THE EFFECTS OF FETAL ALCOHOL EXPOSURE ON MAMMARY EPITHELIAL CELL SUBPOPULATIONS AND TUMORIGENESIS

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ABSTRACT OF THE DISSERTATION

The Effects of Fetal Alcohol Exposure on Mammary Epithelial Cell Subpopulations and Tumorigenesis

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Previous work from our laboratory found that alcohol exposure *in utero* using the Lieber-DeCarli diet increases the risk of carcinogen-induced mammary tumorigenesis in adult rat offspring; however, the underlying mechanism remains unknown. The mature mammary gland is established after birth and maintained throughout adulthood by a mammary cell lineage where stem cells produce progenitor cells that generate differentiated epithelial cells comprising the ductal and secretory structures of the adult gland. Altering the mammary cell composition has been shown to increase susceptibility to tumorigenesis. Therefore, we hypothesized that alcohol exposure *in utero* may target cells along the mammary epithelial cell (MEC) lineage, shifting it towards one that promotes tumorigenesis. To test this hypothesis, we investigated the effects of fetal alcohol exposure (FAE) in normal and hyperplastic mammary glands, utilizing the MMTV-Wnt1 mouse model of breast cancer. FVB/NJ female mice were bred to MMTV-Wnt1 male mice to produce both wild-type (WT) and transgenic (Tg) female offspring. Alcohol dams were given ad-lib access to 5% alcohol in 0.2% saccharin solution from GD9-10 and 10% alcohol in 0.2% saccharin from GD11-GD19. Control dams were given ad lib access to 0.2% saccharin solution from GD9-GD19. Thoracic and inguinal mammary glands from WT and Tg offspring were

harvested at puberty (5 weeks of age) and adulthood (10 weeks of age) and dissociated to yield a single cell suspension enriched for MECs. To determine the effects of FAE on the mammary gland, MECs were analyzed by flow cytometry to characterize the luminal, luminal progenitor and basal epithelial subpopulations. WT glands of FAE animals exhibited a decreased basal cell population and increased luminal:basal ratio at 10 weeks of age. qRT-PCR analysis of total MECs found that Hey1 mRNA expression was increased in the WT FAE group at 10 weeks of age. In Tg glands FAE increased the luminal progenitor cell population at 5 weeks of age but did not alter MEC composition at 10 weeks of age. Total MECs were plated for mammosphere assay and passaged twice to monitor secondary and tertiary mammosphere formation. Tertiary mammosphere forming efficiency was greater in the WT glands of FAE animals at 10 weeks of age; however, this effect was not observed in the WT glands at 5 weeks of age or in Tg glands at either age. To further investigate how an altered MEC composition may affect tumorigenesis, a subset of Tg female offspring was followed for tumor formation. Overall, tumor latency was decreased in the FAE group. Flow cytometry analysis indicated that FAE females developed tumors with an increased basal cell population. These data indicate that FAE can shift MEC subpopulations, increasing the proportion of cells that are potentially vulnerable to transformation and affecting cancer risk.

DEDICATION

I dedicate this thesis to my family – my mother, father and sister. Thank you for your unconditional love, patience and support throughout this journey. I am forever grateful for all that you've done so that I could be here.

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List of Abbreviations

ABC	ATP binding cassette
ADAM	Disintegrin Metalloproteases
BMI	Body mass index
BPA	Bisphenol-A
CD	Cluster differentiation
CSC	Cancer stem cell
DAB	3,3'-diaminobenzidine
DES	Diethylstilbestrol
DMBA	Dimethylbenzathracene
dnIGF1R	Dominant negative IGF1 receptor
DOHAD	Developmental origins of health and disease
E	Embryonic day
EDC	Endocrine disrupting chemicals
ER	Estrogen receptor
FAE	Fetal alcohol exposure
FASD	Fetal alcohol spectrum disorder
GD	Gestational day
GH	Growth hormone
HF	HBSS/2% FBS
IHC	Immunohistochemistry
K14	Cytokeratin 14

K8	Cytokeratin 8
MaSC	Mammary stem cell
MEC	Mammary epithelial cell
MFE	Mammosphere forming efficiency
MMTV	Mouse mammary tumor virus
NBF	Neutral buffered formalin
NEXT	Notch extracellular truncation
NICD	Notch intracellular domain
NMU	N-nitroso-N-methylurea
OD	Optical density
PND	Postnatal day
PR	Progesterone receptor
TEB	Terminal end bud
Tg	Transgenic
TIC	Tumor initiating cell
WT	Wild type

Chapter 1

Review of the literature

Alcohol consumption and breast cancer

There are many known risk factors for breast cancer. These include factors that are out of our control, such as family history and early menarche/late menopause, as well as modifiable lifestyle factors. Alcohol is one such lifestyle factor considered as a risk for breast cancer, with studies showing an increased 7-12% risk of breast cancer for every 10 g of ethanol consumed per day (Hamajima et al., 2002; Quandt et al., 2015). Although there are several factors to consider when investigating the effect of alcohol on breast cancer risk, such as quantity and timing of exposure, several epidemiological and animal studies support the correlation between alcohol exposure and breast cancer risk.

Over several decades, epidemiological studies have investigated the relationship of alcohol consumption and breast cancer risk. The Nurses' Health Study Initiative, which initiated in 1980 and was updated in 2011, analyzed a questionnaire given to nurses between the ages of 34 and 59. Based on the data from this questionnaire, women who drank more than 5 g ethanol per day had a slight increase in risk and this risk did not change with the type of alcoholic drink consumed. Additionally, women who consumed more than 30 g per day had a greater risk of breast cancer (Chen et al., 2011). In another study, women who drank more than 7 servings per week of alcohol had a higher breast density as compared to nondrinkers, indicating an increased risk for developing breast cancer (Quandt et al., 2015). A similar study also found increased breast density with alcohol consumption as well as higher circulating levels of estrogen (Frydenberg et al., 2015). In a cross-sectional analysis, a positive and significant correlation was found between alcohol consumption and urinary estradiol levels in women who drank more than 15 g/day (Hartman et al., 2016). Both breast density as well as estrogen levels are known risk factors for breast cancer and may therefore provide additional reason for women to reduce their alcohol

intake. Investigators have also looked at whether alcohol consumption promotes a specific subtype of breast cancer. The risk for triple-negative breast cancer does not appear to increase with alcohol consumption; however, the risk for HER2 overexpressing breast tumors does increase (Baglia et al., 2018). In post-menopausal women, alcohol consumption was found to be associated specifically with the increased risk for hormone receptor (estrogen and progesterone) positive breast tumors (Baglia et al., 2017; Enger et al., 1999; Suzuki et al., 2005; Zakhari and Hoek, 2015).

In vitro and *in vivo* work investigating the relationship between alcohol and breast cancer has not only supported the epidemiological data reported, but also greatly contributed to the understanding of the mechanisms underlying this effect. Rodent studies have shown that alcohol consumption promotes mammary tumorigenesis (Hong et al., 2010; Singletary and McNary, 1994; Watabiki et al., 2000). In a 1994 study, rats consuming alcohol at 20-30% of calories had an increase in terminal end bud number (Singletary and McNary, 1994). Mice fed a liquid diet containing 3.2% (v/v) ethanol for 4 weeks had an increased number of ductal branch points in the mammary glands compared to control (Masso-Welch et al., 2012). These data are indicative of a proliferative, denser mammary gland as seen in human data. Additionally, alcohol has been shown to promote growth and proliferation in human breast cancer cells (Izevbigie et al., 2002; Singletary et al., 2001). This increase in cell proliferation can go hand in hand with increased cell migration and metastases, as has been shown in several animal and cell culture studies (Meng et al., 2000; Xu et al., 2016; Zhao et al., 2017).

Effects of fetal environment on the mammary gland

For several decades it has been known that a suboptimal fetal environment affects the risk for chronic diseases later in life. An altered fetal environment can bring about permanent changes in tissues that can affect normal tissue function and maintenance and ultimately affect risk for

disease (McMullen and Mostyn, 2009). Both epidemiological studies and animal models have provided evidence for this effect and supports the theory of the Developmental Origins of Health and Disease (DoHAD). While initially this theory applied to evidence of altered maternal nutrition and subsequent metabolic disorders in adult offspring, it has now expanded to encompass other diseases such as cancer (Fenton and Birnbaum, 2015). The mammary gland is one of the numerous organs susceptible to an altered fetal environment and exposure to environmental factors can affect mammary stem cells, normal gland development and tumorigenesis.

Maternal nutrition is known to play a significant role in fetal development both *in utero* as well as later in life. Maternal exposure to a high-fat diet promoted an accelerated mammary gland development as well as increased tumor incidence and decreased latency (Montales et al., 2014). In two separate studies, female rat offspring born from pregnant dams fed a high-fat diet containing corn-oil or n 6-polyunsaturated fatty acids had an increased tumor incidence when induced with DMBA (Hilakivi-Clarke et al., 1997; Stark et al., 2003). Mice exposed to a high-fat/high-sugar diet *in utero* and in adulthood had an expanded stem cell pool as well as the highest incidence of tumorigenesis when induced with DMBA (Lambertz et al., 2017). Together, these data suggest that maternal nutrition not only plays a significant role in fetal growth, but also in normal mammary gland development and can promote an increased risk for tumorigenesis.

Exposure to endocrine disrupting chemicals (EDCs) during fetal development has also been shown to affect normal mammary gland development. EDCs alter hormonal signaling *in utero*, affecting developmental programming and organ development (Kopras et al., 2014). Bisphenol-A (BPA) is an EDC found in a variety of household plastic products which can leach and be consumed unintentionally. BPA is structurally similar to estradiol and has estrogenic effects on the body and mammary glands. Mice exposed to BPA *in utero* showed increased ductal elongation at one month of age and increased number of terminal end buds at 6 months of age

(Markey et al., 2001). In rats, exposure to BPA at ranges of 2.5 μg – 1000 μg / kg bodyweight/ day resulted in hyperplastic ducts compared to the control as evidenced by increased proliferation at PND50 and PND95 based on Ki67 staining (Murray et al., 2007). In rhesus monkeys, exposure to BPA *in utero* caused an accelerated mammary gland development, such as the number of terminal end buds, branching and mammary area (Tharp et al., 2012).

Diethylstilbestrol (DES) was prescribed to pregnant women from 1938 -1971 to prevent miscarriages and premature births. It is a synthetic estrogen that was thought to help with certain pregnancy-related complications, however it was ultimately found that DES did not help carry pregnancies to full-term and caused rare cancers in young girls and women, in addition to increasing breast cancer risk (Fenton and Birnbaum, 2015; Hoover et al., 2011). In rat models, female offspring exposed to DES *in utero* had an increased incidence of DMBA-induced tumors as well as decreased latency (Boylan and Calhoon, 1983; Rothschild et al., 1987). In mice, neonatal exposure also had an effect on mammary gland proliferation and development, as mice exposed to DES had increased ductal growth at 33 days of age and increased dilation of ducts at 12 weeks of age (Hovey et al., 2005). Overall, these data show that EDCs, especially those that simulate increased systemic estrogen, have a significant impact on normal mammary growth and tumorigenesis.

Despite recommendations by the CDC to avoid drinking during pregnancy, women continue to do so. This is evidenced by the proportion of children born with characteristics of Fetal Alcohol Spectrum Disorder (FASD), an umbrella term that describes many of the ramifications of prenatal alcohol exposure (Roozen et al., 2016). Children that fall under this spectrum in the most extreme cases present birth defects including craniofacial abnormalities and growth deficiency to less specific forms of damage to the body and brain that affect the child's quality of life and well-being (Abel and Sokol, 1987, 1991; Hoyme et al., 2005; Roozen et al., 2016). Much of the work on FASD has focused on these immediate cognitive and behavioral abnormalities; however

recently there has been an increased interest in long-term effects of FAE on the offspring. Work from our lab as well as Hilakivi-Clarke has shown that FAE increases risk of mammary tumorigenesis later in life (Hilakivi-Clarke et al., 2004; Polanco et al., 2010). Our data show that females exposed to alcohol *in utero* injected with NMU, to induce mammary tumors, develop more malignant tumors based on ER expression and tumor type classification (Crismale-Gann et al., 2016; Polanco et al., 2010). We also found that mammary glands of FAE rats have increased proliferation in pre-pubertal glands at postnatal day 20. Additionally, serum estradiol levels are higher in FAE pups during proestrus and aromatase expression is increased in mammary glands on PND20 and PND40, before and around puberty (Polanco et al., 2011). These data further support that alcohol may play a role in promoting tumorigenesis through an estrogen-mediated pathway. Another recent study using the MMTV-erbB2 mouse model found that FAE pups had increased ductal extension at 10 weeks of age and upregulation of ERα signaling in the pubertal mammary gland. In this study, tumor development was slightly delayed in FAE animals, but overall tumor multiplicity was greater, suggesting that FAE mediates its effects on tumorigenesis through estrogen and erbB-2 pathways (Ma et al., 2015). Together, these data show that FAE not only has immediate cognitive effects, but can also alter the developing mammary gland, promoting disease later in life.

Mammary gland development

Overview of normal mammary gland development

Mammary gland development begins during embryogenesis around embryonic day 10 (E10) in mice. During embryonic development a single ectoderm layer enlarges to form mammary lines. From E12 – E14 the ectodermal layer becomes mammary placodes, which are the epithelial buds that penetrate the surrounding mesenchyme (Cowin and Wysolmerski, 2010; Richert et al., 2000). Ductal morphogenesis begins around E16, where the epithelial bud grows until it forms

several cell layers and a rudimentary ductal tree. This ductal tree elongates and invades the embryonic mammary fat pad, growing until birth (Richert et al., 2000). A similar developmental sequence occurs in humans, with mammary development beginning at 4 to 6 weeks of gestation with prenatal development of the human breast complete by the end of the third trimester (Javed and Lteif, 2013).

The mammary gland is unique as development mostly occurs postnatally. From birth up through puberty, the mammary gland grows isometrically with the rest of the body. At the onset of puberty mammary gland growth accelerates and grows allometrically with the body with major molecular and cellular changes. At this point in development, several ovarian and pituitary hormones regulate mammary gland development, especially estrogen and growth hormone (GH). Ductal morphogenesis is primarily driven by estrogen (McNally and Stein, 2017). During ductal morphogenesis the mammary ductal tree elongates as terminal end buds (TEB) invade the surrounding mammary fat pad. TEBS contain a top layer of cap cells that drive ductal elongation and branching. Cap cells differentiate into luminal epithelial cells that line the lumen of the mammary ducts and myoepithelial, or basal cells, that surround the luminal cells (Macias and Hinck, 2012; Richert et al., 2000). In humans, pubertal mammary gland development is very similar, with TEBs driving ductal elongation throughout the mammary stroma; however at the completion of pubertal development, lobular structures are evident in human breast tissue, whereas this type of structure is only seen in mice during pregnancy (McNally and Stein, 2017; Silberstein, 2001). GH also plays an important role in ductal morphogenesis through IGF-I. GH acts on stromal cells in the mammary gland, binding to GH receptors that activate IGF-I mRNA expression. IGF-I then acts to stimulate development of terminal end buds that go on to form the adult ductal structure of the mammary gland (Kleinberg et al., 2000). With the fluctuation of hormones in every estrous cycle, the mammary gland continues to proliferate and expand into the fat pad by forming secondary branches that bud from the primary ductal structure. By 10 weeks of age in the mouse and 11-14 years of age in humans, the ductal structures have reached the end

of the mammary fat pad and the terminal end buds stop dividing (Hovey et al., 2002; Macias and Hinck, 2012).

Full development and maturation of the mammary gland does not occur without the influence of pregnancy hormones that cause the mammary gland to undergo extensive remodeling and lobulo-alveolar development to prepare for lactation. These changes begin with the development of additional side branching as well as alveologenesis to form alveolar buds that fill the entire mammary fat pad (Macias and Hinck, 2012; Richert et al., 2000). During the later stages of pregnancy, the alveolar buds differentiate into individual alveoli, which will contain the cells capable of forming milk. At parturition, the mammary gland has achieved functional differentiation, whereby the gland can now produce and secrete milk (McNally and Stein, 2017). Weaning of offspring induces a choreographed apoptosis in the mammary gland. After 48 hours, epithelial cells begin to collapse as a result of apoptosis and extensive remodeling of the remaining cells in the gland (Macias and Hinck, 2012; Radisky and Hartmann, 2009). Extracellular matrix begins to breakdown and proteases aid in the apoptosis of cells. Upon the completion of involution, most of the secretory epithelial cells has been replaced by adipocytes and the gland returns to a prepregnant state. This process is repeated with every new pregnancy and lactation.

Overview of mammary epithelial cell hierarchy

The dynamic capacity of the mammary gland with every pregnancy and lactation is possible through the existence of mammary stem cells (MaSCs). Two hallmark characteristics of adult stem cells are multi-potent differentiation and self-renewal (Tiede and Kang, 2011). Early experiments by DeOme and colleagues found that small pieces of the mammary gland could be transplanted into a cleared fat pad, regenerating an entire ductal tree (Lloyd-Lewis et al., 2017; Young et al., 1971). These results indicated that cells within the gland were capable of differentiating into the multiple epithelial cell lineages found in the mammary gland as well

reconstituting an entire mammary gland through multiple successive transplantations, providing direct evidence that stem cells exist in the mammary gland.

The mammary epithelial cell (MEC) lineage is similar to the characterized hematopoietic lineage where a multi-potent stem cell at the apex asymmetrically divides to produce progeny progenitor cells that function to maintain the mature luminal and myoepithelial cells of the mammary gland (Figure 1). Beginning in the early 2000s, additional studies used more advanced and complex experiments to delineate the MEC hierarchy through flow cytometry, lineage tracing, and transgenic mouse models. Using flow cytometry, several cluster differentiation (CD) cell surface markers have been found to enrich for cells along the MEC lineage (Dontu and Ince, 2015). Although these CD markers are not unique to MECs alone, they have been monumental in characterizing the MEC hierarchy within the gland. Using these cell surface markers, it was first determined in 2006 that stem cells in mice could be enriched based on $CD24^{+}$ (heat stable antigen) and $CD29^{hi}$ (β1 integrin) and $CD49f^{hi}$ (α6 integrin) expression and a single cell was capable of reconstituting an entire mammary gland capable of lobuloalveolar development during pregnancy, when transplanted into the cleared fat pad of a host animal (Shackleton et al., 2006). In mice CD29, or β1 integrin, has been shown to play a role in MaSC maintenance and maintaining a balance between the luminal and basal cell lineages (Visvader, 2009; Visvader and Smith, 2011). CD24 has also been shown to play a role in regulating breast tumor proliferation and invasion (Baumann et al., 2005; Visvader and Smith, 2011). Luminal cells have been characterized based on the expression of CD24 and low expression of CD29 or CD49f in mice. Luminal cells in humans can be identified based on the expression of CD24, CD44, Epcam or CD49f (Visvader and Stingl, 2014). Luminal cells are also distinguishable by their expression of specific proteins, such as cytokeratins 8, 18 and 19, MUC1 and CD133 in both mice and humans (Makarem et al., 2013; McNally and Stein, 2017). Basal/myoepithelial cells are identified by moderate expression of CD24 and high expression of CD29 or CD49f. The MaSC population is

also housed here, although it has been difficult to separate differentiated basal cells from MaSCs due to their similar expression of cell surface markers (Visvader and Smith, 2011). Basal cells express cytokeratin 5, 14 Thy-1 and p63 in mice and humans (Makarem et al., 2013).

Other markers in addition to CD24, CD29 and CD49f described above have served to identify progenitor cells that reside along the cell lineage in mice. CD61 has been shown to identify both ER+ and ER- luminal progenitor cells, which are unable to regenerate a mammary gland *in vivo* but can form spheres in colony forming assays (Vaillant et al., 2008). $CD14^{+}$ and c-$kit^{-/lo}$ expression have been shown to identify colony-forming progenitor cells that form alveolar cells during pregnancy (Asselin-Labat et al., 2011). Sca1+ and c-kit+ expression has been shown to identify a rare subset of ER+ luminal progenitor cells (Regan et al., 2012; Shehata et al., 2012) while cells lacking CD133 and Sca-1 have also been identified as ER- luminal progenitor cells (Sleeman et al., 2007). Together, these markers have served to identify and help visualize the cell hierarchy that resides within the mammary gland.

Breast Cancer

Breast cancer is the second highest cancer-related death in women; it is estimated that 1 in 8 women will develop breast cancer over her lifetime and over 266,000 new cases of breast cancer are expected to be diagnosed in the US (Feng et al., 2018; Siegel et al., 2018). Despite these statistics, incidence rates have been steadily declining since 1989 (Siegel et al., 2018). This is due to advances in research that are elucidating mechanisms underlying the cause of breast cancer, increasing earlier detection as well as identifying treatments such as immunotherapy (Feng et al., 2018).

Overview of Breast Cancer subtypes

Breast cancer is a molecular and phenotypically heterogeneous disease. As such, several subtypes have been identified to classify different types of breast cancer based on

immunohistochemical and molecular markers (Blows et al., 2010; Network, 2012). Additionally, studies nearly twenty years ago first identified several gene clusters that identified and characterized these breast cancer subtypes (Perou et al., 2000; Sørlie et al., 2001). The four main breast cancer subtypes are Luminal A, Luminal B, HER2-enriched and triple-negative/basal-like breast cancer (Feng et al., 2018). Each subtype is characterized based on hormone receptor positivity, patient prognosis, and therapeutic targets and more recently has been associated with specific cell of origins within the mammary cell lineage. This cell of origin hypothesis may be helpful in clinical prognosis of tumor subtypes, although more extensive research needs to be completed as the role of different cell types in tumor subtype formation is not as well understood.

The luminal A breast cancer subtype is classified based on estrogen-receptor (ER) and/or progesterone (PR) positivity but no HER2 expression. Luminal B breast cancer subtypes are also positive for either ER and PR but can also be positive for HER2 and have high expression for the proliferation marker, Ki-67. Although the data remains limited, studies suggest that the Luminal A tumor profile contains a gene signature of mature luminal cells, while the Luminal B subtype gene signature was found to be most similar to bipotent progenitor cell types (Liu et al., 2016; Visvader and Stingl, 2014) The luminal A subtype harbors many gene mutations; the most frequent in PIK3CA, along with mutations in *MAP3K1*, *GATA3*, *TP53*, *NCOR1*, and *RUNX1*. Luminal B tumors also harbor several gene mutations, with the most frequent being TP53, PIK3CA and PTEN (Network, 2012; Visvader and Stingl, 2014).

HER2-enriched tumor subtypes make up about 10-15% of all breast cancers. These tumors do not express ER or PR but have high expression of HER2 (ERBB2) and proliferation markers (Baselga and Swain, 2009). HER2 positive tumors also arise from a luminal cell of origin that acquired the HER2 mutation (Visvader, 2009). The most significantly mutated genes found in HER2 tumors include TP53, PIK3CA, PIK3R1, PTEN, and Cyclin D1 (Network, 2012). Since HER2 tumors express markers of proliferation, they are typically more aggressive than Luminal subtype tumors and have a worse prognosis. However, many targeted therapies aimed at HER2

positive tumors have been successful with the use of monoclonal antibodies such as Herceptin (Trastuzumab) and Perjeta (pertuzumab), tyrosine kinase inhibitors and HSP90 inhibition (Baselga and Swain, 2009).

Basal-like or triple-negative breast cancer is negative for ER, PR and HER2. It accounts for approximately 20% of all breast cancers and has the worst prognosis for patients (Sørlie et al., 2003). Interestingly, despite this subtype name suggesting it originates from basal cells, basal-like breast cancer has a gene signature most closely related to a luminal progenitor cell of origin (Visvader and Stingl, 2014). In basal-like breast cancers, TP53 mutations occur in nearly 80% of all cases, PIK3CA is the next most commonly mutated gene, usually resulting in high PI3K/Akt pathway activity, as well as mutations in RB1, Cyclin E1 and CDK2NA (Network, 2012). Unfortunately, current treatment for triple-negative breast cancer is limited to chemotherapy. However, several new targets may be excellent candidates for new treatment options, such as targeting the EGF receptor or DNA damaging agents for basal tumors with BRCA1 mutations (Reis-Filho et al., 2006; Robson et al., 2003; Yehiely et al., 2006).

Cancer stem cell theory

The Cancer Stem Cell (CSC) theory postulates that within a tumor there are stem cells capable of producing and maintaining the cancer. The concept of CSCs first arose in the 1930s, where cells from leukemic tumors were shown to initiate new tumors when injected in host mice (Clevers, 2011). Then, several decades later, Dick and colleagues found that in acute leukemia, only a small subset of cells were able to initiate leukemia when transplanted into immunodeficient mice (Lapidot et al., 1994). This same group found that human myeloid leukemia was organized in a hierarchy similar to that found in the normal hematopoietic system and therefore it could be said that CSCs were the initiating cell of origin for this specific cancer (Bonnet and Dick, 1997).

The first evidence of CSCs in solid tumors was from a seminal 2003 paper that investigated the existence of such tumor-initiating cells in human breast tumors. In this study, human breast

cancer cells labeled as $CD44^{+}CD24^{-}$ produced tumors when xenografted in nude mice. This $CD44^{+}CD24^{-}$ population was shown to not only have increased tumorigenic properties, but could also be isolated and xenografted into mice a second time, showing that this population of cells could initiate and maintain tumor growth (Al-Hajj et al., 2003). Additionally, only a small number of $CD44^{+}$ CSCs were necessary to initiate tumor growth, showing that CSCs were a small and rare population of cells within tumors. Since then, many other studies have been able to identify CSCs in various mouse models of breast cancer (Liu et al., 2007; Ma et al., 2012; Zhang et al., 2008). In addition to the various cell surface markers that have been found to enrich for CSCs, many experimental systems have been established to characterize them. These assays have been designed to primarily test the self-renewal and differentiation potential of tumor cells. The nonadherent sphere forming assay is based on the idea that CSCs will be capable of surviving in suspension and forming a tumor sphere. In another, similar assay, tumor cells are plated in Matrigel and their ability to form tumor colonies taken as a measure of progenitor activity. The ADELFLUOR assay, which measures aldehyde dehydrogenase activity, has also been shown to serve as a good marker to identify CSCs (Liu and Wicha, 2010). However, the transplantation assay serves as the gold standard assay to determine the CSC traits of a particular cancer cell (Lindeman and Visvader, 2010).

One issue with this theory is the concept that a single stem cell population in the gland is responsible for the formation of breast cancer. This oversimplifies the concept, as there has been much debate over the specific cell of origin for breast cancer. It has been thought that other cells along the stem cell hierarchy have plasticity, acquiring oncogenic mutations and reverting to a stem cell state to initiate the formation of tumors (Chaffer and Weinberg, 2015; Clevers, 2011). Several studies have shown that changes in molecular pathways that maintain the normal stem cell population or differentiated state can cause cells to revert to a stem-like state (Chaffer et al., 2011; Iliopoulos et al., 2011). The idea of a stem-like cell responsible for tumor formation is still

relevant, however the theory has now been revised to include other possible populations of cells in the gland that may possess tumor-initiating properties. As a result, some investigators prefer to use the term tumor-initiating cells (TICs) to indicate cells that serve to maintain the tumor while producing differentiated cells that cannot self-renew but form the bulk of the tumor (Ercan et al., 2011; McDermott and Wicha, 2010).

Regardless of which cell specifically becomes the CSC/TIC within a tumor, there is still interest in the clinical implications of this specific population of cells. Substantial improvements in cancer treatment and detection have improved survival rates. However, there are many challenges in cancer treatment, not only the obvious question of what the root cause is for most cancers, but also how to address cancer heterogeneity and chemoresistance. CSCs are suggested to have drug resistance through ATP binding cassette and multi-drug resistance transporters as compared to their normal counterparts (Ebben et al., 2010). Studies using breast cancer cell lines have shown that breast cancer stem cells are resistant to radiation and chemotherapy (Dave and Chang, 2009). In a study where *BRCA1/p53* mouse tumors were treated with cisplatin, a cytotoxic chemotherapy drug, despite an initial positive response with this treatment, tumor relapse and refraction increased and it was found that these tumors had an increased putative CSC subpopulation based on $CD29^{hi}CD24^{mod}$ expression (Shafee et al., 2008). Additionally, CSCs also seem to be resistant to DNA damage through enhanced DNA repair mechanisms and increased resistance to reactive oxygen species as shown in glioma and breast cancer cells (Bao et al., 2006; Diehn et al., 2009). There has been extensive research on the molecular pathways that regulate CSCs and these have become targets for tumor treatment in breast cancer. Recent clinical trials have taken advantage of these known molecular pathways and targeted specific aspects of the signaling pathways to determine their effects on (Lin et al., 2016). If targeting CSCs will reduce the rate of tumor recurrence and metastasis, then targeting these cells can provide a breakthrough in cancer treatment and improve patient outcome and survival.

Molecular regulation of the mammary epithelial cell lineage

There has been an increased interest in understanding the role of signaling pathways in the self-renewal and multi-potent properties of stem cells. As a result, several signaling pathways that regulate normal mammary gland development were also found to regulate stem cell function (Figure 2). Tumorigenesis can arise when these pathways go awry. Therefore, by understanding the normal molecular regulation of stem cell function, we can begin to understand how in the wrong context these pathways can contribute to the development of cancer.

Overview of Wnt signaling pathway

The Wnt signaling pathway plays a pivotal role in development across many species. This pathway consists of three separate activation methods: the canonical Wnt pathway, the non-canonical Wnt pathway and the non-canonical Wnt/calcium pathway. The mammalian Wnt family consists of 19 glycoprotein members. These glycoproteins bind to the frizzled family of 7-pass transmembrane receptors. There are also co-receptors such as low-density lipoprotein receptor-related protein termed LRP5/6 and non-canonical co-receptors such as Ryk and Ror (Clevers et al., 2014; Ring et al., 2014; Yu et al., 2016). The canonical pathway is activated upon binding to a frizzled receptor, this activates the scaffold protein Dishevelled. When this pathway is not activated, β-catenin is phosphorylated and degraded. The activation of Dishevelled disrupts the β-catenin destruction complex, which prevents the phosphorylation of β-catenin, allowing it to accumulate in the cytoplasm and translocate into the nucleus. β-catenin then activates the expression of Wnt target genes through the formation of a complex with T-cell factor/lymphoid enhancer factor transcription factors (Yu et al., 2016).

Although these various Wnt signaling pathways play critical roles in embryonic development, the canonical Wnt/β-catenin signaling pathway is known to function in stem cell regulation in various tissues including the mammary gland. The Wnt signaling pathway plays a role in cell fate specification and maintenance of stem cells. Wnt signaling increases the self-renewal capacity of

MaSCs as well as their ability to reconstitute a mammary gland when transplanted after several passages (Zeng and Nusse, 2010). Inactivation of the Wnt-controlled transcription factor LBH affects mammary gland tissue expansion during puberty and pregnancy as well as decreases the proportion and activity of MaSCs (Lindley et al., 2015). Basal mammary cells have been shown to express the canonical Wnt signaling receptors Lrp5 and 6 (Ring et al., 2014). Knockdown of these two receptors has shown a decrease in stem cell activity as well as observable decreased ductal branching (Badders et al., 2009; Lindvall et al., 2009). Lineage-tracing experiments found that mammary stem cells are driven by Wnt signaling as they can be labeled based on *Lrg5* and *Axin2* expression, thereby providing evidence that Wnt signaling controls stem cells (Clevers et al., 2014; de Visser et al., 2012; van Amerongen et al., 2012; Van Keymeulen et al., 2011).

Wnt signaling is known to promote tumorigenesis when signaling goes awry. Dysregulated Wnt signaling has been found in colon cancer as well as many other cancers including the breast (Bergstein and Brown, 1999; Howe and Brown, 2004; Polakis, 2000). This effect is largely due to the role of Wnt in stem cell maintenance. When this balance is challenged, stem cell regulation and maintenance are affected, altering differentiation and self-renewal capacities of these cells, leading to the development of TICs and tumor formation (Sacchetti et al., 2013). An example of the effects of altered Wnt signaling is in the MMTV-Wnt1 mouse model, which exhibits mammary ductal hyperplasia and tumor development due to the overexpression of Wnt1 (Li et al., 2003; Many and Brown, 2014; Tsukamoto et al., 1988). As described above, overexpression of Wnt1 activates the canonical Wnt signaling pathway and leads to the accumulation of β-catenin, turning on the activation of genes that promote proliferation (Cho et al., 2008). This also leads to the accumulation of stem/progenitor cells, as evidenced by the increased expression of molecular markers for stem cells that are found in the mammary glands of this model as well as transplantation assays (Li et al., 2003; Liu et al., 2004). As a result of this accumulation of basal/stem cells, mammary tumors spontaneously form in approximately 50% of animals by 6

months of age, with most succumbing to tumors by 1 year of age (Li et al., 2000). These data show that increased Wnt signaling promotes tumorigenesis.

Several other animal studies have contributed to the understanding of Wnt signaling in mammary tumorigenesis (Chakrabarti et al., 2014; Huang et al., 2008; Huang et al., 2005; Lindvall et al., 2006; Zhang et al., 2010). In humans, breast cancer cells and patient-derived samples exhibit increased Wnt signaling as compared to normal breast tissue (Lamb et al., 2013). When breast cells were treated with Wnt1, cells survived longer, formed non-adherent spheres and formed more tumors when injected into mice as compared to control breast cells (Ayyanan et al., 2006). Additionally, about 50% of breast cancer cases have high levels of β-catenin (Feng et al., 2018; Howe and Brown, 2004; Lin et al., 2000). Constitutive activation of the canonical Wnt signaling pathway is commonly found in cases of triple-negative breast cancer and can increase the risk of metastases to the lung and brain (Dey et al., 2013).

Overview of Notch signaling pathway

The Notch gene family consists of 4 transmembrane receptors that interact with 5 membrane-bound ligands from the Delta/Jagged gene family. When a ligand binds to one of these 4 receptors, this initiates a proteolytic cleavage of the receptor by Disintegrin Metalloproteases (ADAM), forming a cleaved form of Notch termed Notch Extracellular Truncation (NEXT) that becomes a substrate for γ-secretase. The cleavage of NEXT by γ-secretase forms a Notch intracellular domain (NICD) that translocates into the nucleus (Kopan and Ilagan, 2009; Raafat et al., 2011). NICD goes into the nucleus and associates with DNA binding proteins which forms a complex and activates target genes such as those from the Hes and Hey family (Kopan and Ilagan, 2009).

Notch signaling has been shown to play a role in cell proliferation, cell death and cell fate specification in the mammary gland. Activated Notch signaling promotes the differentiation of MaSCs into cells of the luminal lineage (Chakrabarti et al., 2012). In this study, Elf5-null mice

exhibit a loss of Elf5 in the mammary gland, which increased Notch signaling and the proportion of $CD61^+$ luminal progenitor cells (Chakrabarti et al., 2012). Alternatively, knockout of the Notch related transcription factor Rbpj in mammary progenitor cells altered cell fate specification and lead to the increase of proliferating basal cells (Buono et al., 2006). *In vitro*, treatment with a Notch activating peptide promoted an increase in mammosphere formation. In this same study, mammospheres with increased Notch signaling also had a greater number of multi-potent cells, based on their ability to form multilineage colonies in collagen (Dontu et al., 2004). Together these data indicate that the Notch pathway plays a significant role in cell fate within the adult mammary gland.

In breast cancer, overactivation of Notch signaling promotes tumorigenesis. Notch1 was shown to interact with a chemokine, CCR7, to promote stemness and increase mammary tumor progression in the MMTV-PYMT model (Boyle et al., 2017). Transgenic mice overexpressing intracellular Notch1 enriched for the basal $CD24^+CD29hi$ cell population in the mammary glands and correlated with an increased number of tumors formed (Ling et al., 2010). In human breast cancers, tumors with high expression of Jag1 or Notch1 correlated with poorer patient outcome (Feng et al., 2018; Reedijk et al., 2005). Several studies have shown that high expression of Notch1 or ligands such as Dll4 and Jag2 are found in triple negative breast cancer, promote metastasis and increase self-renewal of TICs (Kontomanolis et al., 2014; Xing et al., 2011). Overall, these data show that dysregulation along various points in the Notch signaling pathway can promote breast tumorigenesis. Fortunately, this can be a promising area for cancer therapeutics. Inhibition of Notch signaling has shown promising results; gamma-secretase inhibitors have been shown to reduce the proportion of breast CSCs as well as their mammosphere forming efficiency (Grudzien et al., 2010). Anti-Notch1 antibodies have been successful in depleting tumors of CSC populations, thereby increasing anti-tumor efficacies and

offering a promising approach to targeting breast cancer related notch signaling (Qiu et al., 2013; Sharma et al., 2012).

Overview of Hedgehog signaling pathway

The hedgehog signaling pathway regulates organogenesis, cell fate and proliferation, survival and stem cell self-renewal. Canonical hedgehog signaling works through several ligands and receptors to regulate specific target genes. In the absence of a ligand – such as sonic hedgehog, desert hedgehog and Indian hedgehog – the 12-transmembrane receptors Ptch1 and Ptch2 inhibit Smoothened (Smo). This inhibition allows a multiprotein complex in the cytoplasm to keep two transcription factors of the Gli family (Gli2 and Gli3) phosphorylated and cleaved; therefore, their activity is inhibited (Monkkonen and Lewis, 2017; Visbal and Lewis, 2010). When the pathway is activated through ligand binding, the inhibition of Smo is released and can move to the cell surface. Gli transcription factors are no longer inhibited and remain full length, allowing them to translocate to the nucleus. These transcription factors can then go on to activate genes that regulate survival and proliferation (Visbal and Lewis, 2010).

Hedgehog signaling plays many roles in normal organ development in a variety of tissues; however, there is evidence indicative of its role in adult stem cell maintenance as well. Hedgehog signaling is normally activated in stem/progenitor cells and is downregulated as the cell differentiates (Luo et al., 2010). Mammosphere cultures from normal human breast tissue has shown that Ptch1, Gli1 and Gli2 are highly expressed in mammary stem and progenitor cells compared to more differentiated cells. Activation of canonical hedgehog signaling increases the number of mammospheres formed as well as size while inhibition of this pathway reverses these effects (Liu et al., 2006). The predominant form of p63, ΔNp63, known to play a role in stem cell maintenance, was found to regulate stem cells through the hedgehog signaling pathway. More specifically, ΔNp63 was found to regulate Shh, Gli2 and Ptch1 to promote and maintain the self-renewal potential of mammary cancer stem cells (Memmi et al., 2015). The overexpression of

Smo driven by the MMTV promoter in a transgenic mouse model increased mammosphere forming efficiency in transgenic animals as compared to normal wild type. Interestingly, a decrease in cells capable of regeneration was observed based on limiting-dilution transplantation assays, indicating that overexpression of Smo decreases the number of regenerative stem cells but increases the number of cells capable of proliferating in anchorage-independent conditions (Moraes et al., 2007). Together, these studies show that hedgehog signaling plays a critical role in normal stem cell maintenance and function.

Hedgehog signaling has also been shown to play a role in tumorigenesis. Triple negative breast cancer frequently exhibits altered hedgehog signaling (Hui et al., 2018). When treated with Thiostrepton, the proportion of $CD44^{+}CD24^{-}Lin^{-}$ breast cancer stem cell population is reduced as well as the sphere forming capabilities of these cells in TNBC cell lines. This attenuation was mediated through the hedgehog signaling pathway, suggesting a role of hedgehog signaling in cancer stem cell maintenance and function (Yang et al., 2016). The Lin^{-} $CD44^{+}CD24^{-}$ breast cancer cell population has also been shown to exhibit increased expression of Ptch1, Gli1 and Gli2 mRNA (Liu et al., 2006). Ptch and Gli1 expression in tumors is indicative of poorer patient outcome, increased tumor size and increased lymph node metastasis (Zhao et al., 2016).

Overall, it is evident that alcohol consumption influences the risk for breast cancer in both pre and postnatal life. However, for women born from mothers who drank, there is a lack of suitable prevention strategies. By further understanding the molecular mechanisms and pathways that underlie this effect, specific biomarkers or molecular targets of interest could be developed and applied as a more effective treatment strategy.

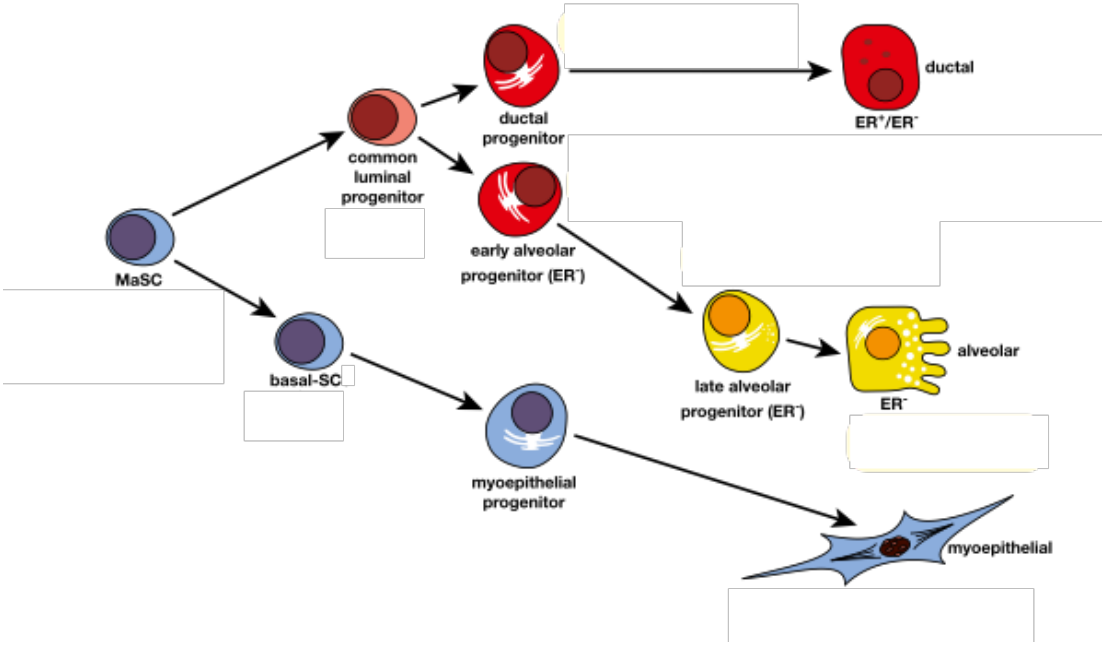

Figure 1. Overview of mammary epithelial cell hierarchy. Schematic diagram of the proposed mammary epithelial cell hierarchy, with mammary stem cells (MaSC) at the apex of the lineage, followed by progenitor cells which produce the mature epithelial cells of the mammary gland.

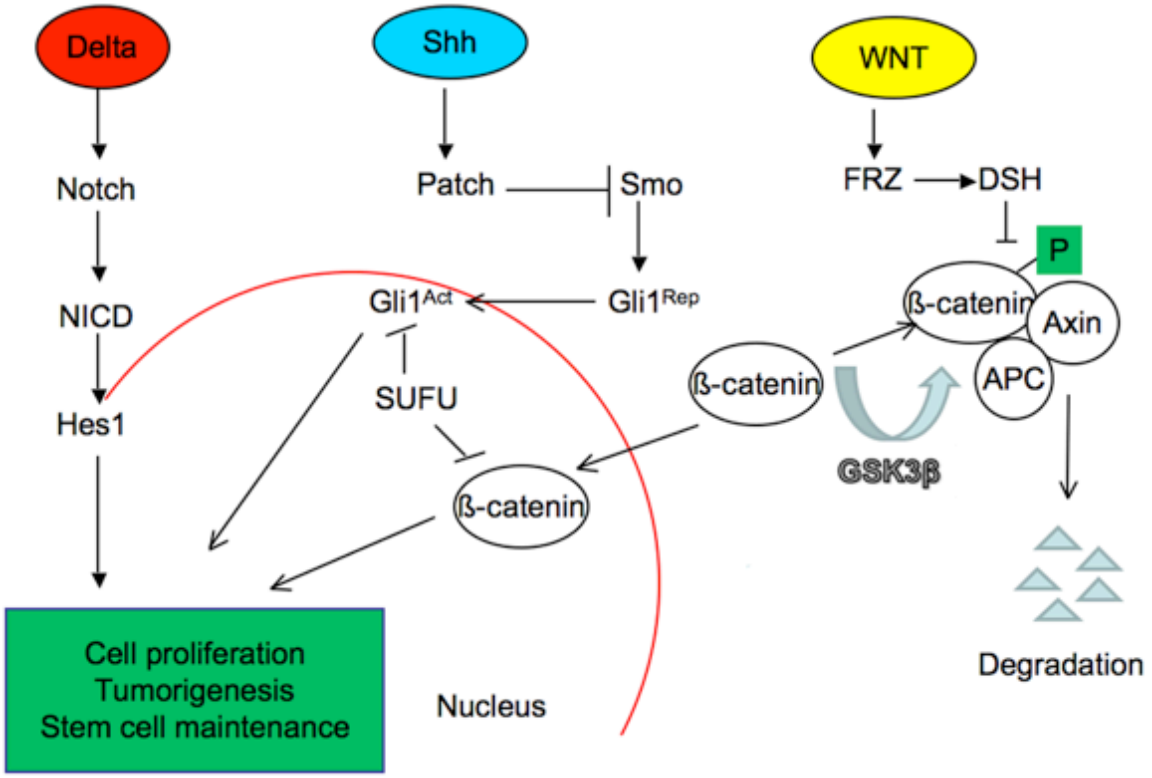

Figure 2. **Molecular pathways that regulate stem cells**. Schematic diagram of Notch (Delta), Hedgehog (Shh) and Wnt pathways that are known to play a role in stem cell maintenance.

Objectives

A growing number of studies have found that alcohol can affect both embryonic and adult stem cells in a number of tissues (Di Rocco et al., 2019). In embryonic stem cells, alcohol can inhibit differentiation (Wang et al., 2017). In adults, alcohol induces the proliferation of liver progenitor cells, worsening liver disease (Sancho-Bru et al., 2012). Although the relationship between alcohol and stem cells has not been thoroughly investigated in the mammary gland, a few studies have shown that alcohol may have a deleterious effect on the mammary stem cell population. For example, alcohol exposure increases the CSC population as well as stem cell-related genes in MCF-7 cells (Gelfand et al., 2016; Xu et al., 2015). A similar study using the MMTV-*neu* mouse model found that direct alcohol exposure increases CD44+ cells and metastases to the lung and colon (Xu et al., 2016). Our previous studies found that FAE increases susceptibility to mammary tumorigenesis (Crismale-Gann et al., 2016; Polanco et al., 2010). However, to date, the effects of FAE on mammary cell composition has not been investigated. Therefore, we hypothesized that FAE increases susceptibility to tumorigenesis by altering the mammary cell composition, shifting it towards one that promotes tumorigenesis.

The goal of this work was to investigate perturbations within MEC subpopulations of normal and preneoplastic glands that may occur from FAE during critical embryonic mammary development. The first objective of this work was to investigate the role of FAE on the MEC composition in the normal, wild type (WT) mammary gland. To study this effect, MEC subpopulations of WT FVB/NJ offspring were evaluated at 5 (puberty) and 10 (adult) weeks of age. Gene expression analysis was conducted to elucidate a possible mechanism by which this may occur. To explore the relationship between mammary cell lineage equilibrium and tumor development, the second objective of this work was to investigate the impact of FAE on transgenic (Tg) MEC subpopulations and tumorigenesis, using the MMTV-Wnt1 mouse model.

Alterations in the MEC composition of hyperplastic MMTV-Wnt1 glands were investigated prior to tumor formation. Tumor development was monitored and upon harvest tumors were analyzed by flow cytometry, sphere formation and histological analysis. Overall, these studies provide a potential mechanism by which FAE promotes mammary tumorigenesis.

Chapter 2

Fetal alcohol exposure alters mammary epithelial cell subpopulations and promotes tumorigenesis

Introduction

Breast cancer is one of the most commonly diagnosed cancers in American women, with 1 in 8 women predicted to develop breast cancer over their lifetime and an estimated 266,120 new cases diagnosed in 2018. There are many known risk factors for breast cancer, and while many of these, such as family history, race, early menarche, and late menopause, cannot be controlled, other risks, such as alcohol consumption, can be managed through lifestyle changes (Feng et al., 2018). Much of breast cancer research has focused on adulthood, but several studies suggest that the risk for breast cancer may begin during fetal development (Hilakivi-Clarke and de Assis, 2006; Simmen and Simmen, 2011; Soto et al., 2008). For example, maternal exposure to diethylstilbestrol (DES) or a high fat diet in rats increases the risk for tumorigenesis in their offspring (Hilakivi-Clarke et al., 1997; Kawaguchi et al., 2009). In the context of alcohol, studies have indicated that fetal alcohol exposure (FAE) promotes tumorigenesis in rodent models (Crismale-Gann et al., 2016; Hilakivi-Clarke et al., 2004; Ma et al., 2015; Polanco et al., 2010). These statistics become particularly alarming when considering that half of women of childbearing age report drinking and 18% of these women binge drink; additionally, 11.5% of women admit to drinking during pregnancy (Denny et al., 2019; Tan et al., 2015).

In recent years, a considerable amount of research has focused on the role of stem and progenitor cells in normal tissue development and breast cancer (Fu et al., 2017; Luo et al., 2010; Oakes et al., 2014; Van Keymeulen et al., 2011). Researchers have found that the gene expression

profiles of certain mammary epithelial cell (MEC) subpopulations are associated with specific breast cancer subtypes, suggesting each has a different cell of origin (Lim et al., 2009; Prat and Perou, 2011). Breast cancer is a heterogeneous disease composed of several subtypes that are characterized by cellular composition and general response to therapy. Examining these cellular hierarchies within each subtype can further elucidate the relationship between normal mammary cell counterparts and their tumor subtypes to help develop new cancer treatments.

Exposure to various exogenous factors *in utero* or during adulthood can alter MEC subpopulations in the mammary gland and subsequently affect tumor incidence in mouse models (Chang et al., 2012; Kim et al., 2013; Rahal et al., 2013; Wang et al., 2014). In addition, embryonic mammary stem cells (MaSC) contribute to the fate of adult MaSC, suggesting that changes in this embryonic cell population can contribute to long-term effects in the mature gland (Boras-Granic et al., 2014; Spike et al., 2012). Data from our lab indicates that FAE affects normal mammary gland growth early in development and alters tumor susceptibility in adulthood (Polanco et al., 2011; Polanco et al., 2010). Therefore, the overall goal of the present work was to evaluate the effects of FAE on normal wild type (WT) and hyperplastic transgenic (Tg) MMTV-Wnt1 MEC subpopulations and their role in promoting tumorigenesis. The MMTV-Wnt1 mouse model spontaneously develops mammary tumors due to the overexpression of Wnt1 driven by the MMTV-LTR (Cho et al., 2008; Tsukamoto et al., 1988). Transgenic mice exhibit hyperproliferative glands from birth, with an expansion of progenitor cells in preneoplastic glands that result in a mixed lineage tumor with an accumulation of tumor initiating cells (Cho et al., 2008; Kim et al., 2011). In the present study, we tested the hypothesis that FAE promotes tumorigenesis in adult progeny through effects on the MEC composition, shifting it towards one that promotes tumorigenesis. Results demonstrate that FAE promotes decreased tumor latency and can shift both normal and preneoplastic MEC subpopulations prior to tumor formation, suggesting a possible mechanism underlying the increased susceptibility to tumorigenesis.

Materials and Methods

Animals and treatment

All animal protocols were approved by Rutgers University Institutional Animal Care and Use Committees (IACUC 15-023). Animals were housed in a controlled environment with a 12-hour light/dark cycle. The MMTV-Wnt1 line on an FVB background (FVB.Cg-Tg(Wnt1)1Hev/J) was obtained as a gift from Dr. Pamela Cowin with permission from The Jackson Laboratories. Female WT FVB/NJ mice (JAX stock #01800) were bred to either WT FVB/NJ males or Tg MMTV-Wnt1 male mice starting at 8 weeks of age and checked for seminal plugs each morning. The day a plug was identified was considered gestational day (GD) 0. On GD9, pregnant dams were randomly assigned to the alcohol or control group. In lieu of water, alcohol dams were given ad-lib access to 5% alcohol in 0.2% saccharin solution for two days and then 10% alcohol in 0.2% saccharin until birth on GD19. Control dams were given ad-lib access to 0.2% saccharin solution from GD9 through birth. The amount consumed by each dam was measured every 24 h. Treatment stopped when dams gave birth and pups remained with the dam until weaning on postnatal day (PND) 21. Litters were normalized to 5 to 9 pups. Pup weight was recorded every three days until weaning, following which body weights were recorded once a week until the end of the study. WT and Tg offspring (n = 10-13) were sacrificed at 5 and 10 weeks of age and mammary glands were harvested (Figure 1).

A separate group of Tg female offspring (one offspring from each of 10-11 dams) were monitored for tumor development. Beginning at 6 weeks of age mice were palpated twice a week for tumors. Tumor growth was measured every 3-4 days using a caliper and mice were sacrificed when the tumor reached 1-1.5 cm in diameter (Figure 1). Primary tumors were harvested and divided in half, with one half fixed in 10% neutral buffered formalin (NBF; ThermoFisher;

Waltham, MA) and the other used for isolation of tumor epithelial cells. At harvest, lungs were drop fixed in 10% NBF to determine the occurrence of lung metastases by histological analysis.

Blood alcohol levels

A separate group of animals (n = 17) were given 10% ethanol with 0.2% saccharin for 1 week. Trunk blood was collected 2 to 4 h after lights off and allowed to clot for 30 min at room temperature then spun at 1500 x g for 10 min at 4°C. Serum was stored at -20°C until analysis. Blood alcohol levels were measured using an Analox Alcohol Analyzer (Analox instruments).

Mammary epithelial cell isolation

At sacrifice, left and right thoracic and inguinal mammary glands were harvested and lymph nodes were removed. For Tg offspring, glands from one female per dam were analyzed while for WT offspring, glands from 2 littermates per dam were pooled. MEC isolation followed procedures as previously described (Prater et al., 2013). All reagents were from Stem Cell Technologies unless otherwise noted. Glands were placed into 15 ml conicals containing a 1X collagenase solution [DMEM/F12 media (Hyclone; Pittsburgh, PA) with 50 μg/ml gentamicin (VWR Amresco; Dublin, Ireland) containing 1 mg/ml collagenase and 10 U/ml hyaluronidase] and dissociated on a rotating incubator at 37°C overnight for 15 h. After dissociation, red blood cells were lysed with a 1:4 mixture of HBSS/2% FBS (HF; Hyclone; Pittsburgh, PA and Atlanta Biologicals; Flowery Branch, GA, respectively) and 0.8% ammonium chloride, resulting in a suspension of mammary organoids. A single cell suspension was obtained by pipetting organoids in 0.25% pre-warmed trypsin-EDTA for 1 min, followed by the addition of 10 ml HF. Cells were spun for 10 min at 1400 x g at 4°C and resuspended in pre-warmed 5 mg/ml dispase plus 1 mg/ml DNAse I and pipetted for 1 min followed by addition of 10 ml of HF and filtered through a 40 μm mesh cell strainer (VWR; Dublin, Ireland). Freshly isolated cells were immediately stained

for flow cytometry analysis, as well as plated for mammosphere culture. Remaining cells were stored at -80 C for qRT-PCR analysis.

Tumors were minced with scissors or blades into a paste-like consistency and dissociated in a rotating incubator at 37°C for 2 h in DMEM/F12 containing 3000 U/ ml collagenase with 1000 U/ml hyaluronidase and 50 μg/ml gentamycin (VWR Amresco). After dissociation, red blood cells were lysed in ammonium chloride as described above. At this point, tumor organoids were frozen in DMEM/F12 media containing 10% FBS and 10% DMSO (Sigma; St. Louis, MO) for a slow freeze and stored at -80°C until further use. Upon thawing, organoids were resuspended in 10 ml pre-warmed DMEM/F12 media and spun at 1200 x g for 10 min and further dissociated into a single cell suspension as described above.

Flow cytometry analysis

Antibodies used for flow cytometry were titrated to determine the optimal concentration (for more information see Appendix). Approximately 0.5-1 x 10^6 freshly isolated mammary or tumor epithelial cells were resuspended in HF. MECs were labeled with the following antibodies, all from BD Pharmingen (San Jose, CA) unless otherwise stated: biotinylated anti-CD31 (1:100, clone 390), anti-CD45 (1:100, clone 30-F11) and anti-TER119 (1:100, clone TER-119), anti-CD24-FITC (1:400, clone M1/69), anti-CD49f-PE-Cy7 (1:200, clone GoH3; BioLegend; San Diego, CA), anti-CD61-PE (1:1000, clone 2C9.G2; Biolegend; San Diego, CA) and streptavidin PerCP-Cy5.5 (1:100). Tumor epithelial cells were similarly labeled with the following exceptions: biotinylated anti-CD31, anti-CD45, and anti-TER119 were used at 1:800 dilutions, anti-CD29-PE (1:160, clone HM B-1) was used instead of anti-CD49f, and anti-CD61-AlexaFluor 647 (1:800, clone 2C9.G2) and streptavidin APC-Cy7 (1:800) were used. Cells were incubated with biotinylated antibodies for 30 min in the dark on ice, then washed with HF and incubated with remaining antibodies and streptavidin for an additional 30 min. After washing,

cells were resuspended at a concentration of 1 x 10^6 cells/ml and at least 100,000 events were acquired for analysis. DAPI (Invitrogen; Waltham, MA) was added just prior to analysis to label dead cells. Single color controls were used to set up compensation at the beginning of each experiment. Data acquisition was performed using a Beckman Coulter Gallios flow cytometer. Gating was based upon single color staining and fluorescence minus one controls. The gating strategy is presented in Figure S1. Data were analyzed using FlowJo software (Tree Star, Inc.).

Mammosphere and tumorsphere formation

To assess mammosphere forming efficiency (MFE), freshly isolated MECs were plated in triplicate on 6-well ultra-low attachment plates (Corning) in serum-free DMEM/F12 medium containing B27 (without vitamin A; Gibco, Waltham, MA), 10 ng/ml epidermal growth factor (Stem Cell Technologies; Vancouver, BC), 10 ng/ml basic fibroblast growth factor (Stem Cell Technologies), 10 μg/ml heparin (Stem Cell Technologies) and 50 μg/ml gentamycin (VWR Amresco; Dublin, Ireland). MECs were plated at 10,000 cells/well in triplicate and cultured for 7 days to allow for sphere formation. Fresh media (1 ml) was added every 3 days. After 7 days in culture, mammospheres larger than 50 μm were counted. MFE was calculated by dividing the number of mammospheres formed by the number of single cells plated. Spheres were passaged by pooling spheres from triplicate wells and dissociating with 0.05% trypsin-EDTA (Gibco) for 10 min at 37°C. Single cells were counted and plated at 5,000 cells/well to monitor secondary and tertiary mammosphere formation.

To assess tumorsphere forming efficiency, freshly isolated tumor cells were plated on 6-well ultra-low attachment plates without serum as described above with the following modification: primary tumor cells were plated at 100,000 cells/well in triplicate. Spheres were dissociated and plated an additional two times at 5,000 cells/well to monitor secondary and tertiary mammosphere formation. For all assays, tertiary mammosphere formation was analyzed.

RNA isolation and analysis

Total RNA was purified from isolated MECs using the Qiagen RNeasy micro kit according to the manufacturer's directions (Qiagen; Germantown, MD). RNA quantity and quality were assessed using the Nanodrop ND-100 (Thermo Scientific; Waltham, MA) and Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano kit (Agilent Technologies; Santa Clara, CA), respectively. RNA (500 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies; Waltham, MA) and used for RT-PCR analysis. Primers were validated as previously described (Agostini-Dreyer et al., 2015) using a pool of RNA obtained from isolated MECs.

qRT-PCR was performed as previously described (Agostini-Dreyer et al., 2015) with the following modification: cDNA samples were diluted 1:20 or 1:200 based on the relative standard curve established for each gene during primer validation. Samples were analyzed by determining the fold-change relative to the calibrator using the $2^{-\Delta\Delta Ct}$ method. The calibrator was a cDNA pool of 2-3 cDNA samples from each treatment group. Cyclophilin A was used as the housekeeping gene. Primer sequences are presented in Table 1.

Mammary gland whole mount analysis

Left or right inguinal mammary glands were harvested from both genotypes and treatments at 5 and 10 weeks of age and processed as previously described (Stires et al., 2016). The number of terminal end buds (TEB) was determined in whole mounts from animals at 5 weeks of age by counting the bulb-like structures at the ends of ducts in the gland. Digital images of whole mounts were obtained using a Leica MDG41 stereomicroscope equipped with a camera and Leica Acquire software. Ductal elongation was analyzed in FIJI by measuring the length of ductal

growth from the distal end of the lymph node to the edge of the epithelial structure in the fat pad. Mammary epithelial area was quantified using FIJI image threshold, highlighting the epithelial structure and calculating the area of that structure in the gland.

Tissue histology and immunohistochemistry (IHC)

Mammary tumors and lungs were drop fixed in 10% NBF for at least 24 h, then subsequently washed in PBS 3 times for 20 min. After washing, fixed tissues were dehydrated in increasing concentrations of ethanol (70%, 80%, 95%, 100%) and xylene twice for 10 min. Tissues were then placed in a 1:1 mix of xylene and paraffin for 1 h and then placed in fresh paraffin overnight and embedded the following morning. Tissues were sectioned at 5 μm thickness and placed on Superfrost® Plus microscope slides (VWR). Slides were baked for 15 min at 60°C and then deparaffinized using xylene and rehydrated through decreasing concentrations of ethanol. Tissue sections were then stained with hematoxylin followed by eosin, dehydrated with increasing concentrations of ethanol followed by xylene, then mounted using Permount (Fisher Scientific; Waltham, MA).

Tumor IHC for estrogen receptor (ER)-α and progesterone receptor (PR) was performed as previously described (Crismale-Gann et al., 2016; Stires et al., 2016). Rabbit ERα primary antibody (MC20; Santa Cruz Biotechnology; Dallas, TX) was diluted to 1:500 in 1% BSA in PBS, respectively, while mouse PR primary antibody (MS-197-P0; Thermo Scientific) was diluted to 1:100 in PBS + 0.5% triton. Rabbit (ERα) and mouse (PR) IgG isotype control served as negative controls. Slides were counterstained with hematoxylin as described above.

Intensity of DAB staining was quantitated as previously described (Stires et al., 2016). Briefly, five random fields from each section per tumor were taken using an Olympus FSX100 microscope at 20X magnification. Images were analyzed using the FIJI color deconvolution plugin (Schindelin et al., 2012). Images were divided into brown and purple images and mean

grey density was calculated from the brown image. Optical density (OD) was then determined using the equation: OD = log (255/mean grey density), where 255 is the maximum intensity for 8-bit images.

Statistical analysis

Differences between FAE and control groups were analyzed using an unpaired t-test or an unpaired t-test with Welch's correction if variances were not equal using GraphPad Prism version 6.0 (La Jolla, CA). Body weights were analyzed using repeated measures one-way ANOVA. Significance of the Kaplan-Meier curve was determined using Log-rank Mantel-Cox test. $P \leq 0.05$ was considered statistically significant. Tendencies were considered for $0.05 < p \leq 0.1$.

Results

Dam and pup parameters

Total fluid consumption of alcohol or saccharin-sweetened water was recorded every 24 h. Dams fed alcohol consumed 5-6 ml of ethanol each day, which equaled approximately 12 g ethanol/kg body weight/day, while control dams consumed 7-9 ml saccharin solution per day (Figure S2A, B). Blood alcohol level was analyzed and found to average 82 mg/dl, which is a level that correlates to legal limit of intoxication in humans. Dam body weights did not differ between treatment groups throughout pregnancy. At birth, neither litter size nor average pup weight was affected by treatment (Figure S2C,D).

FAE decreases the basal epithelial cell population and increases mammosphere forming activity in the normal mammary gland

The effects of FAE on the normal mammary gland were first investigated using whole mounts from 5- and 10-week WT offspring. Glands from FAE offspring did not visibly differ from that of the control group (Figure S3). Quantitation of ductal elongation and ductal area indicated there were no differences between the two groups (data not shown).

To determine if FAE increases the population of stem-like cells in the WT mammary gland, MECs were isolated and analyzed for MFE at 5 and 10 weeks of age. Luminal and basal epithelial cell populations were distinguished based on the expression of CD24 and CD49f within the lineage negative (TER119, CD45 and CD31) population, where luminal cells were labeled as $CD24^{+}CD49f^{lo}$ and basal cells were labeled as $CD24^{+}CD49f^{hi}$ (Figure 2A, B). The cell surface marker CD61 labels luminal progenitor cells in murine mammary glands (Vaillant et al., 2008; Visvader and Smith, 2011). When mammary glands were analyzed at 5 weeks of age to determine if there was an effect on the MEC subpopulations at puberty, flow cytometry analysis

showed comparable proportions of luminal, basal and luminal progenitor epithelial cells in both treatment groups (Figure 2C). At 10 weeks of age FAE significantly reduced the basal cell population ($p < 0.05$; Figure 2D), leading to an increase in the luminal to basal ratio ($p < 0.01$; Figure 2D). There was no treatment effect on the luminal progenitor cell population.

To determine if these changes in MEC populations corresponded with changes in MFE, MECs were plated in anchorage independent conditions to promote the growth of stem-like cells (Pastrana et al., 2011; Shaw et al., 2012). Primary cells were passaged to determine secondary and tertiary MFE (Figure 3A). FAE did not affect tertiary MFE at 5 weeks of age (Figure 3B). Interestingly, tertiary MFE was higher in the FAE group at 10 weeks of age ($p < 0.05$; Figure 3C), indicating an increase in stem-like/progenitor cells. These results suggest that without an oncogenic insult, FAE shifts the normal MEC composition and expands a cell population with increased sphere forming capabilities.

FAE affects expression of genes in the WT mammary gland that promote a shift in cell fate

To further explore the shift in MEC subpopulations in the normal gland at 10 weeks, several markers of interest were measured in total MECs by qRT-PCR analysis. In agreement with the flow data, expression of cytokeratin 14 (K14), a marker of basal MEC, was significantly lower in the FAE group ($p < 0.05$; Figure 4A) while expression of cytokeratin 8 (K8), a marker of luminal cells, was not altered (data not shown). Interestingly, Ki67 expression tended to be lower in the alcohol group ($p \geq 0.05$; Figure 4A). To further explore the increased luminal to basal ratio in the 10-week MEC data, expression of several genes that play a role in luminal cell fate and commitment was analyzed. When investigating key players of the Notch pathway, FAE significantly increased the expression of Hey1 ($p < 0.01$; Figure 4B), a target gene of the Notch pathway, while Notch1, Hes1 and Dll4 were not affected. FAE did not alter the expression of Elf5 and Gata3 (data not shown). Expression of the same set of genes at 5 weeks of age was not

different between the two groups (data not shown). These results suggest that FAE may shift WT MECs towards a luminal cell fate by altering Notch signaling.

FAE alters MEC subpopulations in pubertal transgenic offspring

Since FAE shifted MEC subpopulations in the normal gland, the effects of FAE in the hyperplastic mammary gland of Tg offspring was also investigated (Figure S4). Whole mounts of glands at 5 and 10 weeks of age did not show any morphological differences based on TEB number, ductal elongation and ductal area (data not shown).

MECs were isolated from 5- and 10-week old glands and analyzed by flow cytometry to determine shifts in the mammary epithelial cell subpopulations (Figure 5A, B). At 5 weeks of age, although the luminal and basal populations were not different between the two groups, the luminal progenitor population was significantly higher in the FAE group ($p < 0.01$; Figure 5C). However, by 10 weeks of age the luminal and basal cell populations as well as the luminal progenitor populations were similar between the groups (Figure 5D).

Tg MECs at 5 and 10 weeks of age were also plated for mammosphere culture as described above. FAE did not affect MFE at either age (Figure 5E, F). This may be due to the presence of the Wnt1 oncogene which increases MFE alone without additional insults, as the average tertiary MFE of Tg and WT glands from the control group were 0.33% vs 0.24%, respectively. Overall, these data suggest that FAE targets the gland earlier in Tg animals, increasing the proportion of luminal progenitor cells, which has been shown to be the population of cells vulnerable to tumor development in Wnt1 Tg mice.

FAE does not alter Notch signaling in the presence of the Wnt1 oncogene

To investigate the mechanism underlying the increase in luminal progenitor cells at 5 weeks of age, the expression of several genes was measured. No differences were observed in Notch gene

expression in the 5-week Tg glands, though Ki67 tended to be lower ($p = 0.0575$) (Figure 6A). Although no differences were detected in the 10-week MEC subpopulations, qPCR analysis of Notch pathway genes were also analyzed; however, no differences were found in expression of Notch pathway genes or Ki67 (Figure 6B).

FAE decreases tumor latency and alters tumor phenotype

Previous animal studies have shown that FAE increases the risk for tumorigenesis in rat and mice female offspring (Crismale-Gann et al., 2016; Hilakivi-Clarke et al., 2004; Ma et al., 2015; Polanco et al., 2010). To determine if FAE also affected tumorigenesis in the Wnt1 Tg model, offspring were palpated for tumor development starting at 6 weeks of age. Mammary tumors were first detected at 6 weeks of age in the control group. However, by 30 weeks of age 50% of the FAE group had developed tumors compared to less than 30% of animals in the control group. Overall, tumor latency was significantly decreased in FAE females compared to the control group ($p < 0.5$; Figure 7).

To further investigate whether the decreased latency was due to a shift in the proportions of epithelial cell subpopulations, tumors were harvested when they reached 1 – 1.5 cm in diameter and analyzed by flow cytometry. Overall, FAE increased the basal cell population in the mammary tumors while the luminal and luminal progenitor populations were comparable between the two groups ($p < 0.05$; Figure 8A,B). When cells were plated for tumorsphere culture, tertiary tumorsphere forming efficiency was not statistically different between the groups (Figure 8C).

ER and PR status were evaluated by IHC to assess hormone receptor status in tumors formed from FAE and control offspring. Representative images of ER and PR expression in both treatment groups are shown in Figure 9. ER expression was not significantly different between the two groups, however, PR expression tended to be lower in the FAE group ($p = 0.1$; Figure 9). Histological sections of lungs from animals that formed tumors were stained with H&E for the

detection of metastases. Metastases were not found in either group at the time of tissue harvest (data not shown).

Discussion

Since the establishment of the DOHAD hypothesis many diseases such as diabetes and cancer have been linked to early embryonic insults (Gluckman et al., 2008). Alcohol is a known teratogen; however, the connection between FAE and the mammary gland is minimally explored compared to other fetal tissues. Numerous studies, including several reports from our group, have shown that alcohol acts as a fetal insult on the mammary gland, promoting increased tumorigenesis and tumor multiplicity in female rats exposed to alcohol *in utero* (Crismale-Gann et al., 2016; Hilakivi-Clarke et al., 2004; Ma et al., 2015; Polanco et al., 2010). In the current study, the hypothesis that alcohol may potentially affect mammary gland tumorigenesis by targeting MEC subpopulations was investigated. To test this hypothesis, we utilized the transgenic MMTV-Wnt1 mouse model that spontaneously develops mammary tumors due to an increased stem/progenitor cell pool, as opposed to other mouse models where tumor formation results from alternative tumorigenic events (Mikaelian et al., 2004; Shackleton et al., 2006; Taneja et al., 2009). This allowed for the investigation of FAE on the mammary stem/progenitor cell pool and its role in tumorigenesis. The results show that MMTV-Wnt1 offspring exposed to alcohol *in utero* exhibit decreased tumor latency with tumors that exhibit an expanded basal cell population compared to tumors from the control offspring. Additionally, the use of WT FVB/NJ littermates determined that FAE can target the MEC subpopulations in the normal mammary gland in the absence of additional insults.

Interest in the concept that lifelong cancer risk can begin during fetal development has gained interest over the past several years and brought attention to the fact that tissue development and cell fate can be altered by the environment (Soto et al., 2008). The primary role of the MEC lineage is to develop and maintain the epithelial structure of the mammary gland. However,

studies have shown that both pre- and post-natal insults can shift the lineage and influence the risk for mammary tumorigenesis (Chang et al., 2012; Lambertz et al., 2017; Rahal et al., 2013; Wang et al., 2014). Deregulation of key regulatory pathways can alter the fate of cells along the MaSC lineage and affect normal development; therefore, we investigated changes in the mammary gland prior to tumor formation that may influence tumor risk later in life. Capturing the mammary gland state at 5 and 10 weeks of age in the mouse can identify changes during puberty and in the mature mammary gland, respectively, that could result from insults that occurred early in life. In the present study, the normal WT gland exhibited comparable luminal and basal proportions between the treatment groups at 5 weeks of age; however, by 10 weeks of age the luminal to basal cell ratio was significantly greater in the FAE group. These data corresponded with a greater tertiary MFE at this time point in the FAE glands. The mammosphere forming assay promotes the growth of spherical colonies enriched in stem-like cells and serves as a readout for stem/progenitor cell activity (Pastrana et al., 2011). Similar results were found in a recent study where mice on a high fat diet exhibited a decrease in the basal cell population coupled with an increase in luminal cell proportions. This was suggested to be a contributing factor in the increased risk for breast cancer (Chamberlin et al., 2017). While in the present study the luminal progenitor population did not increase in the WT gland based on CD61 expression, there was a greater ratio of luminal to basal cells, suggesting a shift in the MEC composition. This shift could represent the cell of origin for the developing cancer.

Based on the shift in the WT MEC composition at 10 weeks of age, we hypothesized that signaling pathways that control cell fate would be altered in these glands. Of the genes examined, expression of Hey1 was significantly greater in the FAE group. Hey1 is a target gene of the Notch pathway and increased expression is indicative of Notch signaling activation (Bouras et al., 2008). Notch signaling has been shown to play a role in cell proliferation, cell death and cell fate specification in the mammary gland. Activated Notch signaling promotes MaSC differentiation into cells of the luminal lineage and has been shown to promote tumorigenesis in mice (Callahan

and Egan, 2004; Chakrabarti et al., 2012; Jhappan et al., 1992). Additionally, studies have shown Notch signaling affects mammosphere formation, further supporting its role in stem cell maintenance (Dontu et al., 2004; Grudzien et al., 2010). Therefore, the increased luminal to basal ratio in conjunction with increased MFE may have resulted from the increased Notch signaling, promoting cells along the mammary cell lineage into a luminal cell fate.

The effects of FAE on the developing mammary gland differed in the presence of the Wnt1 oncogene. In the Tg mammary gland, the shift in MEC composition was evident during puberty with an expansion of the luminal progenitor population in the FAE group. By 10 weeks of age no differences were observed in the MEC composition. Similarly, results from our previous work showed advanced mammary development in FAE females, based on increases in MEC proliferation and TEB numbers, at PND20 that was lost by PND40; despite this return to normal FAE animals exhibit an increased susceptibility to carcinogen-induced tumorigenesis (Polanco et al., 2011). In 2014, Wang et al. reported that pubertal BPA exposure increases the basal cell population in Balb/c mammary glands at 6 weeks of age but not later at 2 or 4 months of age; however, increased neoplastic lesions are observed in regenerated mammary glands from cells of animals exposed to BPA (Wang et al., 2014). These results suggest that early alterations in the mammary gland can affect function despite a morphologically normal mature gland.

The luminal progenitor population increased at 5 weeks of age in Tg offspring; however, FAE did not affect tertiary MFE. This may be due to the presence of the Wnt1 oncogene alone which increases the number of stem/progenitor-like cells capable of forming spheres. Furthermore, despite the increased luminal progenitor population in the 5-week old gland, the expression of common Notch ligands, receptors and target genes were not different in the FAE group. The canonical Wnt signaling pathway alone expands the mammary progenitor population (Li et al., 2003; Liu et al., 2004; Teissedre et al., 2009). Therefore, one could speculate that FAE may further enhance the Wnt signaling pathway, increasing the proportion of luminal progenitors

susceptible to tumorigenesis. Future work will explore Wnt signaling to determine if it has been enhanced by FAE.

To determine if the shift in MEC subpopulations at 5 weeks translates to an increase in susceptibility to tumorigenesis, tumor latency was followed in an additional set of Tg offspring. FAE females exhibited a decrease in tumor latency and tumors exhibited an expansion of the $CD24^{+}CD29^{hi}$ basal cell population, suggesting that the luminal progenitor expansion found at 5 weeks of age may contribute to the increased tumorigenesis. In contrast to our data, other studies investigating early windows of exposure and breast cancer risk found an expansion of stem cells in the mammary gland (Chang et al., 2012; Lambertz et al., 2017; Wang et al., 2014). In two of these studies where tumor development was monitored, the increased stem cell population resulted in increased tumor risk (Lambertz et al., 2017; Wang et al., 2014). However, these studies induced cancer formation by DMBA, as opposed to a transgenic model such as the Wnt1 model. This raises the question of whether the outcomes would be the same in these studies if a transgenic model was used instead. These data also suggest that different cell types within the same tissue can be susceptible to oncogenic transformation and lead to tumor formation. Breast cancer subtypes are proposed to arise from different cells along the mammary gland lineage, with basal-like breast cancers arising from luminal progenitor cells (Visvader and Stingl, 2014). A portion of BRCA1-associated breast cancers are characterized as basal-like breast cancer and in BRCA1-deficient mice it was the luminal progenitor population and not the MaSC population that was altered in the mammary gland (Lim et al., 2009). This indicates that luminal progenitors are the cell of origin for basal-like and BRCA1-associated breast cancers. Luminal cells have been shown to have plasticity, where luminal progenitor cells are capable of complete regeneration of the mammary gland in transplantation assays (Vaillant et al., 2008). Upon oncogenic stress by either PYMT or ErbB2 signaling, luminal cells were shown to have the ability to transform into basal cells during tumorigenesis (Hein et al., 2016). Together, these data

suggest that luminal cells can transform into basal cells and in the present study may be the cell of origin for these tumors.

Tumors developed from FAE offspring had a tendency for decreased PR expression compared to the control group. PR expression is indicative of active estrogen signaling, which suggests that there may be a loss of ER activity in the FAE animals despite comparable ER status between the two groups (Arpino et al., 2005; Clarke et al., 2003; Horwitz et al., 1978). However, other factors can also contribute to downregulation of PR including alterations to the PR promoter or growth factors such as IGF or EGF that downregulate PR (Cui et al., 2005). ER+ PR- tumors are indicative of an aggressive tumor type that is tamoxifen resistant (Thakkar and Mehta, 2011). These data suggest that additional modifications in estrogen or growth factor signaling that decrease PR may promote a more aggressive tumor phenotype in FAE animals and calls for further investigation.

Interestingly, Ki67 tended to be lower in FAE offspring at 10 weeks for WT animals and at 5 weeks for Tg offspring. Alcohol exposure has been shown to decrease proliferation in a variety of tissues (Di Rocco et al., 2019). Neural progenitor cell proliferation is transiently decreased in mice chronically exposed to alcohol (Rice et al., 2004). Human liver stem cells treated with ethanol showed decreased cell proliferation based on BrDU incorporation and promoted mesenchymal transformation, thereby inhibiting differentiation as well (Shi et al., 2014). Neurospheres from fetal rat ganglionic eminence regions had decreased proliferation and differentiation when cultured at a high concentration of ethanol (Vemuri and Chetty, 2005). However, Ki67 has been suggested to be a prognostic marker for cancer and disruption of Ki67 depletes the cancer stem cell niche and delays tumor formation, suggesting a role for Ki67 in cancer progression and growth (Cidado et al., 2016; Sun and Kaufman, 2018). While mammary glands from FAE offspring may not have displayed increased proliferation, we found an expansion of a MEC subpopulation that may be susceptible to tumorigenesis. This suggests that

decreased tumor latency did not result from a hyperproliferative gland, but rather a shift in cell composition that promotes a more susceptible environment to tumorigenesis.

Our findings suggest that MEC subpopulations are susceptible to FAE, which may provide a possible mechanism by which FAE increases susceptibility to breast cancer. Alcohol consumption promotes the expansion of stem and progenitor cells in certain organ systems. In the liver, alcohol consumption affects liver stem cells by altering cell fate and inhibiting differentiation, thereby promoting the expansion of the liver stem cell pool (Di Rocco et al., 2019; Shi et al., 2014). The small intestine of mice given an alcohol diet exhibit altered cell composition, where chronic alcohol consumption promotes the shift to a more secretory intestinal cell fate, thereby increasing gut hyperpermeability (Forsyth et al., 2017). Additionally, FAE affects embryonic neural tissue by diverting cells from neuroectodermal formation, which may contribute to neurodevelopmental deficits in children with fetal alcohol spectrum disorder (Sanchez-Alvarez et al., 2013). This effect is not as extensively studied in the mammary gland. Recently, alcohol exposure has been shown to increase the cancer stem cell population and metastasis in the MMTV-neu tumor mouse model, indicating that alcohol exposure can target stem-like cells in the mammary gland (Xu et al., 2016). To the best of our knowledge, our study is the first to investigate the effect of FAE on mammary and tumor development in a transgenic mouse model. Future studies will be aimed at further elucidating the mechanism underlying this MEC subpopulation shift and its role in tumor development.

Genes	Forward primer 5' – 3'	Reverse primer 5' – 3'
K8	AGTTCGCCTCCTCATTGAC	GTCGCAACAGGCTCCACT
K14	AGCGGCAAGAGTGAGATTT	AATAACCTGGAGGAGACCAAAG
Elf5	GAGACCAAGACTGGCATCAA	TCCAAAGTTCTCACCTGTGG
Ki67	CTGCCTGTTTGGAAGGAGTAT	AAGTCAAAGAGCAAGAGGCA
Gata3	CGAGATGGTACCGGGCACTA	GACAGTTCGCGCAGGATGT
Notch1	ACAACAACGAGTGTGAGTCC	ACACGTGGCTCCTGTATATG
Dll4	GGAACCTTCTCACTCAACATCC	CTCGTCTGTTCGCCAAATCT
Hey1	TGAGCTGAGAAGGCTGGTAC	ACCCCAAACTCCGATAGTCC
Hes1	CCCCAGCCAGTGTCAACAC	ACACGTGGCTCCTGTATATG
Cyclophillin	TGCTGGACCAAACACAAACGGTTC	CAAAGACCACATGCTTGCCAT

Table 1. Primer sequences for genes analyzed by qRTPCR.

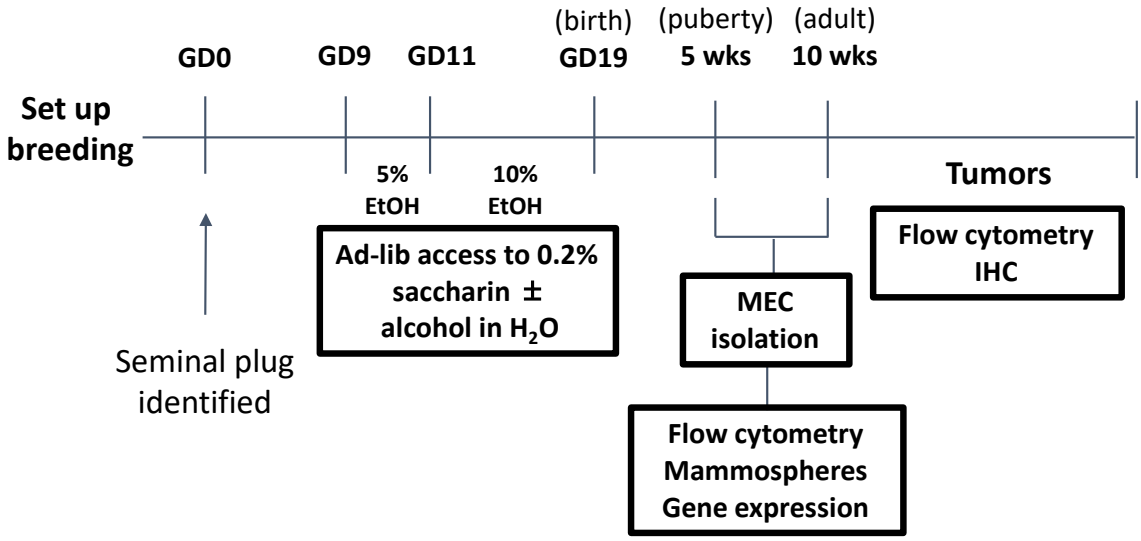

Figure 1. Experimental design. Pregnant dams were given ad libitum access to ethanol in 0.2% saccharin (5% from gestational day (GD) 9-10 and 10% from GD11-19) or water with 0.2% saccharin from GD 9-19. Treatments were stopped at birth. Mammary glands from wild type (WT) and transgenic (Tg) female offspring were harvested at 5 and 10 weeks of age and an enriched population of mammary epithelial cells was isolated. A separate group of Tg females were monitored for tumor formation.

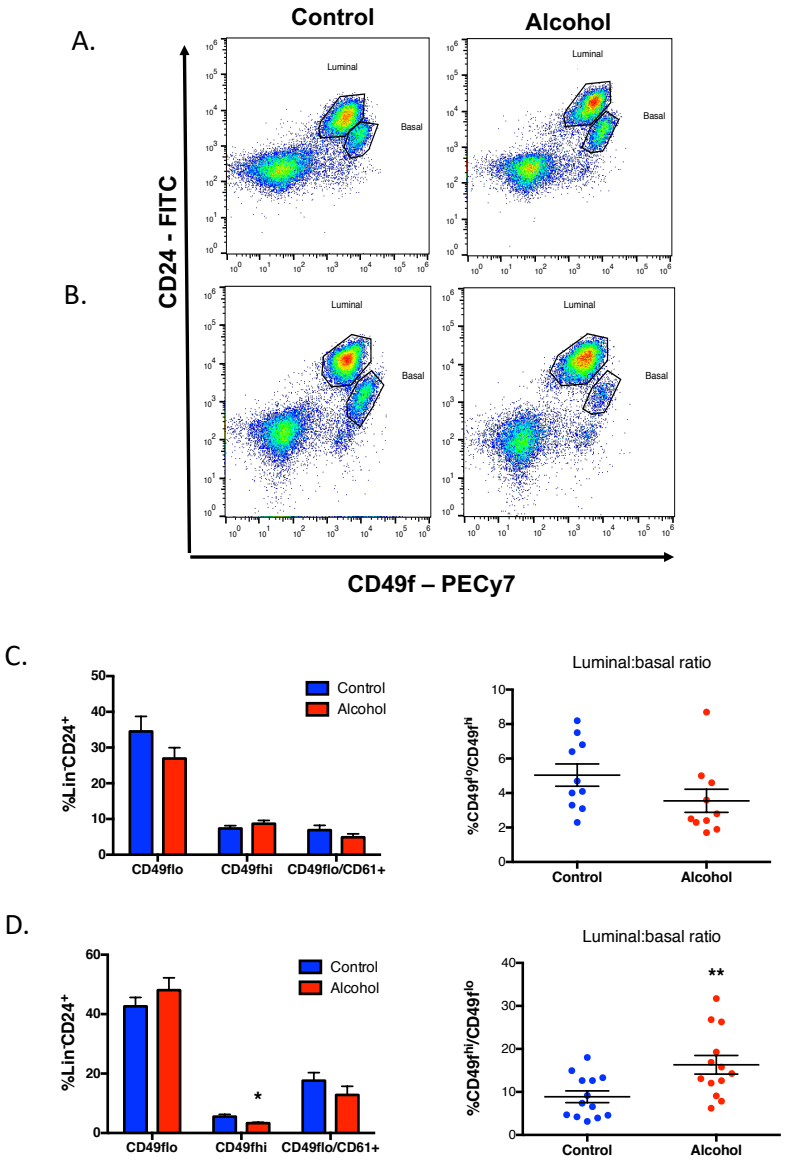

Figure 2. Fetal alcohol exposure decreases the wild type (WT) basal cell population at 10 weeks of age. Mammary glands of WT offspring exposed to alcohol or saccharin (control) in utero were harvested and total mammary epithelial cells (MECs) were analyzed by flow cytometry. Representative flow cytometry pseudocolor plots of WT MECs from mice at A) 5 and B) 10 weeks of age. Lineage negative (Lin-) cells were separated into $CD24^{+}CD49^{lo}$ (luminal) and $CD24^{+}CD49f^{hi}$ (basal) expression. The luminal $CD24^{+}CD49f^{lo}$ population was further divided into $CD61^{+}$ cells to determine the proportion of luminal progenitors within the luminal cell population (n = 7 at 5 weeks, n = 7-8 at 10 weeks). Proportion of MECs in luminal and basal cell populations as well as the luminal to basal cell ratio at C) 5 (n = 10) and D) 10 weeks of age (n = 13). Bars represent mean ± SEM. Student's t test, *$p < 0.05$, **$p<0.01$.

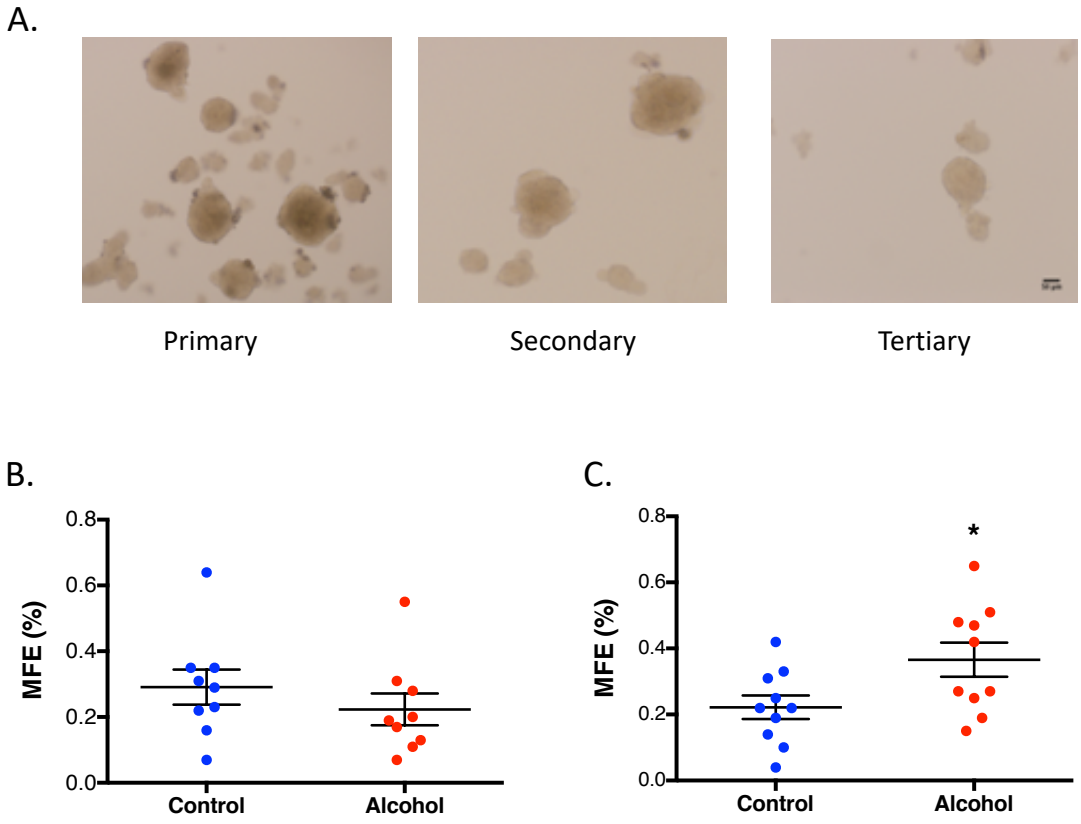

Figure 3. Mammosphere forming efficiency (MFE) is increased in wild type (WT) fetal alcohol exposed (FAE) offspring at 10 weeks of age. Mammary glands of WT offspring exposed to alcohol or saccharin (control) in utero were harvested and total mammary epithelial cells (MECs) were isolated and plated for mammosphere culture. A) Representative images of primary through tertiary spheres. Tertiary MFE was determined in WT control and alcohol MECs from mice at B) 5 and C) 10 weeks of age by dividing the number of spheres formed over the total number of cells plated (n = 9-10). MFE is calculated based on the number of spheres formed over the number of cells originally plated. Bars represent mean ± SEM. Student's t test, *$p < 0.05$.

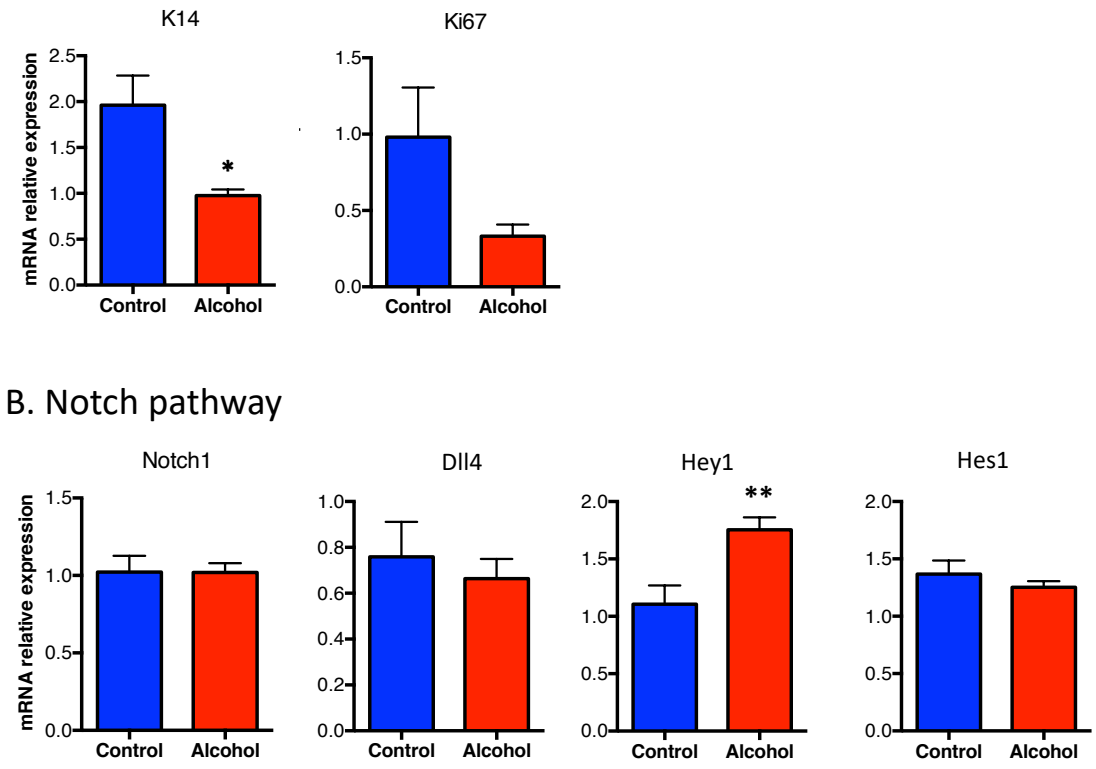

Figure 4. Hey1 expression is increased in 10-week wild type (WT) MECs. Mammary glands of WT offspring exposed to alcohol or saccharin (control) in utero were harvested and total mammary epithelial cells (MECs) were isolated and used for RNA isolation. Relative mRNA expression was determined by qRT-PCR in 10-week WT MECs for markers of A) basal cells and proliferation and B) Notch pathway. Bars represent mean ± SEM, n = 7 – 9 individual samples consisting of 2 pooled littermate MECs per sample. Student's t test *$p < 0.05$, **$p < 0.01$.

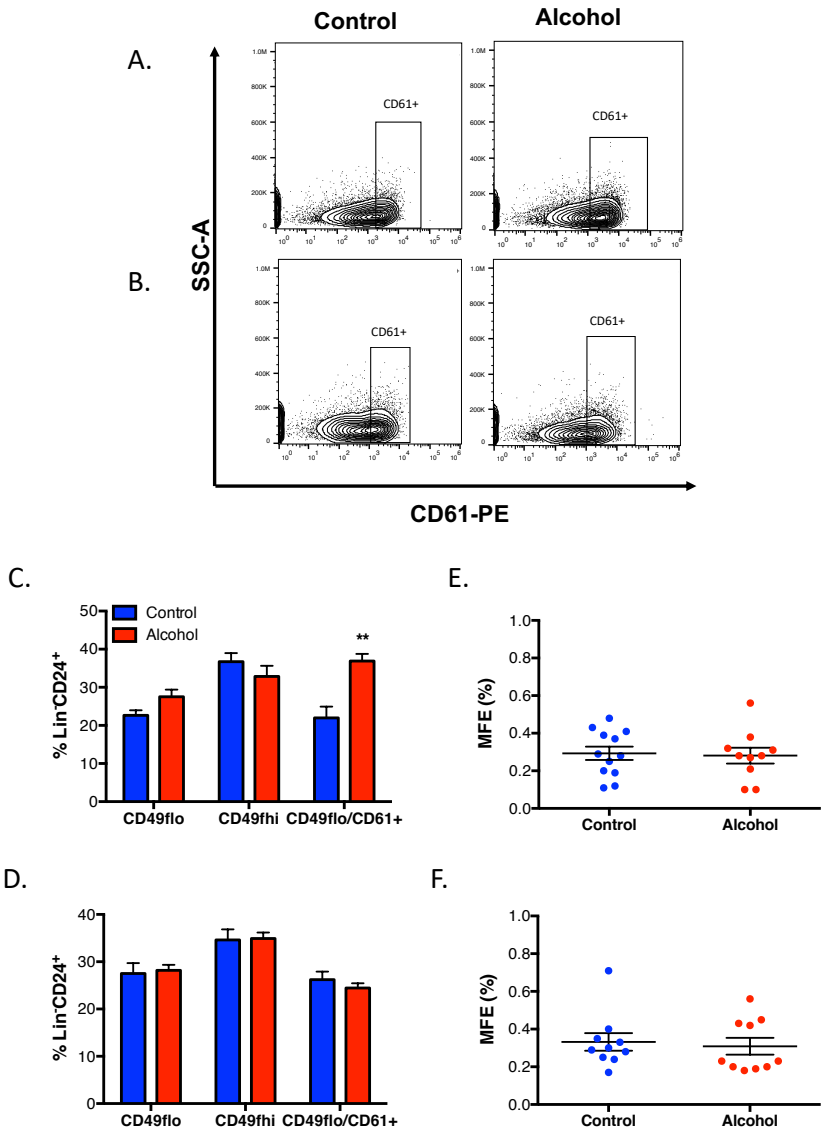

Figure 5. Fetal alcohol exposure alters transgenic (Tg) mammary epithelial cell (MEC) composition at puberty. Mammary glands of Tg offspring exposed to alcohol or saccharin (control) in utero were harvested at 5 and 10 weeks of age and total MECs were isolated and analyzed by flow cytometry. Representative images of $CD61^+$ luminal progenitor populations in MECs from Tg offspring at A) 5 and B) 10 weeks of age. Proportion of MECs in luminal ($CD24^+CD49f^{lo}$), luminal progenitor ($CD24^+CD49f^{lo}CD61^+$) and basal ($CD24^+CD49f^{hi}$) cell populations C) at 5 and D) 10 weeks of age. Tertiary MFE of E) 5 week and F) 10-week Tg MECs. Bars represent mean ± SEM, n = 10-12; Student's t test, $^{**}p < 0.01$.

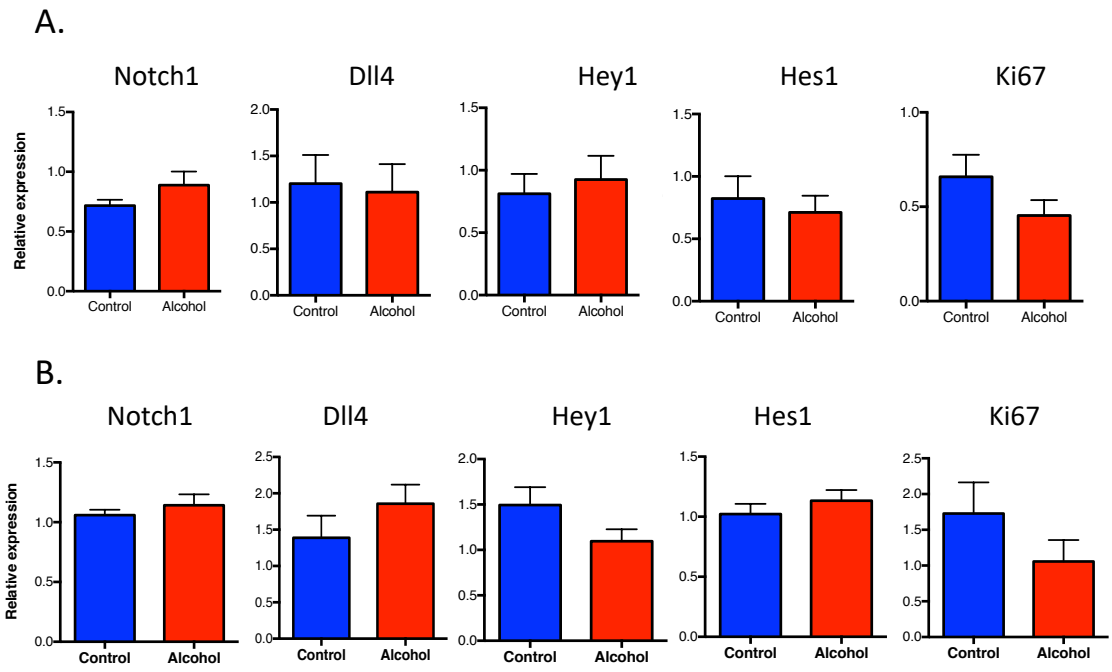

Figure 6. Fetal alcohol exposure did not affect Notch gene expression in transgenic (Tg) mammary glands. Mammary epithelial cells (MECs) isolated from glands of Tg offspring exposed to alcohol or saccharin (control) in utero were analyzed by qRT-PCR. Relative mRNA expression of Notch genes and Ki67 in A) 5-week Tg MECs (n = 6-8) and B) 10-week Tg MECs (n = 10). Bars represent mean ± SEM.

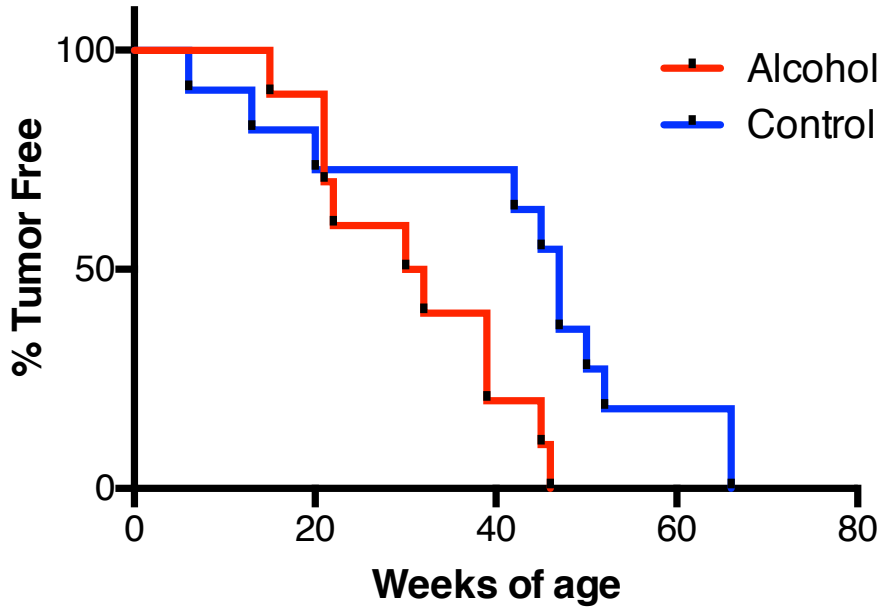

Figure 7. Fetal alcohol exposure decreases tumor latency in transgenic (Tg) offspring. Tumor formation was monitored in Tg offspring exposed to alcohol or saccharin (control) in utero. Mice were palpated for tumors twice a week starting at 6 weeks of age. A) Kaplan-Meier curve showing tumor latency (n = 10 per group). Log-rank Mantel-Cox test, *$p < 0.05$.

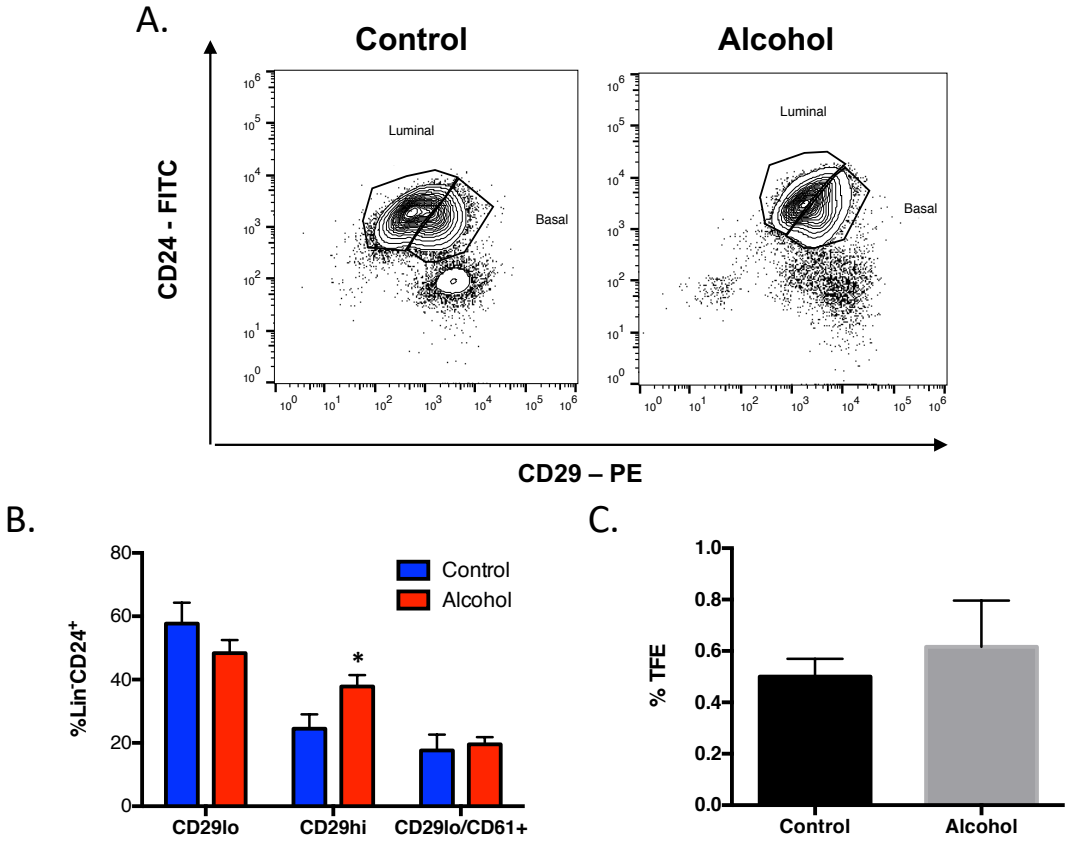

Figure 8. Fetal alcohol exposure increases basal epithelial cell population in tumors. Tumor formation was monitored in transgenic offspring exposed to alcohol or saccharin (control) in utero. Mice were palpated for tumors twice a week starting at 6 weeks of age and tumors were harvested at 1- 1.5 cm in diameter for flow cytometric analysis. A) Representative flow cytometry contour plots of control and alcohol tumors. B) Bar graph analysis of proportion of luminal ($CD24^{+}CD29^{hi}$), basal ($CD24^{+}CD29^{lo}$) and luminal progenitor ($CD24^{+}CD29^{lo}CD61^{+}$) tumor cells from alcohol and control groups (n = 8-10). C) Bar graph analysis of tertiary sphere forming efficiencies (TFE) of tumor cells (n = 5-6). Bars represent mean ± SEM. Student's t test, *$p < 0.05$.

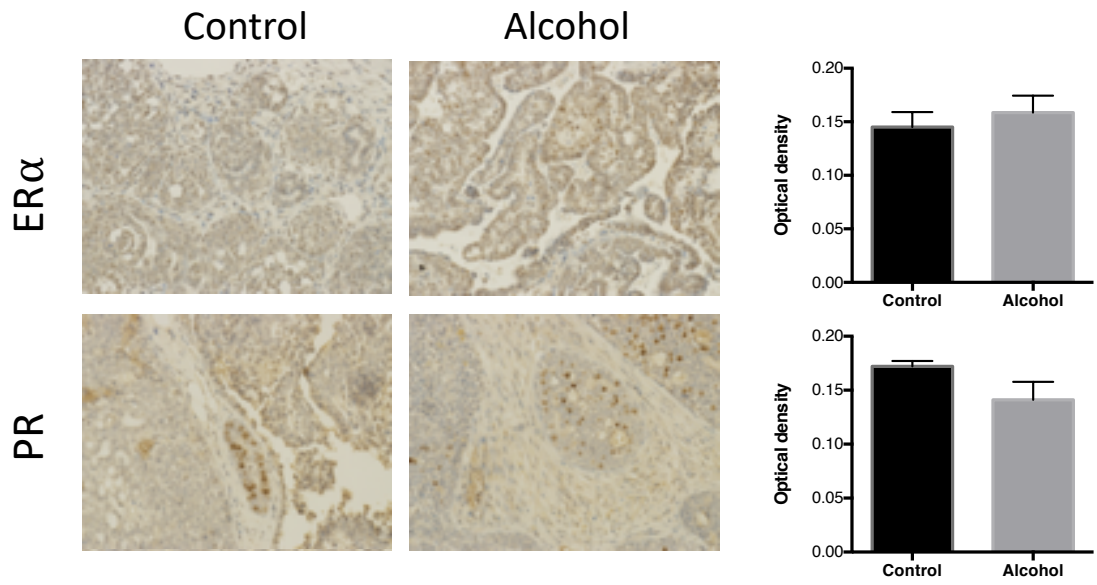

Figure 9. Effects of fetal alcohol exposure on ER and PR status in mammary tumors. Tumor formation was monitored in transgenic offspring exposed to alcohol or saccharin (control) in utero. Mice were palpated for tumors twice a week starting at 6 weeks of age and tumors were harvested at 1- 1.5 cm in diameter. Representative images depicting ER and PR expression in alcohol and control tumors. Both treatment groups had a range of low and high ER and PR expression. Overall expression of each tumor was measured by optical density analysis (n = 7-9 per treatment). Bars represent mean ± SEM.

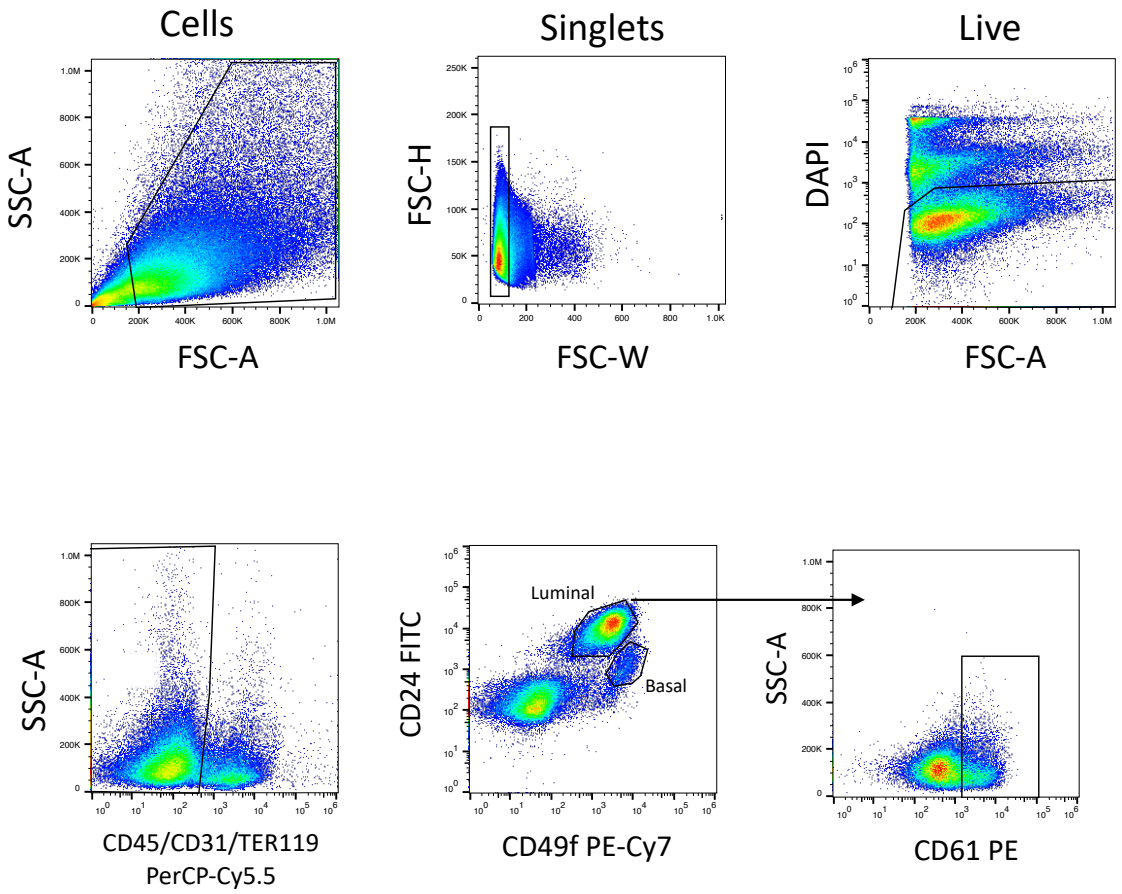

Figure S1. Gating strategy for mammary epithelial cells (MECs). Isolated MECs were labeled with the following antibodies conjugated to their respective fluorophores: CD24-FITC, CD49f-PE-Cy7, CD61-PE, biotinylated TER119, CD45 and CD31 and streptavidin-PerCP-Cy5.5. Cells were excluded for debris, dead cells (using viability marker DAPI) and lineage cells (positive for TER119, CD45 and CD31). Lineage negative cells were separated into $CD24^{+}CD49f^{lo}$ (luminal) and $CD24^{+}CD49f^{hi}$ (basal) expression. The $CD24^{+}CD49f^{lo}$ luminal population was further divided into $CD61^{+}$ cells to determine the proportion of luminal progenitors.

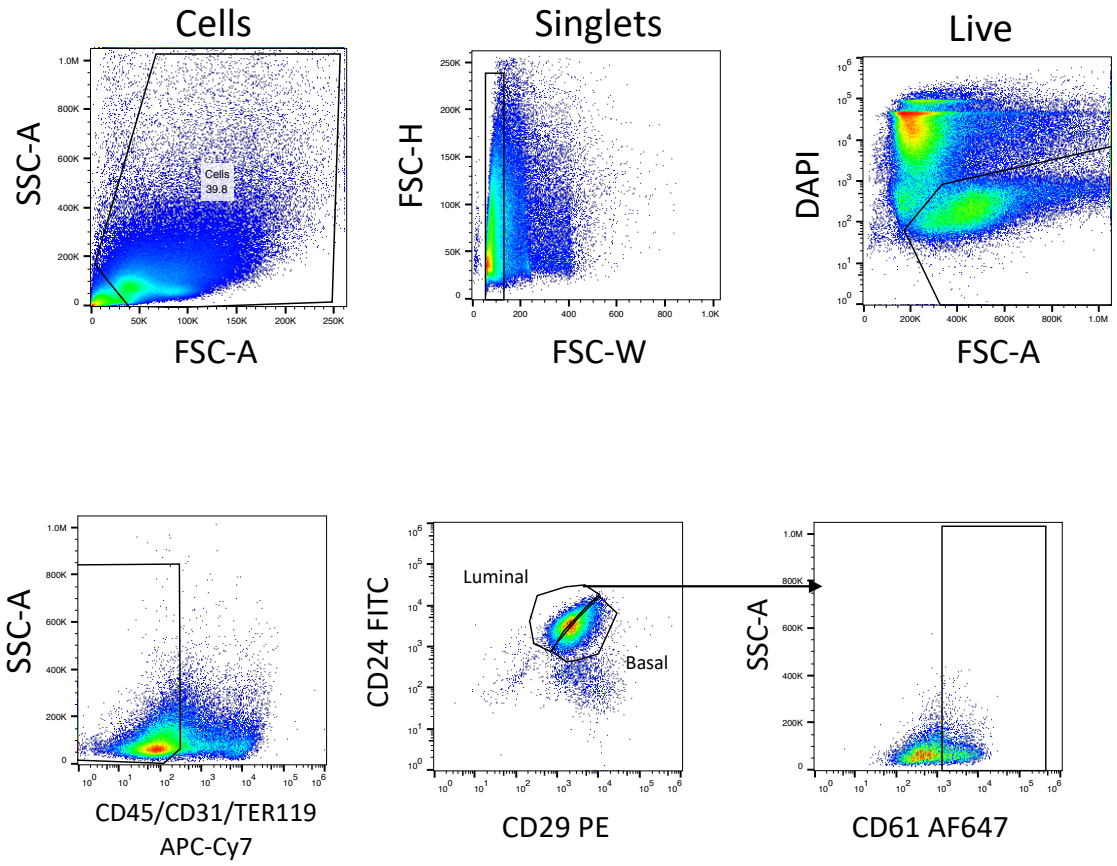

Figure S1 (continued). Gating strategy for tumors. Isolated tumor epithelial cells were labeled with the following antibodies conjugated to their respective fluorophores: CD24-FITC, CD29-PE, CD61-Alexa Fluor 647, biotinylated TER119, CD45 and CD31 and streptavidin-APC-Cy7. Cells were excluded for debris, dead cells (using viability marker DAPI) and lineage cells (positive for TER119, CD45 and CD31). Lineage negative cells were separated into $CD24^{+}CD29^{lo}$ (luminal) and $CD24^{+}CD29^{hi}$ (basal) expression. The $CD24^{+}CD29^{lo}$ luminal population was further divided into $CD61^{+}$ cells to determine the proportion of luminal progenitors.

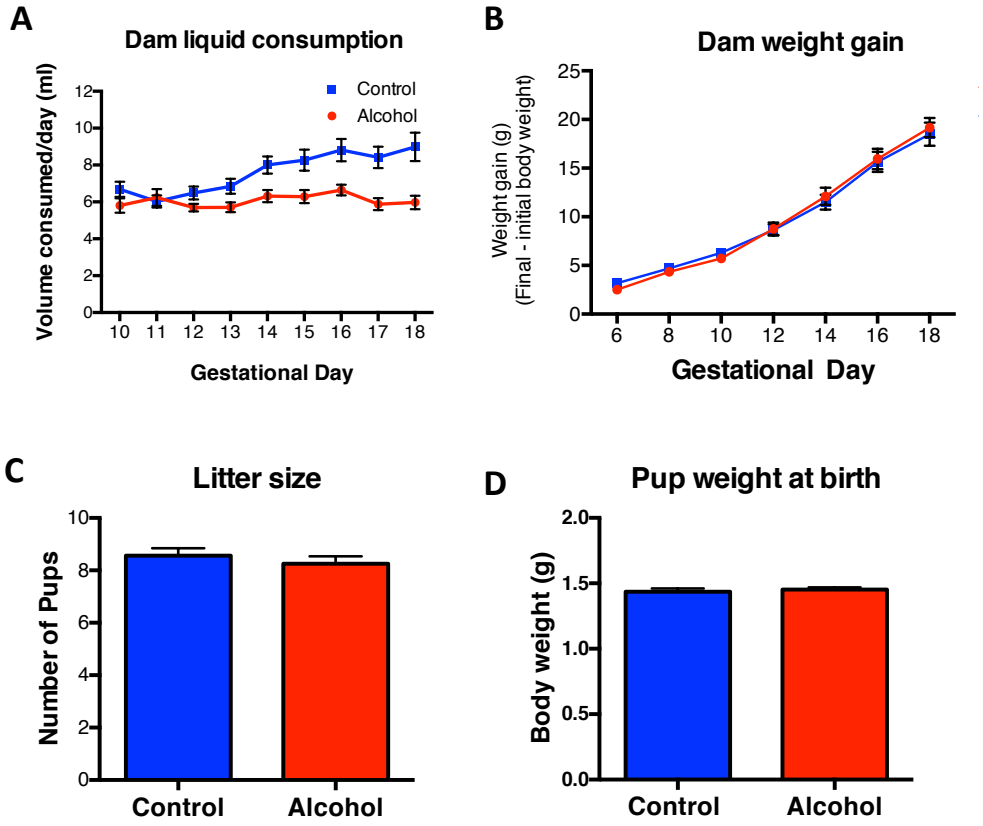

Figure S2. Dam and pup parameters. Pregnant dams were given alcohol or saccharin control solution from GD 9-19 and daily fluid consumption, weight gain and pup parameters were recorded. A) Daily consumption of alcohol or saccharin (control) solution in dams from GD10 – 18. Each point on the line graph represent the mean ± SEM for 38-40 dams. B) Daily weight gain of dams from GD6-18. Each point represents the mean ± SEM of 10 dams. C) Average litter size and D) average weight per pup at birth (38-40 litters per group). Bar graphs represent mean ± SEM.

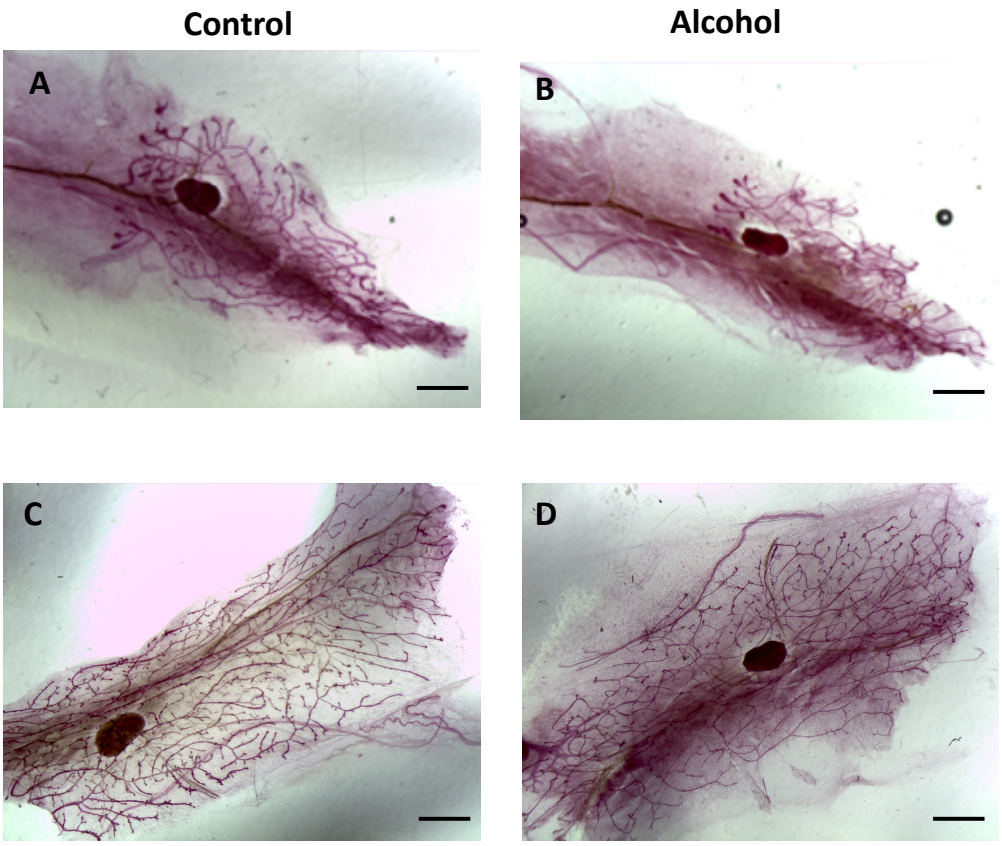

Figure S3. Representative wild type (WT) mammary gland whole mounts. Mammary glands of WT offspring exposed to alcohol or saccharin (control) in utero were harvested and processed as whole mounts as described in materials and methods. Whole mounts of the right inguinal mammary gland from WT control and alcohol exposed offspring at 5 (A, B) and 10 (C, D) weeks of age. Scale bars, 2 mm.

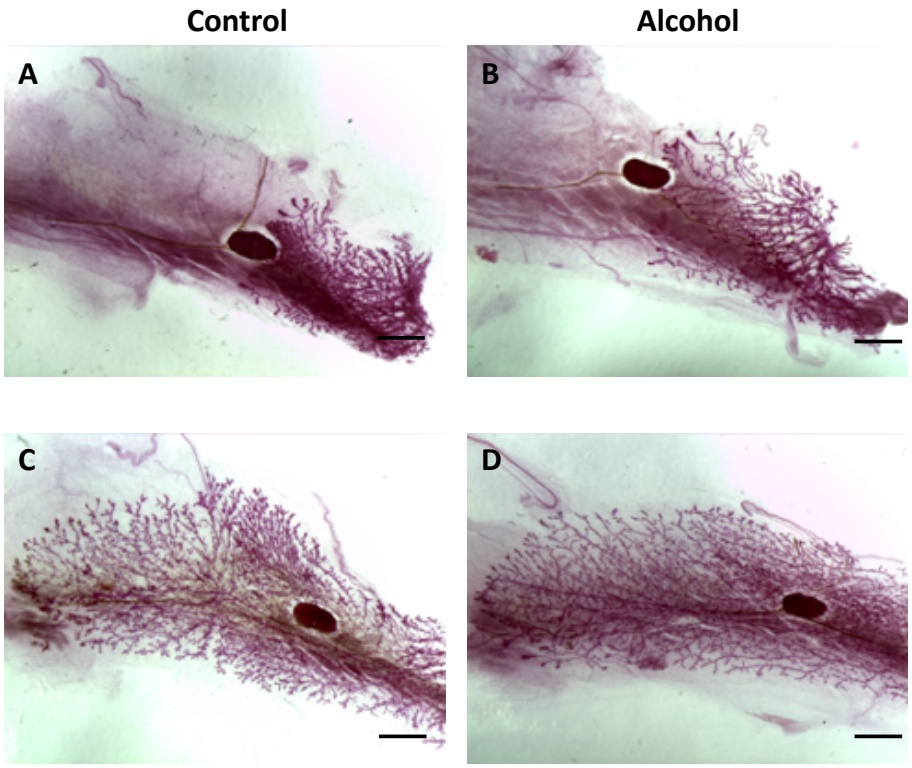

Figure S4. Representative transgenic (Tg) mammary gland whole mounts. Mammary glands of Tg offspring exposed to alcohol or saccharin (control) in utero were harvested and processed as whole mounts as described in materials and methods. Whole mounts of the right inguinal mammary gland from Tg control and alcohol exposed offspring at 5 (A, B) and 10 (C, D) weeks of age. Scale bars, 2 mm.

Conclusions and Future Directions

Several genetic and external factors contribute to the development of cancer. Recently the concept that the fetal environment may also contribute to cancer susceptibility has become a major area of interest. Alcohol is a known risk factor for breast cancer and our work has previously shown that fetal alcohol exposure (FAE) increases susceptibility to breast cancer in a Sprague-Dawley NMU model; however, the underlying mechanisms are not well understood (Crismale-Gann et al., 2016; Polanco et al., 2010). Since the establishment of the mammary stem cell (MaSC) lineage concept, many researchers have provided evidence for its role in cancer formation (Ercan et al., 2011; Visvader, 2009; Visvader and Stingl, 2014). Therefore, the present work tested the hypothesis that FAE increases susceptibility to tumorigenesis through shifts in the mammary epithelial cell (MEC) composition, suggesting an effect on the lineage. The effect of FAE with a focus on MEC subpopulations was investigated in the normal wild type (WT) gland, MMTV-Wnt1 transgenic (Tg) mammary gland and tumors. The results have laid the groundwork for future studies on how FAE can target cells during early development which can eventually become cancer initiating cells later in life.

Analysis of normal WT glands found a decreased basal cell population and increased luminal:basal cell ratio in the FAE group at 10 weeks of age. Although this effect was not observed at 5 weeks of age, this may be due to the many cellular and structural changes occurring during puberty, which could mask the subtle effects of FAE on the MEC subpopulations. Initially, we predicted that FAE increases tumorigenesis through the expansion of the basal cell population, since MaSCs are known to be housed here and hold tumor-initiating capabilities. However, as the mammary hierarchy continues to be further defined both basal and luminal cells have been found to have repopulating cells, a known characteristic of true stem/progenitor-like cells (Keller et al., 2012). Cells within the MEC lineage also exhibit cell plasticity, as differentiated cells are capable of acquiring stem-like properties (Chaffer et al., 2011).

Additionally, progenitor cells housed in the luminal population are proposed to be the tumor initiating cell in triple-negative breast cancer and are capable of undergoing luminal to basal transformation (Chiche et al., 2019). Similar to findings of the current study, the loss of IGF1 receptor (IGFR) decreased the basal cell population and increased luminal progenitors in the developing mammary gland. When the loss of IGF1R was investigated in Wnt1-induced tumorigenesis, tumor latency was reduced in bigenic MMTV-dominant-negative igf1r (dnigf1r)/Wnt1 animals (Rota et al., 2014), Therefore, these data support the current hypothesis that FAE may shift the MEC composition towards a more luminal cell fate and increase a population of cells susceptible to tumorigenesis.

Interestingly, at 10 weeks of age WT MECs also exhibited increased mammosphere formation as well as expression of Hey1, a target gene of the Notch pathway, in the FAE group. Notch signaling is known to play a role in cell fate and in mammary glands it specifically promotes luminal cell fate commitment (Artavanis-Tsakonas et al., 1999; Bouras et al., 2008). Hey1 is a known luminal cell fate regulator and has been shown to be expressed in the $CD61^{+}$ luminal progenitor cell population in mice (Bouras et al., 2008; Chiche et al., 2019). Therefore, Hey1 may be increased in committed luminal progenitor cells in the WT glands and result in the increased luminal:basal cell ratio observed. Notch signaling is also known to influence mammosphere forming efficiency (MFE) (Dontu et al., 2004). In the present study, tertiary spheres from FAE animals exhibited greater MFE compared to the control group. Given that stem and progenitor cells are both capable of forming mammospheres the challenge with plating a heterogeneous population of total MECs is identifying the origin of the sphere-forming cell (Dontu et al., 2003; Pastrana et al., 2011). Since total MECs were analyzed for this present study, future studies would determine which cell is responsible for increased MFE as well as Notch signaling. Luminal and basal cells would be sorted from total MECs and plated for the mammosphere assay to determine which cell type yields a greater MFE. Hey1 expression in the sorted cell populations

would also be determined by qRT-PCR analysis to identify the origin of the higher Hey1 expression. Additional functional assays such as the colony formation assay could also be implemented. This assay has frequently been used in other studies as a readout for progenitor activity (Chamberlin et al., 2017; Visvader and Lindeman, 2008). When plated in Matrigel, luminal epithelial cells form round colonies while myoepithelial cells form ductal colonies (Joshi et al., 2010; Stingl et al., 2006). To further support the current data, total MECs isolated from 10-week WT glands could be plated for the colony forming assay to assess the proportion of colonies that form from basal and luminal lineages. If more round colonies form, suggesting a luminal cell origin, then this would support the hypothesis that FAE promoted a luminal cell shift at this timepoint. Finally, to test if FAE directly affects mammosphere formation via increased Notch signaling, total MECs from the FAE group could be treated with a Notch inhibitor such as γ-secretase *in vitro*. Treatment of cells with γ-secretase has been shown to decrease mammosphere formation in human breast cancer cells (Farnie et al., 2007) and therefore this experiment would help determine if Notch signaling was responsible for the increased MFE. Overall, these additional experiments would further delineate the MEC composition shift in the normal wild-type gland.

Another factor to consider is characterization of the decreased basal cell population at 10 weeks of age. Additional studies would investigate molecular changes that contribute to a decrease in basal cells. qPCR analysis of 10-week MECs demonstrated a tendency for decreased Ki67 expression in the FAE group. However, Ki67 expression was measured from the total, heterogenous population of MECs, which includes luminal and basal epithelial cells as well as stromal cells. Luminal and basal epithelial cells should be sorted to measure Ki67 expression and determine if the decreased Ki67 originated from the basal cell population. If Ki67 is decreased in the basal cell population, then the reduced proliferation may contribute to the observed decrease in this population. Another possible scenario is that mature, differentiated basal cells have

decreased, leaving only the small stem cell population in the remaining basal cell population. Since cytokeratin 14 (K14) mRNA expression was also lower in the FAE group, it is possible that K14 expression decreased due to the overall decrease in basal cells. Alternatively, K14 expression may have decreased due to the majority of the basal cell population consisting of more stem-like cells, some of which have low K14 expression (Wang et al., 2015). Zhang and Rosen have found that the basal $CD24^{lo}CD29^{hi}$ stem cells are capable of forming mammospheres, suggesting another population of cells that may be responsible for the increased MFE observed in this study (Zhang et al., 2015). While a single marker cannot specifically identify MaSCs, several have been shown to enrich for them. Low Sca-1 expression has been used to enrich for MaSCs within the basal cell population (Shackleton et al., 2006; Stingl et al., 2006). However, more recently additional markers have been shown to label MaSCs within the normal mammary gland, such as Lgr5, Procr and Sship (Bai and Rohrschneider, 2010; Plaks et al., 2013; Rios et al., 2014). Assessing the expression level of these genes in the 10-week WT basal cell population could indicate the relative proportion of stem cells in the basal cell population and give insight into additional cell populations that may be susceptible to oncogenic transformation.

In the preneoplastic Tg mammary gland, luminal progenitors increased at 5 weeks of age. When these animals were monitored for tumor formation, FAE animals developed tumors sooner than the control group. In contrast to what was observed in the normal WT gland, MFE at 5 and 10 weeks of age did not differ nor did Notch signaling change. One possibility is that the strong oncogenic potential of the Wnt1 model may mask subtle effects that arise from FAE. The MMTV-Wnt1 model has been extensively characterized over the past 30 years and the luminal progenitor population has been suggested to be the target cell for Wnt signaling and subsequent tumor formation (Li et al., 2003; Liu et al., 2004). Considering the results from this study, this suggests that FAE may further enhance the Wnt signaling pathway in this model. Future studies should be aimed at determining if increased Wnt activity played a role in the observed phenotype

at 5 weeks of age in Tg glands. Activation of the canonical Wnt signaling pathway stabilizes β-catenin levels. Therefore, β-catenin protein levels and nuclear localization could be measured in the Tg glands by western blot. Gene expression analysis of the Wnt1 ligand and Wnt target genes, such as *c-myc*, could also be used to assess enhanced Wnt signaling.

Several epidemiological studies have investigated the association of direct alcohol consumption in the development of specific breast cancer subtypes. In two studies, alcohol consumption was more strongly associated with risk of ER+ or PR+ tumors (Chen et al., 2011; Zhang et al., 2007). In contrast, others have found that alcohol consumption increased the risk for ER- tumors in pre and post-menopausal women (Britton et al., 2002; Potter et al., 1995). In this current study, tumor ER expression was comparable between the two groups based on IHC analysis; however, PR tended to be lower in alcohol tumors. ER +/PR - tumors characterize a subset of mammary tumors that are aggressive and tamoxifen resistant (Thakkar and Mehta, 2011). Tumors from MMTV-Wnt1 mice are characterized as basal-like. Considering that tumors from FAE progeny also had a larger basal cell population, it would be interesting to further characterize these tumors since FAE may promote the development of more aggressive tumors, as seen in our previous studies (Polanco et al., 2010). The lack of significance with PR expression may be due to a small sample size and therefore future studies with a larger sample size would help explore the role of PR expression in these tumors.

Tertiary tumorsphere formation was not significantly different between the alcohol and control groups. Unfortunately, due to a small sample size and variation in sphere formation between samples, it raises the question of whether this assay is sensitive enough to measure tumorsphere formation. The use of multi-sphere culture comes with caveats such as cell aggregation which can result in inaccurate sphere formation (Pastrana et al., 2011). An alternative approach would be to plate tumor cells in a sphere limiting dilution assay, as described in (Rota et al., 2012), as a more accurate readout of stem/progenitor cells in tumors. The sphere limiting dilution assay would

allow us to continue assessing tertiary sphere formation but with an ability to plate each sample at various densities, allowing for a more confident readout of stem/progenitor cell frequency.

Overall, the results in this thesis suggest a possible mechanism by which FAE shifts the MEC composition towards one that promotes tumorigenesis. This the first study of its kind to assess the effects of FAE on the MEC composition. Evidence dating back to the Dutch famine support the hypothesis that a suboptimal fetal environment affects an individual's risk for disease later in life (Barker, 2007; Barker, 2012; Gluckman et al., 2008; Wood and Keller-Wood, 2016). If the findings from this study and others translates to humans, then it is important that pregnant women understand the lasting effects that alcohol consumption may have on the developing fetus, beyond the risk for fetal alcohol spectrum disorder. As breast cancer subtypes have been proposed to arise from different tumor initiating cell subpopulations, it would be important to know which mammary cells are capable of transformation and tumor formation following FAE. By further understanding how these TICs work, more progress can be made towards developing effective treatments and prevention strategies, especially if FAE does play a role in the development of TIC. Women born from mothers who drank should be aware of their increased risk for breast cancer and undergo additional screenings. If future studies can elucidate the specific pathways that increase tumor susceptibility, then preventative treatments can be given to women at higher risk for developing this disease.

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APPENDIX

Flow cytometry antibody titration and protocol validation

Antibody titration was done by aliquoting 0.5 - 1 x 10^6 mammary epithelial cells (MECs) into separate 12 x 75 mm polypropylene tubes and stained with a single antibody at serial dilutions of 1:50, 1:100, 1:200, 1:400 and 1:800 as described in materials and methods. MECs were also stained with DAPI to gate out dead cells during titration analysis. MECs were analyzed by acquiring at least 100,000 events using a Beckman Coulter Gallios flow cytometer at the Rutgers EOHSI Flow Cytometry core facility. Data were analyzed using FlowJo version 10 with the methods described in (Hulspas, 2010). Briefly, for each tube a dot plot for side light scatter versus forward light scatter was used to exclude debris and side light scatter versus DAPI staining was used to exclude dead cells. After exclusion of debris and dead cells, each antibody fluorophore versus forward scatter was plotted in a dot plot to analyze fluorescence intensity. One region was drawn around the negative population, based on the maximum autofluorescence observed in unstained cells and another was drawn around the positive population. The median fluorescence intensity was determined for each negative and positive population of each sample using the FlowJo statistics tool. The signal-to-noise ratio, indicating the strongest signal with minimal background interference, was calculated by dividing the median fluorescence intensity of the positive cells over that of the negative cells. These values were plotted for each dilution of an antibody. The dilution where the signal-to-noise ratio was highest was chosen as the optimal antibody dilution to use.

To validate the optimized antibody panel, cells were stained with the antibody panel at the determined concentrations and gated for cell debris, cell aggregates, dead cells and lineage positive cells, to enrich for the luminal and basal epithelial cells. Luminal and basal cells were sorted into separate 12 x 75 mm polypropylene tubes containing HBSS/2% FBS (HF) + 50% FBS on a Beckman Coulter Moflo XDP cell sorter. Collected cells were pelleted, snap frozen and

stored at -80°C until further use. RNA was isolated from sorted cells using the Qiagen RNAeasy Micro kit as per manufacturer's instructions (Qiagen; Germantown, MD). RNA quantity and quality were assessed using the Nanodrop ND-100 (Thermo Scientific; Waltham, MA) and Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano kit (Agilent Technologies; Santa Clara, CA), respectively. 0.25 μg of RNA was reverse transcribed to cDNA using a high capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA). Primers for cytokeratin 8 (K8), a marker for luminal cells, and cytokeratin 14 (K14), a marker for basal/myoepithelial cells, were used for qRT-PCR (see Table 1 for primer sequences). qRT-PCR was performed using Power SYBR Green MasterMix using a StepOne Plus™ Real-Time PCR system (Thermo Scientific; Waltham, MA). Results were analyzed using Step One software and exported into excel. mRNA expression was normalized to the housekeeping gene cyclophilin and analyzed with the $2^{-\Delta\Delta C_t}$ method. Final qRT-PCR analysis confirmed enrichment for K8 expression in the luminal sorted population and K14 expression in the basal sorted population (Figure 1).

Gene	Accession Number	Primer Sequence	Product length
K8	NM_031170.2	F: AGTTCGCCTCCTCATTGAC R: GTCGCAACAGGCTCCACT	77 bp
K14	NM_016958.2	F: AGCGGCAAGAGTGAGATTT R: CTTTGGTCTCCTCCAGGTTATT	115 bp

Table 1. Accession numbers and primer sequences for cytokeratin genes used for qRT-PCR validation.

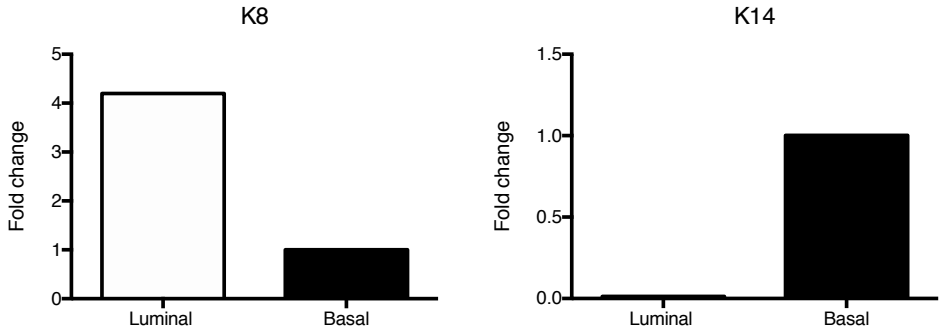

Figure 1. PCR validation of FACS protocol. RNA isolated from lineage negative luminal ($CD24^{+}CD49f^{lo}$) and basal ($CD24^{+}CD49f^{hi}$) MECs sorted cells was used for qRT-PCR to detect K8 and K14 expression. Expression is relative to that of basal cells, set at 1.