	207114_at	ENSRNOG00000000843
Col7a1	-1.88047	204136_at	ENSRNOG00000020579
Sfn	-1.86846	209260_at	ENSRNOG00000033153
Tpsab1	-1.86223	205683_x_at	ENSRNOG00000024181
Tm6sf2	-1.84362	219892_at	ENSRNOG00000042237
Fam111a	-1.82931	218248_at	ENSRNOG00000012067
Kif26b	-1.80358	220002_at	ENSRNOG00000028624
Cryba4	-1.80038	206843_at	ENSRNOG00000049770
Sox11	-1.77881	204913_s_at	ENSRNOG00000030034
Slc22a3	-1.73609	205421_at	ENSRNOG00000022946

Rat Gene ID	PND 02 v 10 log2fold	Affymetrix ID	Rat Ensembl
Pmepa1	-1.73399	217875_s_at	ENSRNOG00000050404
Clstn3	-1.73191	204375_at	ENSRNOG00000011156
Aldh1a7	-1.6919	212224_at	ENSRNOG00000017878
Crtac1	-1.68709	221204_s_at	ENSRNOG00000015220
Retsat	-1.6575	218124_at	ENSRNOG00000014090
Cyb5r2	-1.65631	220230_s_at	ENSRNOG00000019751
Ogdhl	-1.65394	219277_s_at	ENSRNOG00000019955
Cdkn1a	-1.64231	202284_s_at	ENSRNOG00000000521
Gbe1	-1.62632	203282_at	ENSRNOG00000051232
Lpin1	-1.6179	212272_at	ENSRNOG00000004377
Acpp	-1.6071	204393_s_at	ENSRNOG00000011820
Slc16a1	-1.60664	202234_s_at	ENSRNOG00000019996
Elovl6	-1.58821	204256_at	ENSRNOG00000048949
Pkp3	-1.57892	209872_s_at	ENSRNOG00000015152
Sctr	-1.578	210382_at	ENSRNOG00000049766
Srd5a1	-1.57102	204675_at	ENSRNOG00000017601
Apoc2	-1.54749	204561_x_at	ENSRNOG00000018402
Fbn2	-1.52829	203184_at	ENSRNOG00000043219
Ghr	-1.52184	205498_at	ENSRNOG00000015654
Apoc3	-1.52053	205820_s_at	ENSRNOG00000047503
Mt2A	-1.50196	212185_x_at	ENSRNOG00000043098
Pfkfb3	-1.4955	202464_s_at	ENSRNOG00000018911
RT1-Da	1.50092	208894_at	ENSRNOG00000032844
Siglec1	1.50231	219519_s_at	ENSRNOG00000021243
Icos	1.50291	210439_at	ENSRNOG00000046196
Mybpc1	1.50832	214087_s_at	ENSRNOG00000056493
Hlf	1.51172	204753_s_at	ENSRNOG00000002456
Tnnt3	1.51636	205693_at	ENSRNOG00000020332
Sh3bgr	1.52102	204979_s_at	ENSRNOG00000028238
Fgf16	1.52409	221374_at	ENSRNOG00000061530
Mylpf	1.52437	205163_at	ENSRNOG00000017645
Lsp1	1.5248	203523_at	ENSRNOG00000020300
Runx3	1.52799	204197_s_at	ENSRNOG00000054217
Myl1	1.5301	209888_s_at	ENSRNOG00000013262
Scn7a	1.53532	207864_at	ENSRNOG00000029342
Apobec2	1.53569	206160_at	ENSRNOG00000012303
Cxcr2	1.53849	207008_at	ENSRNOG00000014269
Rergl	1.54173	220276_at	ENSRNOG00000008130
Cmah	1.54367	205518_s_at	ENSRNOG00000003094
Htra1	1.54626	201185_at	ENSRNOG00000020533
Myom2	1.55041	205826_at	ENSRNOG00000011754

Rat Gene ID	PND 02 v 10 log2fold	Affymetrix ID	Rat Ensembl
Itgb7	1.55143	205718_at	ENSRNOG00000012208
Tmem156	1.55359	220169_at	ENSRNOG00000026518
RT1-Bb	1.56075	209480_at	ENSRNOG00000032708
RT1-Bb	1.56075	209480_at	ENSRNOG00000032708
Sema4d	1.56133	203528_at	ENSRNOG00000013679
RGD1559731	1.56148	219652_s_at	ENSRNOG00000004265
Clu	1.56798	208791_at	ENSRNOG00000016460
Inhbb	1.56876	205258_at	ENSRNOG00000060237
Usp18	1.5708	219211_at	ENSRNOG00000037198
Spib	1.57379	205861_at	ENSRNOG00000019660
Eln	1.58057	212670_at	ENSRNOG00000001469
Gfra1	1.58491	205696_s_at	ENSRNOG00000017438
Il10ra	1.58824	204912_at	ENSRNOG00000016308
Rcan2	1.58956	203498_at	ENSRNOG00000010350
Clec11a	1.59534	205131_x_at	ENSRNOG00000019138
Tnnc2	1.59789	205388_at	ENSRNOG00000015155
Pik3cd	1.60217	203879_at	ENSRNOG00000016846
Tnni2	1.61362	206393_at	ENSRNOG00000020276
Gpr171	1.61998	207651_at	ENSRNOG00000025297
Pygm	1.62234	205577_at	ENSRNOG00000021090
Ctse	1.62263	205927_s_at	ENSRNOG00000006963
Fmod	1.62415	202709_at	ENSRNOG00000003183
B4galnt1	1.62437	206435_at	ENSRNOG00000004839
RT1-Ba	1.62459	203290_at	ENSRNOG00000000451
Igfbp6	1.62858	203851_at	ENSRNOG00000010977
LOC100911572	1.62954	214641_at	ENSRNOG00000015365
Pla2g2d	1.63018	220423_at	ENSRNOG00000016826
Ntrk3	1.63456	206462_s_at	ENSRNOG00000018674
Lef1	1.63735	210948_s_at	ENSRNOG00000010121
Ikzf1	1.64585	205038_at	ENSRNOG00000004444
Synpo2l	1.64676	219804_at	ENSRNOG00000008949
P2ry10	1.64879	214615_at	ENSRNOG00000037839
Tcf7	1.65102	205254_x_at	ENSRNOG00000005872
Cbx7	1.65669	212914_at	ENSRNOG00000016875
Arhgap22	1.66168	206298_at	ENSRNOG00000024728
Grb14	1.66564	206204_at	ENSRNOG00000052498
Rhoh	1.66676	204951_at	ENSRNOG00000002540
Il27ra	1.66899	205926_at	ENSRNOG00000005747
Myl3	1.67031	205589_at	ENSRNOG00000020955
Chrdl1	1.67185	209763_at	ENSRNOG00000004330
Il2rb	1.67266	205291_at	ENSRNOG00000048636

Rat Gene ID	PND 02 v 10 log2fold	Affymetrix ID	Rat Ensembl
Osgin1	1.6775	219475_at	ENSRNOG00000014948
Sash3	1.67785	204923_at	ENSRNOG00000004409
Gpr132	1.68589	221140_s_at	ENSRNOG00000013914
Ptprcap	1.68724	204960_at	ENSRNOG00000021724
Actn2	1.69087	203861_s_at	ENSRNOG00000017833
Bcas1	1.69413	204378_at	ENSRNOG00000012906
Tspan32	1.69494	220558_x_at	ENSRNOG00000026039
Dbp	1.69769	209782_s_at	ENSRNOG00000021027
Cpa1	1.70147	205615_at	ENSRNOG00000010725
Grap2	1.70213	208406_s_at	ENSRNOG00000018316
Eef1a2	1.70219	204540_at	ENSRNOG00000012477
Pvalb	1.71706	205336_at	ENSRNOG00000006471
Plxnc1	1.71745	206470_at	ENSRNOG00000007970
Kif21b	1.73248	204411_at	ENSRNOG00000008471
Ccl21	1.74073	204606_at	ENSRNOG00000034290
Il7r	1.75247	205798_at	ENSRNOG00000058446
Ccr6	1.7551	206983_at	ENSRNOG00000012964
Gpx3	1.75666	201348_at	ENSRNOG00000052564
Acta1	1.76098	203872_at	ENSRNOG00000017786
Fam129a	1.76294	217966_s_at	ENSRNOG00000002403
Cytip	1.76411	209606_at	ENSRNOG00000004772
Acsm5	1.7642	220061_at	ENSRNOG00000031211
Camk4	1.77644	210349_at	ENSRNOG00000020478
Ampd1	1.77773	206121_at	ENSRNOG00000018656
Cd74	1.78496	209619_at	ENSRNOG00000018735
Esr1	1.79068	205225_at	ENSRNOG00000019358
Spn	1.79068	206056_x_at	ENSRNOG00000036711
Gzmm	1.79555	207460_at	ENSRNOG00000030530
Casq1	1.79555	219645_at	ENSRNOG00000006930
Cox6a2	1.7998	206353_at	ENSRNOG00000019851
Pou2f2	1.80412	211660_at	ENSRNOG00000055650
Skap1	1.80658	205790_at	ENSRNOG00000023881
Col4a4	1.81084	214602_at	ENSRNOG00000014851
Ptprc	1.82104	207238_s_at	ENSRNOG00000000655
Fhl5	1.82569	220170_at	ENSRNOG00000007680
Adamts5	1.82932	219935_at	ENSRNOG00000057794
Lck	1.83017	204890_s_at	ENSRNOG00000009705
Cd7	1.84241	214049_x_at	ENSRNOG00000036674
Npy1r	1.84285	205440_s_at	ENSRNOG00000014149
Alox15	1.86083	207328_at	ENSRNOG00000019183
Dnase1l3	1.86114	205554_s_at	ENSRNOG00000009291

Rat Gene ID	PND 02 v 10 log2fold	Affymetrix ID	Rat Ensembl
Pi15	1.86145	207938_at	ENSRNOG00000017686
Sh2d1a	1.88072	210116_at	ENSRNOG00000006263
Cd8a	1.88423	205758_at	ENSRNOG00000007178
Prx	1.88826	220024_s_at	ENSRNOG00000018369
Cd8b	1.88873	207979_s_at	ENSRNOG00000007129
Sema3b	1.89703	203070_at	ENSRNOG00000016512
Fcho1	1.91523	213669_at	ENSRNOG00000033912
Cd22	1.93479	204581_at	ENSRNOG00000024000
Ccl5	1.94669	1405_i_at	ENSRNOG00000010906
Cd3d	1.95124	213539_at	ENSRNOG00000015994
Cyfip2	1.95193	215785_s_at	ENSRNOG00000006557
Prps2	1.95924	203401_at	ENSRNOG00000004160
Sh2d2a	1.96372	207351_s_at	ENSRNOG00000013294
Ntf3	1.96394	206706_at	ENSRNOG00000019716
Mal	1.96423	204777_s_at	ENSRNOG00000015445
Gsn	1.97154	200696_s_at	ENSRNOG00000018991
Atp2a1	1.97432	205444_at	ENSRNOG00000047124
Cxcl14	1.99696	218002_s_at	ENSRNOG00000011984
Cd69	2.00581	209795_at	ENSRNOG00000056783
Acsm1	2.01245	215432_at	ENSRNOG00000042084
Il2ra	2.01868	206341_at	ENSRNOG00000047647
Trpm2	2.04287	205708_s_at	ENSRNOG00000001216
Faim3	2.04786	221601_s_at	ENSRNOG00000004441
Zap70	2.05213	214032_at	ENSRNOG00000016995
Cd28	2.05553	206545_at	ENSRNOG00000010283
Pllp	2.05719	204519_s_at	ENSRNOG00000016558
Mt3	2.07608	205970_at	ENSRNOG00000018958
Lsamp	2.08803	214460_at	ENSRNOG00000031852
Treml2	2.0922	219748_at	ENSRNOG00000013554
Tgfbi	2.10049	201506_at	ENSRNOG00000012216
Cd247	2.11662	210031_at	ENSRNOG00000003298
Cd3e	2.12302	205456_at	ENSRNOG00000016069
Trdn	2.15036	222287_at	ENSRNOG00000012609
Il21r	2.15179	219971_at	ENSRNOG00000015773
Cd6	2.15194	208602_x_at	ENSRNOG00000020884
Myl2	2.15549	209742_s_at	ENSRNOG00000030848
Art3	2.15734	210147_at	ENSRNOG00000002256
Rasgrp1	2.16315	205590_at	ENSRNOG00000005404
Acap1	2.1699	205212_s_at	ENSRNOG00000015674
Pmp2	2.17662	206826_at	ENSRNOG00000022707
Cd2	2.19211	205831_at	ENSRNOG00000015821

Rat Gene ID	PND 02 v 10 log2fold	Affymetrix ID	Rat Ensembl
Cd3g	2.20166	206804_at	ENSRNOG00000015945
Ctsw	2.21632	214450_at	ENSRNOG00000027096
Prf1	2.22031	214617_at	ENSRNOG00000000562
Ptpn22	2.23551	206060_s_at	ENSRNOG00000019614
Sit1	2.29774	205484_at	ENSRNOG00000021546
Mx1	2.29837	202086_at	ENSRNOG00000001959
Agap2	2.30736	206152_at	ENSRNOG00000025584
Itk	2.3331	211339_s_at	ENSRNOG00000006860
Akr1c3	2.33909	209160_at	ENSRNOG00000017531
Per3	2.34801	221045_s_at	ENSRNOG00000018413
C7	2.3737	202992_at	ENSRNOG00000061379
Igj	2.37429	212592_at	ENSRNOG00000003666
Tnfrsf14	2.42371	209354_at	ENSRNOG00000013820
Sstr3	2.43725	214491_at	ENSRNOG00000007332
Ccl22	2.44114	207861_at	ENSRNOG00000016535
AABR07072539.1	2.45142	206101_at	ENSRNOG00000031716
Sell	2.4575	204563_at	ENSRNOG00000002776
Camk2b	2.48263	209956_s_at	ENSRNOG00000052080
Nrxn1	2.53096	209914_s_at	ENSRNOG00000050220
Cd27	2.53284	206150_at	ENSRNOG00000027466
Myh1	2.54665	205951_at	ENSRNOG00000049695
Itgbl1	2.57072	205422_s_at	ENSRNOG00000004516
Ccr7	2.57809	206337_at	ENSRNOG00000010665
Tcap	2.70791	205766_at	ENSRNOG00000060511
Npr3	2.79908	219789_at	ENSRNOG00000019184
PCOLCE2	2.82327	219295_s_at	ENSRNOG00000046848
Sfrp4	2.8647	204051_s_at	ENSRNOG00000054957
Pcp4	2.92795	205549_at	ENSRNOG00000001628
Sncg	2.97089	208584_at	ENSRNOG00000058006
Cd79a	3.1886	205049_s_at	ENSRNOG00000020125
Cd79b	3.21407	205297_s_at	ENSRNOG00000011917
RT1-DOa	3.22637	206313_at	ENSRNOG00000026762
Ighm	3.32047	209374_s_at	ENSRNOG00000034190
Apod	3.53073	201525_at	ENSRNOG00000048273
Igkc	3.59043	214669_x_at	ENSRNOG00000049829
Rab3b	3.64829	205924_at	ENSRNOG00000008001
Ccl17	3.67571	207900_at	ENSRNOG00000016278
RT1-DOb	3.70142	205671_s_at	ENSRNOG00000000454
Stc2	3.77287	203438_at	ENSRNOG00000020729
Mpz	4.02051	210280_at	ENSRNOG00000003171

Table 2. List of top 100 pathways that changed from PND 2 to PND 10 in mammary glands.

Ingenuity Canonical Pathways	-log(p-value)	Ratio
iCOS-iCOSL Signaling in T Helper Cells	1.9E01	2.04E-01
CD28 Signaling in T Helper Cells	1.45E01	1.61E-01
Calcium-induced T Lymphocyte Apoptosis	1.27E01	2.19E-01
T Cell Receptor Signaling	1.24E01	1.65E-01
B Cell Development	1.23E01	3.33E-01
PKCθ Signaling in T Lymphocytes	1.22E01	1.44E-01
Role of NFAT in Regulation of the Immune Response	1.15E01	1.11E-01
Nur77 Signaling in T Lymphocytes	1.08E01	2.11E-01
CTLA4 Signaling in Cytotoxic T Lymphocytes	8.47E00	1.36E-01
T Helper Cell Differentiation	8.42E00	1.55E-01
Cdc42 Signaling	7.89E00	8.98E-02
Primary Immunodeficiency Signaling	7.75E00	1.88E-01
Phospholipase C Signaling	7.39E00	7.17E-02
Type I Diabetes Mellitus Signaling	7.36E00	1.09E-01
Autoimmune Thyroid Disease Signaling	6.6E00	1.70E-01
Graft-versus-Host Disease Signaling	6.53E00	1.67E-01
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	6.39E00	1.14E-01
OX40 Signaling Pathway	6.35E00	1.12E-01
Calcium Signaling	5.88E00	7.30E-02
Agranulocyte Adhesion and Diapedesis	5.58E00	6.88E-02
Crosstalk between Dendritic Cells and Natural Killer Cells	5.38E00	1.01E-01
Systemic Lupus Erythematosus Signaling	4.99E00	6.07E-02

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Antigen Presentation Pathway	4.95E00	1.62E-01
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	4.85E00	1.01E-01
Allograft Rejection Signaling	4.65E00	9.52E-02
Hematopoiesis from Pluripotent Stem Cells	4.34E00	1.28E-01
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	4.13E00	1.56E-01
IL-4 Signaling	4.08E00	9.33E-02
Communication between Innate and Adaptive Immune Cells	3.61E00	7.87E-02
Hepatic Fibrosis / Hepatic Stellate Cell Activation	3.6E00	5.46E-02
ILK Signaling	3.56E00	5.41E-02
Regulation of Actin-based Motility by Rho	3.55E00	7.69E-02
Protein Kinase A Signaling	3.45E00	3.89E-02
CCR5 Signaling in Macrophages	3.4E00	8.70E-02
Epithelial Adherens Junction Signaling	2.99E00	5.48E-02
TR/RXR Activation	2.92E00	7.06E-02
Role of JAK1 and JAK3 in γc Cytokine Signaling	2.77E00	8.06E-02
Thyroid Cancer Signaling	2.65E00	1.00E-01
Glycogen Biosynthesis II (from UDP-D-Glucose)	2.55E00	3.33E-01
B Cell Receptor Signaling	2.51E00	4.60E-02
G-Protein Coupled Receptor Signaling	2.49E00	3.91E-02
Actin Cytoskeleton Signaling	2.47E00	4.17E-02
Dendritic Cell Maturation	2.46E00	4.52E-02
Clathrin-mediated Endocytosis Signaling	2.35E00	4.32E-02
VDR/RXR Activation	2.33E00	6.41E-02

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Thrombin Signaling	2.28E00	4.21E-02
IL-2 Signaling	2.2E00	7.55E-02
RhoA Signaling	2.14E00	4.92E-02
Atherosclerosis Signaling	2.11E00	4.84E-02
PAK Signaling	2.09E00	5.62E-02
PI3K Signaling in B Lymphocytes	2.06E00	4.72E-02
Cellular Effects of Sildenafil (Viagra)	2.03E00	4.65E-02
VEGF Signaling	2.03E00	5.43E-02
RhoGDI Signaling	1.97E00	4.05E-02
cAMP-mediated signaling	1.93E00	3.65E-02
Granulocyte Adhesion and Diapedesis	1.92E00	3.95E-02
Glucocorticoid Receptor Signaling	1.82E00	3.27E-02
Androgen Biosynthesis	1.8E00	1.43E-01
Signaling by Rho Family GTPases	1.77E00	3.42E-02
Chemokine Signaling	1.76E00	5.63E-02
FcγRIIB Signaling in B Lymphocytes	1.72E00	7.32E-02
Axonal Guidance Signaling	1.72E00	2.76E-02
Natural Killer Cell Signaling	1.72E00	4.55E-02
CXCR4 Signaling	1.7E00	3.95E-02
Leukocyte Extravasation Signaling	1.67E00	3.54E-02
Gα12/13 Signaling	1.62E00	4.27E-02
Germ Cell-Sertoli Cell Junction Signaling	1.61E00	3.75E-02
Gαi Signaling	1.58E00	4.17E-02
Integrin Signaling	1.58E00	3.38E-02

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Prostate Cancer Signaling	1.56E00	4.88E-02
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	1.47E00	3.20E-02
NF-κB Signaling	1.47E00	3.49E-02
Cardiac Hypertrophy Signaling	1.43E00	3.14E-02
IL-12 Signaling and Production in Macrophages	1.41E00	3.76E-02
Human Embryonic Stem Cell Pluripotency	1.4E00	3.73E-02
SAPK/JNK Signaling	1.37E00	4.26E-02
Estrogen-mediated S-phase Entry	1.36E00	8.33E-02
Glioma Signaling	1.32E00	4.08E-02
p53 Signaling	1.32E00	4.08E-02
Telomerase Signaling	1.31E00	4.04E-02
Paxillin Signaling	1.28E00	3.96E-02
PRPP Biosynthesis I	1.26E00	2.50E-01
IL-15 Signaling	1.24E00	4.76E-02
Eicosanoid Signaling	1.22E00	4.69E-02
Neurotrophin/TRK Signaling	1.18E00	4.48E-02
Remodeling of Epithelial Adherens Junctions	1.16E00	4.41E-02
Tec Kinase Signaling	1.16E00	3.18E-02
Circadian Rhythm Signaling	1.11E00	6.06E-02
MIF-mediated Glucocorticoid Regulation	1.11E00	6.06E-02
Retinol Biosynthesis	1.11E00	6.06E-02
NF-κB Activation by Viruses	1.09E00	4.11E-02
STAT3 Pathway	1.09E00	4.11E-02

Ingenuity Canonical Pathways	-log(p-value)	Ratio
PTEN Signaling	1.08E00	3.39E-02
Triacylglycerol Biosynthesis	1.07E00	5.71E-02
p70S6K Signaling	1.07E00	3.36E-02
Wnt/β-catenin Signaling	1.06E00	2.96E-02
phagosome maturation	1.06E00	3.33E-02
HER-2 Signaling in Breast Cancer	1.05E00	3.95E-02
PI3K/AKT Signaling	1.03E00	3.25E-02
Estrogen Biosynthesis	1.01E00	5.26E-02