THE EFFECT OF ALCOHOL CONSUMPTION ON MAMMARY EPITHELIAL CELL COMPOSITION AND MAMMARY TUMORIGENESIS

By

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ABSTRACT OF THE THESIS

The Effect of Alcohol Consumption on Mammary Epithelial Cell Composition and Mammary Tumorigenesis

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Breast cancer is the most common cancer in women worldwide and in 2019 it is estimated that approximately 41,000 women will die from the disease. There are a variety of factors that increase risk for breast cancer one of which is alcohol consumption. However, the mechanism that underlies this increased risk is unknown. The mammary gland is a dynamic organ composed of a multiple cell types including adipose cells, fibroblasts, immune cells, and epithelial cells. The epithelial cells can be categorized into luminal and basal epithelial cells, whose composition is maintained and controlled by a pool of mammary stem cells. Mammary stem cells are quiescent and long lived, and therefore have the potential to accumulate mutations and transform into breast cancer stem cells. Breast cancer stem cells have the potential to maintain a tumor and may not be irradiated by conventional therapies, leading to relapse. Therefore, understanding what regulates the overall mammary epithelial cell hierarchy is key to improving breast cancer treatments. The goal of this project was to determine whether alcohol consumption alters the mammary epithelial cell composition to favor a tumorigenic state, whether alcohol consumption affects tumor latency, and whether alcohol alters the mammary tumor epithelial cell composition.

The MMTV-Wnt1 mouse model is a useful model for studying the role of mammary stem cells in breast cancer, as the tumors that develop in this model arise from a stem or stem-like cell, and downstream targets of the Wnt signaling cascade have been found to be upregulated in breast cancer. To investigate the effect of alcohol on mammary epithelial cell composition and tumorigenesis, 7- week old MMTV-Wnt1 female mice were given a 20% alcohol solution in place of drinking water sweetened with 0.2% saccharin. Control animals were given a 0.12% saccharin solution for the entire duration of the study. Animals were weighed once per week and were sacrificed after either 8 weeks to analyze the preneoplastic mammary gland or after the first tumor had reached 1.5cm in diameter. Animals in the alcohol group gained more weight compared to the controls, and this difference in weight gain was due to an increase in overall caloric intake due to alcohol consumption. Mammary epithelial cells were isolated and analyzed by flow cytometry and plated for mammosphere/tumorsphere assays. Mammary glands from the alcohol group exhibited an increase in the luminal progenitor population, but a decrease in mammosphere forming efficiency. Alcohol consumption decreased tumor latency in animals that presented with tumors by 43 weeks of age, however, alcohol consumption did not effect on the tumor epithelial cell composition nor the tumorsphere forming efficiency. Alcohol consumption decreased the expression of the epithelial-mesenchymal transition (EMT) factors Snail and Twist in the mammary gland mRNA, and the proliferation marker Ki67. It also decreased expression of Snail in mRNA from the mammary tumor. Alcohol did not affect estrogen receptor positivity in the mammary tumors, suggesting an estrogen-independent mechanism in this model.

In conclusion, alcohol consumption in adulthood altered the mammary epithelial cell composition by increasing the number of luminal progenitor cells, which have been implicated as tumor initiating cells in basal like breast tumors. Alcohol consumption also decreased mammosphere forming efficiency, suggesting a decrease in the stem cell population. Analysis of gene expression further suggested that alcohol decreased the stem cell population in the mammary gland due to a decrease in Snail and Twist expression. It is also possible that alcohol affected the mammary epithelial cell composition by increasing body weight and altering composition, which has also been identified as a risk factor for breast cancer risk.

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LIST OF ABBREVIATIONS

ADH- alcohol dehydrogenase

ALDH- aldehyde dehydrogenase

CSC- cancer stem cell

DAB- 3,3′-diaminobenzidine

EMT- epithelial-mesenchymal transition

E2- estradiol

ERα- estrogen receptor alpha

H&E- hematoxylin and eosin

HGF- hepatocyte growth factor

IHC- immunohistochemistry

MMTV- mouse mammary tumor virus

PR- progesterone receptor

TEB- terminal end buds

CHAPTER 1: REVIEW OF THE LITERATURE

A. Alcohol as a risk factor for breast cancer

Epidemiological studies: In 2019, it is expected that approximately 268,000 new cases of breast cancer in women will be diagnosed in the United States, and over 41,000 women will die from breast cancer (Siegel, Miller and Jemal 2019). Breast cancer is the most common cancer worldwide in women, with 2 million new cases diagnosed in 2018 (World Cancer Research Fund). There are a variety of factors that play a role in a woman's chance of developing breast cancer, one of which is alcohol consumption (Singletary and Gapstur 2001, Smith-Warner et al. 1998, Chen et al. 2011). Various epidemiological studies have found that women who drink are at a greater risk for developing breast cancer. The first report that alcohol is a risk for breast cancer was in 1977. This case control study involved interviews with people with various types of cancer and assessed various external factors including alcohol use. A significant dose-dependent association between breast cancer and alcohol consumption was found in females (Williams and Horm 1977). This concept has been further studied to determine and affirm alcohol as a risk factor. In 1977, a patient survey was conducted by nurses in hospitals in the United States and Canada. Results of the survey showed that of all female patients who consumed alcohol, the largest proportion had breast cancer versus the controls and those with non-malignant diseases, and the risk for breast cancer was 1.9 times higher for alcohol drinkers versus nondrinkers (Rosenberg et al. 1982). This report, however, did not find a difference with age of drinking. Following these studies, in 1984, a cohort study was published which included over 95,000 women in the Kaiser Foundation Health Plan in Northern California. These

women had multiphasic health examinations and answered questionnaires regarding alcohol consumption, including type of alcohol and amount. Health records of these women were used to identify cases of breast cancer. This study did not find an association between general alcohol consumption and breast cancer. However, when a dose dependent response was examined, women who drank 3 or more drinks per day had a significantly higher risk of breast cancer than those who drank less than 2 drinks per day or did not drink at all (Hiatt and Bawol 1984). In 1987, using the data from the first National Health and Nutrition Examination survey in the United States, Schatzkin conducted a follow up epidemiologic study. They found a 40 to 50% increased risk for breast cancer among women who drank 3 drinks in a week; this was the first time that low levels of alcohol consumption were reported to increase breast cancer risk (Schatzkin et al. 1987). Alcohol consumption increases breast cancer risk regardless of menopausal status and also increases risk with prolonged exposure of alcohol resulting from the initiation of drinking at an earlier age (Bowlin et al. 1997). Studies have found that long term drinking increases the chances for breast cancer diagnosis at a later age (Stoll 1999, Vaeth and Satariano 1998). The Million Woman Study was one of the first large cohort studies that was conducted in the UK that recruited women from 1996 through 2001 and followed their health over a 3-year period. This study found an increase in breast cancer risk associated with increased alcohol consumption, with a 12% increase for every 10 grams of alcohol consumed per day (Allen et al. 2009). A large epidemiological study similar to the Million Woman Study was conducted in the United States. The Nurses' Health Study started recruitment in 1976 and analysis in 1980 and Nurses' Health Study II was conducted

from 1989 through 2003. These studies followed a large group of female nurses who were given questionnaires regarding their health habits, family history, as well as breast cancer diagnosis. Analysis of these studies found that there was an increased risk for breast cancer among women who binge drink, that drinking before the first pregnancy increased breast cancer risk, and that women who drank as adolescents had an increased risk of benign breast disease (Chen et al. 2011, Liu et al. 2012, Liu, Nguyen and Colditz 2015, Liu et al. 2013). These epidemiological data indicate that alcohol is a risk factor for breast cancer and as a result of this body of work, the American Cancer Society has listed alcohol as a risk factor for breast cancer.

Animal Studies: Animal studies have confirmed that alcohol consumption may be a risk factor for breast cancer. Alcohol exposure during puberty causes morphological changes in the mammary gland such as epithelial proliferation and branching (Masso-Welch et al. 2012, Singletary 1997). This has been confirmed using rodent models as well as a pig model which found that alcohol consumption increased proliferation in the mammary gland, indicated by an increase in terminal ductal units, as well as an increase in pSTAT5 (Schennink et al. 2015). One of the earliest studies involving breast cancer risk and exposure to alcohol in rodents used C3H/St inbred mice, which spontaneously form tumors by transmission of the Bittner virus during nursing. Offspring were exposed to a 12% ethanol solution that replaced drinking water. The animals exposed to alcohol developed tumors at a much earlier age compared to their control counter parts, indicating a decrease in tumor latency when exposed to alcohol (Schrauzer et al. 1979). In 2000, Watabiki et al. conducted a study using ICR female mice that were exposed to 15% ethanol in place of drinking water. 45% of the mice had tumors before the end of

the study which lasted 25 months, while the control counter parts did not have any tumors by the end of the study (Watabiki et al. 2000). Using a rat model and NMU to induce mammary tumor formation, one group was interested in how ethanol consumption influenced initiation and promotion of tumor formation. They found that ethanol consumption at 20% of calories significantly increased palpable tumor incidence, and that ethanol consumption at 15% and 20% of calories increased tumor progression compared to the control counterparts. However, ethanol consumption at 30% of calories did not exhibit the same effects (Singletary, Nelshoppen and Wallig 1995). While many studies conclude that alcohol may enhance tumorigenesis in animals, there are some studies that find that alcohol does not have this effect. These studies include ones that used models that spontaneously form tumors which found that alcohol in place of drinking water did not increase tumor incidents (Hackney, Engelman and Good 1992), as well as models with induced tumor formation which found that rats given alcohol in place of drinking water had fewer tumors than the controls (McDermott, O'Dwyer and O'Higgins 1992). These varied results suggest that this is an area of research that requires more research.

Proposed mechanisms:

Alcohol as a carcinogen: When alcohol is consumed, it is metabolized to acetaldehyde by alcohol dehydrogenase (ADH), and then further metabolized to acetate by acetaldehyde dehydrogenase 2 (ALDH2). While most alcohol is metabolized in the liver, ADH is also expressed in human breast epithelial cells (Saleem et al. 1984), suggesting that mammary tissue also has the ability to metabolize alcohol to acetaldehyde, which is carcinogenic (Castro and Castro 2014). Specifically, it has been confirmed that class I ADH is found in the mammary gland (Triano et al. 2003). A

portion of consumed alcohol can enter the bloodstream, which can distribute to breast tissue. There it is metabolized and due to a lack of efficiency, there is a build-up of acetaldehyde (Fanelli et al. 2011). This can lead to an inhibition of DNA repair by the formation of alkylating agents, and formation of cross links in DNA (Seitz and Maurer 2007). Alcohol consumption leads to the production of reactive oxygen species and oxidative stress. The processes involved in cellular respiration are affected by the levels of oxygen and levels of these metabolites and enzymes, therefore acting as a carcinogen (Hoek, Cahill and Pastorino 2002). The capacity is limited for the amount of reactive oxygen species to be present in a tissue, therefore a buildup of acetaldehyde enhances toxicity (Liu et al. 2015). While a certain amount of expression is normally expressed in the mammary gland to maintain homeostasis of ethanol levels caused naturally by the body, alcohol consumption can cause a large increase in the amount of enzyme necessary to metabolize ethanol (Triano et al. 2003).

Alcohol and estrogen*:* Approximately 60% of breast cancers are hormone receptor positive and overexpress estrogen receptor (Clarke, Dickson and Lippman 1992). Studies in humans and rodents have demonstrated that alcohol consumption increases serum levels of estrogen (Dorgan et al. 2001, Gavaler and Rosenblum 1987), suggesting a possible mechanism by which alcohol may promote tumorigenesis. The latter study also found an increase in uterine weights in rats exposed to 5% alcohol, providing support that the increase in endogenous estrogen production is physiologically relevant (Gavaler and Rosenblum 1987). However, both of these studies have focused solely on understanding the effects of alcohol consumption on estrogen levels in postmenopausal women. A 1998 study found that consuming 0.7 g/kg of ethanol

increased circulating estrogen levels by more than 50% when women were in the follicular phase of the menstrual cycle (Mendelson et al. 1988), suggesting that estrogen status is not only relevant to women who have gone through menopause. In vivo and in vitro studies have further elucidated this concept and have found that long term alcohol exposure increases tumor incidence as well as increased systemic estrogen, aromatase, and estrogen receptor alpha (ERα) positive tumors in mice expressing the HER2 oncogene (Wong et al. 2012), and that exposure to ethanol increases the proliferation of ERα positive breast cancer cells (Singletary, Frey and Yan 2001). Another study confirmed this finding with MCF7 breast cancer cells exposed to ethanol and E2 reporting an increase in ERα and E2 activity (Fan et al. 2000).

B. Mammary epithelial cell hierarchy

Overview of mammary gland development: The mammary gland is a dynamic organ that does not complete development until after birth (Figure 1). Development starts in the embryo, with the formation of mammary lines at embryonic day 10.5 which continue to invade the fat pad and form buds (Hens and Wysolmerski 2005, Propper 1978, Robinson 2007). Epithelial cell proliferation and elongation into the fat pad occurs beginning on embryonic day 15.5 with continued nipple formation and invasion and branching into the fat pad (Hogg, Harrison and Tickle 1983, Sakakura et al. 1987). During and after puberty, there is further invasion by epithelial cells resulting in branching morphogenesis. The terminal end buds continue to invade the fat pad and stop once the entire fat pad is filled (Lyons 1958, Silberstein and Daniel 1982, Williams and Daniel 1983). The resulting structure is comprised of luminal epithelial cells which line ducts, myoepithelial (basal) cells which have contractile functions, and supporting stromal tissue which

contain adipocytes, fibroblasts, and immune cells. The mammary gland continues to develop to a fully differentiated state during pregnancy and lactation. The ability of the mammary gland to develop alveoli during pregnancy to produce milk, then undergo involution after weaning to return to the virgin state, and repeat this process for each new pregnancy suggests that mammary stem cells exist and contribute to the glands' morphology and alterations during different stages over time.

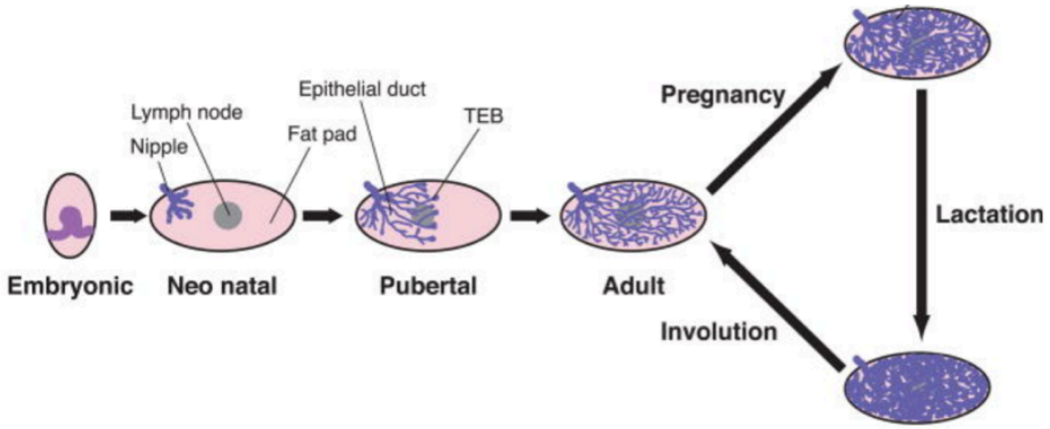


Figure 1: Overview of mammary gland development, starting from the prepubertal stage through adulthood, including the processes that occur during pregnancy, lactation, and involution. From Wiseman and Werb 2002. Reprinted with permission from AAAS.

Mammary stem cells: The earliest evidence of the existence of mammary stem cells came from experiments that involved injecting cleared mouse mammary fat pads with a small portion of an epithelial tree or epithelial cells, and found that the entire mammary tree was able to be reconstructed (Daniel 1975, Faulkin and Deome 1960). In 1998, Kordon and Smith found that a single mammary cell could regenerate an entire mammary epithelium, confirming the presence of mammary stem cells (Kordon and Smith 1998). From then on, others conducted studies to confirm the presence and function of stem and

stem-like cells in the mammary gland. Other groups have reproduced Kordon and Smith's work demonstrating that a single cell has the ability to re-constitute the entire mammary gland (Shackleton et al. 2006, Stingl et al. 2006a).

The unipotent mammary stem cells maintain the luminal and basal lineages postnatally. This was shown by Van Keymeulen et al. who transferred tagged basal epithelial cells and luminal epithelial cells into fat pads, and found that during puberty, stem cell activity was reduced, and that both luminal and basal cells were compartmentalized and had progenitor activity after birth (Van Keymeulen et al. 2011). Lineage tracing studies have demonstrated clonal expansion of mammary stem cells through development, by labeling a single cell and identifying its progeny. Further labeling found that basal and luminal clones were distributed throughout the branches, contributing to elongation of the ducts through puberty (Davis et al. 2016). Evidence from these studies have suggested and confirmed the role and presence of mammary stem cells.

Mammary stem cells can be identified during embryonic development, starting at embryonic day 12.5 through birth, at which time fetal mammary stem cells exist, and have the ability to form spheres in Matrigel (Spike et al. 2012). These cells are identified as $CD24^{hi}/CD49f^{hi}$, which is different from the markers of adult mammary stem cells; however, a fetal mammary stem cell has the ability to give rise to a new functional mammary gland (Sreekumar, Roarty and Rosen 2015). Mammary stem cells are ER negative and progesterone receptor (PR) negative, yet are influenced by paracrine signaling (Asselin-Labat et al. 2006), and are affected by the estrous cycle, showing an expansion during the diestrous phase, in concordance with an increase in progesterone

levels (Joshi et al. 2010). p53 has been shown to be a negative regulator of mammary stem cells (Cicalese et al. 2009). As the mammary gland grows, there are cap cells located at the ends of the terminal end buds (TEBs). It has been hypothesized that some of these cap cells have stem like activity and will migrate from the TEBs to the inner cell layers of the gland and go on to form luminal and basal cells (Williams and Daniel 1983). At the end of puberty, the TEBs disappear, and he gland is now considered mature.

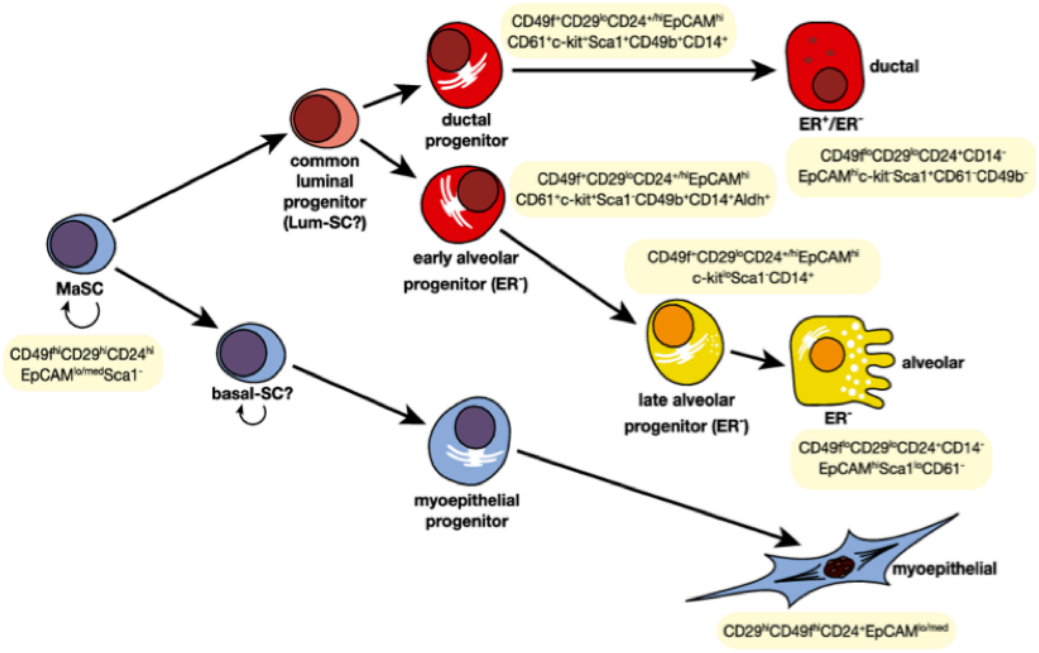


Figure 2: Mammary epithelial cell hierarchy, and markers of prospectively identified mammary epithelial cells. From Visvader and Stingl 2014. Reprinted with permission.

The cells of the mammary epithelial lineage have been analyzed with respect to cell surface markers that can enrich for a given population (see Figure 2). Early studies identified and established CD24 as a surface marker of the mouse mammary epithelium (Shackleton et al. 2006, Stingl et al. 2006a, Sleeman et al. 2006). Recent evidence suggests that although epithelial cell fate can be determined, the programming is

reversible. Expression of Slug and Sox9 together has been found to convert differentiated luminal cells into mammary stem cells (Guo et al. 2012). Cell surface marker analysis has revealed that mammary stem cells occupy the basal compartment of the mammary gland exclusively, however other stem like cells called progenitor cells exist as well (Shackleton et al. 2006, Stingl et al. 2006b, Sleeman et al. 2006).

Basal cell compartment: Mammary stem cells reside in the basal compartment of the mammary epithelial cell population, as evidenced by similarity in expression of cell surface markers CD49f and CD29 (Stingl et al. 2006a). Mammary stem cells possess the ability to switch to a bipotent stem cell during pregnancy (van Amerongen, Bowman and Nusse 2012) and are able to form precursor cells for the luminal and basal lineages (Rios et al. 2014, Wang et al. 2015). Identification of signaling pathways and factors in the basal cell compartment that promote stemness has been an active area of research. Basal stem cells express higher levels of epithelial-mesenchymal transition (EMT) transcription factors that promote stemness as well as Hedgehog signaling (Guen et al. 2017). Hedgehog signaling has been found to play a role that affects cilia that are found in the basal compartment exclusively, and when dysregulated causes disruption in the alveolar development during pregnancy and decreased branching during development (McDermott et al. 2010). The stemness factor BCL11B has been shown to be restricted to the basal compartment and to block basal lineage commitment and promote stemness within the basal compartment (Miller et al. 2018). Id1 is another stemness factor associated with breast cancer that is found to be only expressed in the basal compartment, and has been shown to deregulate mammary basal stem cells (Shin et al. 2015). This

evidence suggests that the unipotent mammary stem cells found in the basal compartment may be a target for dysregulation and perhaps oncogenesis. Further research should be conducted to further establish their role and factors that affect their regulation.

Luminal progenitor cells: Luminal progenitor cells make up a portion of the luminal epithelial cell population. They possess stem cell properties but are committed to forming cells of the luminal lineage. Luminal progenitor cells express the transcription factor Elf5 and have clonogenic activity. Lineage tracing experiments in adult mice using Elf5 as a marker demonstrated no change in the frequency of luminal progenitor cells at 2 days and 8 weeks post-injection, but a reduction at 20 weeks. These data confirm the role of the luminal progenitor cells as a progenitor cell which further differentiates into a functional luminal cell. There is also a corresponding change in the size of the luminal progenitor population during each round of alveogenesis (Rios et al. 2014).

Elf5 is a transcription factor that is implicated in luminal cell fate. Elf5 null mammary glands from pregnant female mice have an expanded luminal progenitor population (Oakes et al. 2008, Chakrabarti et al. 2012). This difference however was not found in the glands of virgin mice (Chakrabarti et al. 2012). Mammosphere assays showed that mammary epithelial cells from Elf5 null mice formed more secondary spheres compared to wild type cells, suggesting that the luminal progenitor cells from the Elf5 null mice maintained their progenitor status rather than progressing to differentiated luminal cells (Chakrabarti et al. 2012).

C. Breast cancer stem cells

Cancer stem cell overview: The cancer stem cell (CSC) model describes a tumor that contains or may have originated from a stem cell, which has the ability to self-renew and differentiate into different cell types, or may have originated from a differentiated cell which has acquired stem cell characteristics due to genetic alterations (Wicha, Liu and Dontu 2006, Visvader and Lindeman 2012, Kakarala and Wicha 2008). Stem cells are slow dividing and long lived and are therefore susceptible to oncogenic mutations (Moore and Lyle 2011, Coller, Sang and Roberts 2006). While stem cells contribute to the cellular hierarchy and maintain the morphology of a tissue, cancer stem cells maintain the cellular hierarchy of the tumor, and are able to evade targeted therapies that target the bulk of the tumor and are therefore able to allow for tumor regrowth and regeneration, which has been seen in breast cancer (Visvader 2011, Ricci-Vitiani et al. 2009). Various studies from epidermal and hematopoietic cell systems have found that cancers arising from stem or progenitor cells usually express the same markers as their cell of origin, and that tumors that arise from stem or progenitor cells tend to be heterogeneous and contain a mixed lineage of cell differentiation (Owens and Watt 2003, Perez-Losada and Balmain 2003).

Identification of breast cancer stem cells: The earliest evidence to support the CSC hypothesis was from studies involving human leukemias which suggested that this form of cancer was driven by a small population of leukemic stem cells capable of transferring the disease to NOD/scid mice (Bonnet and Dick 1997). In 2003, this concept was tested for breast cancer by sorting breast cancer cells by flow cytometry into $CD44^{+}CD24^{-}$ or $CD44^{+}CD24^{+}$ populations and implanting them into mammary fat pads of

immunocompromised mice. Only the cells expressing stem cell markers $CD44^{+}CD24^{-}$, were able to form tumors. (Al-Hajj et al. 2003). The idea that tumor initiating cells are $CD44^{+}CD24^{-}$ was supported by showing that tumors formed when murine fat pads were injected with low concentrations of the putative stem cells (Ponti et al. 2005). In addition, cancer stem cells have phenotypes similar to their stem or progenitor cell of origin (Jamieson et al. 2004, Kelly and Gilliland 2002). Studies using ALDH1 as a marker for breast cancer stem cells showed that cells from breast tumors expressing high amounts of ALDH contained a subset of cells with the ability to self-renew and form the parental tumor upon transplantation (Ginestier et al. 2007). This evidence provides support for the theory that breast cancer may arise from stem or stem like cells, and that existing tumors can be maintained through a stem cell mechanism.

Role of luminal progenitor cells in breast cancer: Luminal progenitor cells may significantly contribute to breast cancer (Lim et al. 2009, Shehata et al. 2012). Luminal progenitors are altered in BRCA1 tumors, with these tumors arising from luminal progenitors that may have undergone dedifferentiation (Molyneux et al. 2010). When epithelial cells were isolated from breast tissue of patients with BRCA1 mutations, a decrease in the mammary stem cell and basal population, but an increase in the luminal progenitor fraction was observed, and this cell fraction had a higher colony forming activity than luminal progenitors from non-mutation carriers (Lim et al. 2009). Microarray profiling to assess the relationship between luminal progenitor cells and breast cancer subtype has demonstrated that luminal progenitor gene signatures more closely associate with basal like breast cancers (Lim et al. 2009). BRCA1 mutation-

induced tumors tend to have a basal like phenotype (Lakhani et al. 2005, Palacios et al. 2005). While it was originally suggested that BRCA1 breast cancers arise from a mammary epithelial stem cell (Foulkes 2004, Liu et al. 2008, Vassilopoulos et al. 2008), further analysis suggested that it was actually the luminal progenitor cells that are the tumor initiating cell, with analyses of the luminal progenitor cells demonstrating that a loss of BRCA1 in these cells induces the formation of human BRCA1 tumors (Molyneux et al. 2010). To further understand and analyze the luminal progenitor cell lineage and its potential differentiation abilities, a search for signaling genes that may be associated with this differentiation process and the formation of basal breast tumors found that gene sets regulated by the oncogene Met and its ligand hepatocyte growth factor (HGF), were particularly upregulated in basal breast cancer. To investigate the role of Met signaling, epithelial cells were isolated from FVB virgin mice and were transfected with HGF. Overexpression of Met signaling was found predominantly in the luminal progenitor cells, leading to hyperproliferation and disorganization of the mammary glands, and an increased luminal progenitor population which had the ability to form colonies. The findings also suggest that constitutive activation of Met signaling causes luminal progenitor cells to differentiate abnormally; rather they have basal like characteristics, further suggesting the possibility of dedifferentiation and a challenge to the concept of the mammary epithelial cell hierarchy which demonstrates unidirectionality of luminal progenitor cells (Gastaldi et al. 2013). Gata3 plays a key role in luminal cell differentiation and luminal progenitor maintenance, and is found to be restricted to only the luminal lineages of the mammary gland (Asselin-Labat et al. 2007). Gata3 has also been implicated as a breast cancer transcription factor, with mutations in Gata3 being

found in breast cancers suggesting a tumor suppressor role (Usary et al. 2004). These findings indicate that the luminal progenitor population of the mammary gland is a target of tumorigenesis and can be sufficient to cause alterations to the mammary hierarchy.

D. MMTV-Wnt1 mouse model of breast cancer

The role of Wnt signaling in the mammary gland: The Wnt signaling system is important for both mammary development and breast cancer. Wnt signaling is driven by a family of receptors and multiple Wnt ligands (Gavin and McMahon 1992, Kouros-Mehr and Werb 2006). Wnt signaling is present in very early embryonic development (Chu et al. 2004), with Wnt10 being expressed at the earliest timepoint suggesting that Wnt signaling is essential for skin cells to initiate mammary specific programming (Veltmaat et al. 2004, Boras-Granic and Wysolmerski 2008). Wnt signaling also regulates mammary stem cells and promotes self-renewal (Clevers, Loh and Nusse 2014), and continuous Wnt signaling under a mouse mammary tumor virus (MMTV) promoter can expand the stem cell and progenitor cell populations (Incassati et al. 2010). Along with increased Wnt1 signaling can coincide increased expression of Wnt1 receptors. ΔNp63, an isoform variant of p63 and the primary isoform in basal breast tumors, is necessary for maintaining the basal cell lineage of mammary epithelial cells (Yalcin-Ozuysal et al. 2010) and modulates Wnt signaling and the mammary stem population by increasing the expression of the Wnt receptor Fzd7 and by converting luminal cells into a stem-like state (Chakrabarti et al. 2014). The Wnt receptors LRP5/6 are necessary for ductal stem cell induction and maintenance, and the over expression of LRP5 has been implicated in basal breast cancer (Lindvall et al. 2006, Badders et al. 2009). Further,

LRP6 has also been implicated in basal breast cancer (Lindvall et al. 2006, Yang et al. 2011). While mammary gland growth usually takes about 7 weeks to complete, ductal outgrowth is accelerated in the MMTV-Wnt1 mouse, starting as early as 1 to 2 weeks of age (Lin et al. 1992).

Wnt signaling pathway: The canonical Wnt signaling pathway is initiated by the binding of Wnt ligands to receptors LRP5/6 or Frizzled, causing β-catenin to translocate to the nucleus and forming an adhesion complex with E-cadherin, α-catenin and actin, and associating with T-cell factor (TCF) and lymphoid enhancer factor (LEF) (Hoogeboom and Burgering 2009). β-catenin levels are controlled by phosphorylation followed by degradation by a multi-component complex composed of proteins including Axin and GSK-3β . However, the activation of a Wnt ligand can lead to phosphorylation of LRP5/6 preventing formation of this complex (Braune, Seshire and Lendahl 2018), as cytosolic β-catenin is regulated by interactions with proteins such as APC, GSK-3β, and axin (Howe and Brown 2004, Clevers 2004, Hatsell et al. 2003, Rowlands et al. 2004). Nuclear β-catenin has been found in breast tumors (Geyer et al. 2011), and β-catenin stabilization and amplification of the target cyclin D1 has been found in more than 50% of breast carcinomas (Lin et al. 2000, Ryo et al. 2001). Integrin-linked kinase (ILK) has also been shown to cooperate with and accelerate Wnt1 tumor formation. ILK is a cytoplasmic effector of integrin receptors that is involved in antiapoptotic signaling, promotion of cell cycling (Hannigan, Troussard and Dedhar 2005, McDonald et al. 2008), and promotion of nuclearization of β-catenin, the same as Wnt1 expression. The use of a bi-transgenic mouse model with overexpression of Wnt1 along with overexpression of ILK demonstrates that these cooperating factors promotes mammary

tumor formation with respect to latency, growth rate, and the proportion of proliferating cells (Oloumi et al. 2010). Wnt1 tumors have been shown to have enhanced expression of ILK, which is necessary for the enhanced expression of cyclin D1, suggesting that perhaps ILK overexpression may be necessary for the malignant Wnt1 phenotype (D'Amico et al. 2000).

The MMTV-Wnt1 model for breast cancer: The mouse mammary tumor virus (MMTV) induces mammary tumors by activating proto-oncogenes via mutagenesis. The Int-1 gene was identified as one of these frequently targeted genes (Nusse and Varmus 1982), and was later renamed Wnt-1 because of the genetic similarities with the Drosophila Wingless gene (Nusse et al. 1991). The transgenic mouse model was created by Tsukamoto et al. in 1998, who inserted the MMTV-LTR upstream of the Wnt-1 promoter and gene. In this model, mammary ductal hyperplasias are very prominent by gestational day 18 (Cunha and Hom 1996) and become more prominent 2 weeks after birth in the TG females (Lin et al. 1992). Approximately 50% of virgin animals of the original strain present with tumors by 6 months of age, with the remainder of animals developing tumors by 1 year of age (Shackleford et al. 1993, Tsukamoto et al. 1988). .

Wnt1 tumors are described as a basal type tumor (Herschkowitz et al. 2007); overall, basal like tumors in humans tend to be more aggressive and difficult to treat, and have high cellular diversity and heterogeneity (Rakha et al. 2009, Kim, Goel and Alexander 2011). Overall, Wnt1 tumors tend to be composed of luminal and basal epithelial cells (Rosner et al. 2002, Cui and Donehower 2000). Wnt1 tumors contain a larger amount of luminal cells than basal cells, however when analyzing only tumor

initiating cells, the tumor initiating activity was enhanced largely in the basal cell compartment compared to the luminal cell compartment (Kim et al. 2011). However, using a dilution assay and analyzing both cell types, it was found that luminal cells were able to reconstitute the original tumor with the same proportion of luminal and basal cells (Kim et al. 2011). This suggests that activity within the luminal cell population allows for these cells to generate these tumors. Keratin 6 and Sca-1, both markers of mammary stem or progenitor cells, are more highly expressed in mammary glands as well as tumors from Wnt1 mice versus their control counterparts, suggesting that Wnt1 tumors may originate from a progenitor cell (Li et al. 2003). Although basal like tumors are often described as triple negative, Wnt1 tumors have been found to express hormone receptors, such as PR and ERα (Zhang et al. 2005).

Notch signaling pathway: The Notch pathway maintains development of organs and maintains stem and progenitor cell state. Signaling is initiated when a ligand, such as Jagged or Delta-like, from one cell interacts with the receptor of another cell, causing cleavage of the notch receptor. The C terminus of the receptor translocates to the nucleus, where it regulates downstream signaling. Some of these downstream targets include Hes and Hey1, which can be activated by constitutive Notch1 signaling (Nishimura et al. 1998, Jarriault et al. 1995, Maier and Gessler 2000). The Notch pathway has been identified as a necessary component for Wnt1 mediated tumorigenesis in human mammary epithelial cells (Ayyanan et al. 2006). The reason that Notch signaling plays a role in Wnt signaling is not completely clear; however, there are overlapping factors and interactions that occur that could explain the relationship. These

include increased expression of Notch ligands and receptors coinciding with increased Wnt signaling, and vice versa (Estrach et al. 2006, Corada et al. 2010, Ungerback et al. 2011, Chakrabarti et al. 2018). Notch signaling has been shown to promote renewal of stem cells such as human mammary epithelial cells (Dontu et al. 2004, Crosnier, Stamataki and Lewis 2006). It has also been found that constitutive Notch signaling increases the luminal progenitor population (Bouras et al. 2008). Notch signaling has been implicated in breast cancer, with evidence suggesting that the Notch inhibitor Numb is decreased or lost in breast cancer (Pece et al. 2004), and Notch1 and Notch3 have been implicated in basal breast cancer (Lee et al. 2008, Yamaguchi et al. 2008). Because of the role the Notch pathway plays in stem cell maintenance and cell fate, it is an important pathway to analyze while studying the mammary gland composition.

In summary, there is overwhelming evidence to suggest that alcohol consumption plays a role in affecting breast cancer risk. However, some of the postulated mechanisms are under examined and outdated. The dynamic activities of the mammary epithelial cell hierarchy suggest that cells within the hierarchy may be targets of alcohol metabolism which promotes tumorigenesis. The overall aim of this study was to determine if alcohol can affect mammary tumorigenesis by targeting the mammary epithelial cell hierarchy. The specific aims were to determine (1) whether alcohol consumption affects the mammary epithelial cell composition in MMTV-Wnt1 transgenic mice, (2) whether alcohol consumption affects tumor latency, (3) whether alcohol affects mammary tumor epithelial composition, and (4) whether alcohol affects gene expression of EMT, stem related, and cell-fate related factors in mammary glands and tumors from MMTV-Wnt1 mice. Our hypothesis was that alcohol consumption would alter the mammary epithelial

cell composition to favor tumorigenesis, decrease tumor latency, and alter the tumor epithelial cell composition.

CHAPTER 2: THE EFFECT OF ALCOHOL CONSUMPTION ON MAMMARY EPITHELIAL CELL COMPOSITION AND MAMMARY TUMORIGENESIS

Introduction

Breast cancer is the most common cancer worldwide in women, and it is estimated in 2019 over 268,000 women will be diagnosed with breast cancer and over 41,000 will die from breast cancer (Siegel et al. 2019). Alcohol consumption has been shown to be a risk factor for the disease, as evidenced by epidemiology studies and animal studies (Singletary and Gapstur 2001, Zakhari and Hoek 2015). However, the mechanism that accounts for this increased risk is unknown. The mammary gland is a dynamic organ, that grows and develops through puberty until it reaches an adult state. Complete development occurs during pregnancy and lactation, after which the mammary gland morphology returns to the adult virgin state (Visvader and Stingl 2014, Inman et al. 2015). This plasticity has been an argument and basis for the mammary stem cell theory, which suggests that the major epithelial components of the gland arise from a mammary stem cell. Flow cytometry has used cell surface markers to identify luminal, basal, luminal progenitor, and mammary stem cells, and it has been shown that the mammary stem cells are located within the basal cell population and maintain the cellular hierarchy (Shackleton et al. 2006, Sleeman et al. 2006, Stingl et al. 2006a, Vaillant et al. 2008). Mammary stem cells and the mammary epithelial hierarchy have been implicated in breast tumorigenesis as they are long lived and slow dividing, making them susceptible to tumorigenesis (Celia-Terrassa 2018, Di Rocco et al. 2019). It is possible that alcohol may induce oncogenic mutations in mammary stem cells, promoting the formation of

breast cancer stem cells, as neural, liver, and intestinal stem cells have been shown to be affected by alcohol consumption (Di Rocco et al. 2019). Therefore, the overall aim of this study was to determine if alcohol can affect mammary tumorigenesis by targeting the mammary epithelial cell hierarchy.

Materials and Methods

Reagents*:* Dulbecco's Modified Eagle Medium (DMEM)/F12 and Hanks' Balanced Salt Solution (HBSS) were purchased from GE Life Sciences/HyClone (Pittsburg, PA). 0.8% ammonium chloride, 0.05% and 0.25% Trypsin-EDTA, Dispase (5 U/ml) in HBSS, DNase I, Human Recombinant EGF, Human Recombinant bFGF, Heparin Solution, 10X Gentle Collagenase/Hyaluronidase in DMEM, 10X Collagenase/Hyaluronidase in DMEM, and Fetal Bovine Serum (FBS) for Human Myeloid Colony-Forming Cells were purchased from Stem Cell Technologies (Vancouver, BC, Canada). 50 mg/ml Gentamicin and all primers for PCR and Reverse Transcription Quantitative PCR (qRT-PCR) were purchased from Sigma-Aldrich (St. Louis, MO). All Applied Biosystems products, B27 without Vitamin A, and Rabbit IgG isotype control were purchased from Thermo-Fisher Scientific (Waltham, MA). Estrogen Receptor alpha (ERα) antibody was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX). All antibodies used for flow cytometry were obtained from BD Biosciences (San Jose, CA) except for CD49f and CD61 which were purchased from BioLegend (San Diego, CA). Clone identification for each antibody was follows: Biotin Rat Anti-Mouse TER-119: TER-119; Biotin Rat Anti-Mouse CD45: 30-F11; Biotin Rat Anti-Mouse CD31: 390; FITC Rat Anti-Mouse CD24: M1/69; PE Hamster Anti-Mouse CD29: HM β1-1; PE-Cy7 anti-human/house

CD49f: GoH3; Alexa Fluor 647 Hamster Anti-Mouse CD61: 2C9.G2. The catalogue number for Streptavidin APC-Cy7 was 554063 and the catalogue number for PerCP-Cy5.5 Streptavidin was 551419.

Study design*:* Wild type FVB/NJ female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). This strain also does not spontaneously form tumors, which is important for this study. MMTV-Wnt1 female mice on an FVB/NJ background were bred in-house by crossing MMTV-Wnt1 transgenic males on an FVB/NJ background (provided by Dr. Pamela Cowin, NYU Department of Cell Biology and Ronald O. Perelman Department of Dermatology) to wild type FVB/NJ females. Females were genotyped as described below and those expressing the Wnt1 oncogene were assigned to the study. Mice were group housed with a limit of 4 mice per cage and were given ad-libitum access to LabDiet 5001 mouse chow (PMI Nutrition International, LLC). At 7 weeks of age, females were assigned to either the alcohol or control group. Animals in the alcohol group were acclimated to treatment by providing 5% alcohol with 0.2% saccharin solution in place of water from days 1 to 3 and 10% alcohol with 0.2% saccharin solution from days 4 to 9. Starting on day 10, females in the alcohol group were given a 20% alcohol with 0.2% saccharin solution for the remainder of the study. Control animals were given a 0.12% saccharin solution in place of water for the entire study. Mice were sacrificed after 8 weeks on treatment (including 9 days of acclimation, n=10 per group) to assess mammary epithelial cell composition. A second group of mice (n=23 per group) were treated ± alcohol until a tumor was found and reached an average diameter of 1.5 cm. Females were palpated twice per week, and all animals were

weighed once per week. Females in the tumor study (n=10 for alcohol and n=14 for control) were placed in an EchoMRI 3-in-1 Body Composition Analyzer (Houston, TX) to determine body composition after 16 weeks on treatment. All females were euthanized by rapid decapitation. For females sacrificed after 8 weeks of treatment, the thoracic and inguinal mammary glands and one of the abdominal mammary glands were harvested for mammary epithelial cell isolation. Isolated cells were immediately plated for mammosphere assays and analyzed by flow cytometry or stored at -80°C for subsequent analysis of gene expression. The contralateral abdominal gland was fixed in 10% NBF and used for immunohistochemistry (IHC) analysis. For animals on the tumor study, one half of each tumor was fixed in 10% NBF. The other half was either flash frozen in liquid nitrogen and stored at -80°C for subsequent analysis of gene expression or partially digested and stored at -80°C as described below. Organoids were then further digested to obtain a single cell suspension on the day of plating for tumorsphere assays and flow cytometry analysis. The contralateral mammary gland was fixed in 10% NBF and used for histology and IHC analysis while one of the remaining tumor-free thoracic or abdominal glands was flash frozen in liquid nitrogen for additional analyses. Lungs and livers were also fixed in 10% NBF.

Genotyping*:* Ear notch pieces were obtained from female mice at 18 days of age. Ear notch pieces were dissociated in an alkaline lysis reagent (25 mM NaOH and 0.2 mM EDTA) at 95°C for one hour. Genomic DNA was then obtained by adding neutralization reagent (40 mM Tris HCl). PCR was performed with 10X PCR buffer, 10X 2 mM dNTPs (Applied Biosystems), Wnt-1 primers and internal control primers (10 pmol/μl), and Taq polymerase to make a 1:10 dilution of the genomic DNA samples. PCR

products were analyzed by running samples on a 2% agarose gel to determine the presence of Wnt bands.

Mammary epithelial cell isolation: Mammary glands were dissociated in a 1X gentle collagenase/hyaluronidase solution prepared with DMEM/F12 media for 15 hours, while tumors were dissociated in a 1X collagenase/hyaluronidase solution prepared in DMEM/F12 media for 2 hours. Mammary glands and tumors were dissociated in their respective solutions in a rotator oven at 37°C. Once the tissues were dissociated, the organoids were resuspended in HBSS with 2% FBS and HEPES (referred to as HF), followed by the addition of 0.8% ammonium chloride solution to lyse red blood cells. Dissociated tumor organoids were stored in liquid nitrogen in a cryotube with media consisting of DMEM/F12, 10% FBS, and 10% DMSO. On the day of analysis, tumors and mammary organoids were further dissociated with 0.25% trypsin-EDTA, dispase, and DNaseI (1 mg/ml). Dissociated tissue was then filtered into a new conical using a VWR 40 μm strainer (Randor, PA) to obtain a single cell suspension. The isolated mammary epithelial cells and mammary tumor epithelial cells were then used for flow cytometry and sphere forming assays. Remaining cells were stored at -80°C.

Flow cytometry: Approximately 500,000 isolated mammary epithelial cells or tumor epithelial cells were resuspended in HF and first stained with biotinylated anti-Ter-119, CD45 and CD31 to label hematopoietic/endothelial cells for 30 minutes on ice in the dark. Samples were then washed with HF and stained with Streptavidin PerCP-Cy5.5 (mammary epithelial cells, 1:100) or Streptavidin APC-Cy7 (tumor epithelial cells, 1:800), CD24-FITC (1:400), CD29-PE (1:160) or CD49f-PE/Cy7 (1:200) and CD61-

Alexa Fluor 647 (1:800) for 30 minutes in the dark. DAPI (1:10,000) was added prior to flow analysis. Single color controls were used for compensation to ensure accurate gating methods, using cells stained only with DAPI, Lin^+ antibodies (biotinylated) with Streptavidin, FITC, PE, or Alexa Fluor 647. Gating was set to include only single, viable, and lineage negative cells, and the proportion of luminal, basal, and luminal progenitor epithelial cells was determined. Samples were analyzed using a Beckman Coulter Gallios Flow Cytometer (Brea, CA). Data was analyzed using the Tree Star software FlowJo (Ashland, OR).

Sphere forming assays: Mammary epithelial cells isolated from hyperplastic mammary glands or mammary tumors were plated in triplicate at 10,000 cells/well and 100,000 cells/well, respectively, on 6 well ultra-low attachment plates (Corning Incorporated, Corning NY), in DMEM/F12 solution with 2% B27, 20 ng/ml EGF, 20 ng/ml FGF, 50 μg/ml gentamicin, and 10 μg/ml heparin (referred to as MS media). Cells were fed every 3 days with MS media, passaged after one week to obtain secondary spheres, re-plated, and passaged after another week to obtain tertiary spheres. Secondary and tertiary spheres were plated at 5,000 cells/well in MS media. For passaging, spheres were pipetted up and collected into a 15 ml conical, centrifuged at 600 rpm for 4 min, resuspended in 0.05% trypsin-EDTA, and incubated at 37°C for 5 minutes. Samples were then gently pipetted up and down for 1 minute to obtain a single-cell suspension then incubated again at 37°C for 5 min. Cells were neutralized with HF and centrifuged again at 600 rpm for 4 min. Mammosphere/tumorsphere forming efficiency (MFE/TFE) was calculated as average number of spheres/well divided by the number of cells plated/well times 100. Tertiary spheres were collected and stored at -80ºC.

Hematoxylin and Eosin (H&E) Staining: Tumors and lungs were sectioned at 5 μm and placed on glass slides. Slides were rehydrated in xylene and decreasing concentrations of ethanol (100, 95, 90, 80, 70, and 50%) followed by tap water. Slides were then stained with hematoxylin and eosin and dehydrated in increasing concentrations of ethanol and xylene.

IHC analysis: Tissue samples were sectioned at 5 μm, placed on glass slides, and baked at 55°C degrees for 30 minutes. Slides were dehydrated in xylene and decreasing concentrations of ethanol (as described above) and Millipore water, placed in boiling 0.01M sodium citrate antigen retrieval buffer (pH 6) for 30 minutes and cooled to 45°C. Endogenous peroxidase activity was inhibited using 3% H_2O_2 for 10 minutes.
For ERα: All blocking and incubation steps took place in a humidified box using reagents from the Rabbit IgG Vectastain kit (Vector Laboratories, Burlingame, CA). Slides were blocked with normal goat serum for 20 minutes then incubated overnight in 1:500 or 1:1000 primary ERα antibody (Rabbit polyclonal IgG) diluted in 1% BSA. The rabbit isotype IgG was used as a negative control. Slides were then incubated with secondary antibody and ABC reagent for 40 minutes each. 3,3′-Diaminobenzidine (DAB) was used to detect specific staining, and hematoxylin was used as a counterstain. Images were analyzed for optical density (OD) and were calculated using Fiji (ImageJ) software.

RNA isolation and qRT-PCR: For RNA isolation from flash frozen tumor tissue, 10 mg frozen tumor tissue was weighed out and placed in Beadbug tubes with 1ml Trizol and dissociated in a Benchmark Scientific, Inc. Beadbug Microtube Homogenizer (Sayreville,

NJ). Samples were then dissociated further using the Machery-Nagle NucleoSpin® RNA kit (Bethlehem, PA). For RNA isolation from mammary epithelial cells and tumor epithelial cells, RNA was isolated using the Qiagen RNeasy® micro kit (Hilden, Germany). Two kits were used because the Qiagen micro kit can isolate RNA from much smaller amounts of starting material compared to the Machery-Nagle kit. RNA concentration was measured using a Nanodrop 1000 Spectrophotometer and integrity was measured using an Agilent Bioanalyzer. RNA was reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA RT kit. All quantitative PCR analysis was done using Applied Biosystems SYBR® green reagents and was analyzed as $2^{(-\Delta\Delta Ct)}$. Primers are listed in Table 1.

Table 1: List of PCR and qPCR primers

Gene Name	Primer Sequence
Wnt1	F: GGACTTGCTTCTCTTCTCATAGCC R: CCACACAGGCATAGAGTGTCTGC
Internal Control	F: CAAATGTTGCTTGTCTGGYG R: GTCAGTCGAGTGCACAGTTT
Elf5	F: GAGACCAAGACTGGCATCAA R: CCACAGGTGAGAACTTTGGA
ERa	F: GCGCAAGTGTTACGAAGTG R: TTCGGCCTTCCAAGTCATC
Hey1	F: TGAGCTGAGAAGGCTGGTAC R: ACCCCAAACTCCGATAGTCC
Gata3	F: CGAGATGGTACCGGGCACTA R: GACAGTTCGCGCAGGATGT
Ki67	F: CTGCCTGTTTGGAAGGAGTAT R: TGCCTCTTGCTCTTTGACTT
Nanog	F: TGCAAGAACTCTCCTCCATTC R: CGCTTGCACTTCATCCTTTG
IGFBP5	F: TTGAGGAAACTGAGGACCTCGGAA R: CCTTCTCTGTCCGTTCAACTTGCT
Snail	F: GCCGGAAGCCCAACTATAGC R: AGGGCTGCTGGAAGGTGAA
Slug (Snail2)	F: AACTACAGCGAACTGGACAC R: ACTGGGTAAAGGAGAGTGGA
Twist	F: CCGGAGACCTAGATGTCATTG R: CGCCCTGATTCTTGTGAATTT

Cyclophilin	F: TGCTGGACCAAACACAAACGGTTC R: CAAAGACCACATGCTTGCCAT

Results

Alcohol consumption increases body weight gain, decreases feed intake, and tends to alter body composition after 16 weeks on treatment: Individual animals were weighed once per week. After 16 weeks of treatment animals consuming alcohol gained more weight than control animals ($p<0.05$) (Figure 3a). Animals consuming alcohol decreased their intake of solid chow relative to the control group (Figure 3b), however, when alcohol intake was accounted for, total caloric intake (kcal per day) was increased ($p<0.05$) in the alcohol group (Figure 3c). Analysis of body composition using the EchoMRI 3-in-1 Body Composition Analyzer indicated that alcohol animals tended to exhibit a higher body fat percentage compared to the control group ($p=0.06$) (Figure 3d).

Alcohol consumption increases luminal progenitor cells in hyperplastic mammary glands: To determine whether alcohol consumption alters mammary epithelial cell composition in preneoplastic glands, mammary epithelial cells were isolated after 8 weeks of treatment and analyzed by flow cytometry. Alcohol treatment significantly increased the percentage of luminal progenitor cells ($p<0.05$) as indicated by a higher percentage of cells staining positive (+) for CD61 within the luminal ($CD24^{+}CD49f^{lo}$) population (Figure 4). There were no differences in the luminal and basal populations between the two treatment groups.

Alcohol consumption decreases mammosphere forming efficiency: The formation of mammospheres under non-adherent conditions is an assay used to demonstrate the presence and persistence of stem and stem-like cells. Only cells that maintain their stem cell properties are able to form a mammosphere while stem-like cells that eventually differentiate will not survive in the given conditions, and each true mammosphere is suggested to originate from one single stem cell (Dontu et al. 2003). Mammary epithelial cells isolated from animals in the alcohol group had a lower tertiary mammosphere forming efficiency compared to the control animals ($p<0.05$), indicating a smaller population of stem like cells with the ability to form colonies (Figure 5).

Alcohol consumption decreases tumor latency in animals that developed tumors by 43 weeks of age: Animals were palpated twice per week to detect tumor appearance. As shown in Figure 6a, Kaplan Meier analysis indicated that overall tumor latency was not affected by treatment. However, the alcohol group appeared to exhibit a biphasic curve in terms of when tumors developed. An analysis of animals that developed tumors by 43 weeks indicated that tumors appeared faster in animals consuming alcohol when only this time period was examined (Figure 6b; $p<0.05$). These data indicate that alcohol consumption decreases tumor latency in tumors that form early on but does not affect overall tumor latency.

Alcohol consumption does not affect tumor epithelial cell composition or tumorsphere forming ability: To determine whether alcohol consumption alters tumor epithelial cell composition, isolated tumor epithelial cells were analyzed by flow cytometry. There

were no differences in the luminal, basal, or luminal progenitor cell populations within the tumor epithelial cell population (Figure 7). There was also no difference in tumorsphere forming efficiency between the two treatment groups (Figure 8).

Alcohol consumption decreases expression of genes involved in EMT: A number of different genes that are involved in mammary cell lineage or tumor formation were analyzed by qRT-PCR from mammary gland (Figure 9) and tumor tissue (Figure 10) to determine whether differences that were observed in the cell populations may be due to changes in cellular regulatory pathways. Elf5 was analyzed since it is a marker of luminal cells (Oakes et al. 2008, Choi et al. 2009), and Gata3 was analyzed due to its role in luminal differentiation (Asselin-Labat et al. 2007). ERα was analyzed since MMTV-Wnt1 tumors have been shown to be ERα positive (Zhang et al. 2005). Hey1 was analyzed since it is a downstream target of Notch signaling, which has been shown to play a role in luminal cell commitment and differentiation (Bouras et al. 2008). IGFBP-5 has been shown to be involved in breast cancer in processes related to cell adhesion and survival (Akkiprik et al. 2008, Sureshbabu et al. 2012). Nanog was analyzed since it is a marker of stem cells and has been found to be expressed in MMTV-Wnt1 tumors (Rota et al. 2014, Katoh 2011). Ki67 was analyzed since it is a marker of proliferation, and has been shown to be upregulated in cells that have a loss of p53 (Chiche et al. 2013). EMT is a process that is necessary for metastasis and has been found to be expressed in stem cells, therefore 3 EMT genes were analyzed (Batlle et al. 2000, Cano et al. 2000, Mani et al. 2008, Nassour et al. 2012). Of the genes studied, ki67 expression was decreased in mammary glands from the alcohol group compared to controls ($p=0.053$) though no

difference was observed in the tumors. Both mammary glands and tumors from animals in the alcohol group had lower expression of Snail, a gene that promotes EMT, compared to the control group ($p<0.05$). The EMT factor Twist also tended to be down-regulated in the mammary glands from the alcohol group ($p=0.064$). No differences were found between the mammary glands or tumors of alcohol and control fed animals for the other genes analyzed (Figures 9 and 10).

Alcohol consumption does not promote metastases to the lungs: Gross morphology of a small subset of lungs was analyzed by staining with H&E to determine micro metastases. None were observed in the analyzed samples (Figure 11a and 11b).

Alcohol consumption does not alter estrogen receptor alpha (ERα) positivity of tumors: While ERα mRNA expression was not affected by alcohol treatment (Figure 10), ERα positivity of the tumors was assessed by IHC to determine if protein expression was altered. There was a wide range of expression in both treatment groups (Figure 12a and 12b). Alcohol did not affect ERα protein expression in the tumors when analyzed by optical density (Figure 12c).

Discussion

This study asked the question of whether alcohol can promote breast cancer by affecting the mammary stem cell system. Alcohol consumption alone does not induce tumorigenesis in animal models, therefore an oncogenic model is needed to determine if alcohol consumption affects tumorigenesis. For the present work, we used the MMTV-Wnt1 mouse model, which spontaneously develops mammary tumors that arise from an

alteration in the mammary epithelial hierarchy (Tsukamoto et al. 1988). The MMTV-Wnt1 mouse model is a beneficial model for studying breast cancer, as these mice develop mammary tumors composed of luminal and basal cells, and the tumors are believed to originate from a stem-like cell (Li et al. 2003). Wnt signaling is also an essential process for stem cell promotion and maintenance and some of the downstream targets of Wnt signaling have been implicated in breast cancer (Pohl et al. 2017, Howe and Brown 2004).

To study the effects of alcohol on the preneoplastic stages of tumorigenesis, we looked at the mammary epithelial composition after 8 weeks of alcohol consumption starting at 7 weeks of age. While the percentages of luminal and basal cells were not affected by alcohol, the mammary glands from the alcohol animals had an increase in the luminal progenitor population ($p<0.05$). Luminal progenitor cells are stem-like cells that are committed to forming cells of the luminal lineage. Both basal and luminal cells isolated from MMTV-Wnt1 tumors are able to reconstitute the original tumor, although with a lower efficiency for the luminal cells (Kim et al. 2011), suggesting that there is sufficient activity within the luminal population capable for tumor formation, which may be the luminal progenitor population. Luminal progenitor cells are susceptible to DNA damage due to shorter telomere length than mature luminal cells (Kannan et al. 2013).

We also conducted mammosphere assays, which allow for only stem and stem-like cells to proliferate and propogate (Dontu et al. 2003, Singh et al. 2003, Ponti et al. 2005). Primary spheres most likely consist of luminal, basal, and stromal cells can be formed due to aggregation by the stromal and luminal cells rather than stem cell persistence, and passaging eliminates this potential and allows only for true sphere

formation by stem cells (Dong et al. 2013). Spheres were passed to the tertiary stage to ensure that cells were true stem-like cells. Interestingly, mammosphere forming efficiency of tertiary spheres was decreased in the alcohol group compared to the control group ($p<0.05$), suggesting that the overall mammary stem-like cell population may be lower in the alcohol group. While mammary stem cells are thought to reside in the basal compartment (Shackleton et al. 2006, Stingl et al. 2006a, Sleeman et al. 2006), we did not specifically identify the mammary stem cell population with our cell surface markers used for flow cytometry, since our markers also identify mature myoepithelial cells and presumptive basal progenitor cells. Cells that are analyzed by flow cytometry can be sorted based on expression of luminal and basal cell surface markers and replated to follow subsequent mammosphere formation. This approach has been used to show that isolated mammary stem cells form mammospheres in culture (Chiche et al. 2013, Dong et al. 2013). The latter established that the mammospheres formed from basal cells exclusively formed mammary structures which is the gold standard for identifying mammary stem cells (Dong et al. 2013, Stingl et al. 2006a). Furthermore, Cicalese et al. found that the only cells within the mammosphere that could self renew were the slow dividing mammary stem cells, not the more rapidly dividing progenitor cells (Cicalese et al. 2009). This is supported by Chen et al. who found that cells that were in the center of an individual mammosphere expressed cell surface markers that identify mammary stem cells rather than progenitor cells (Chen et al. 2007). If mammosphere forming efficiency does reflect stem cell number, our data suggest that alcohol treatment may decrease stem cells but increase the number of luminal progenitor cells which are further down in the

mammary epithelial lineage. Further studies will determine what specific cell types comprise the mammospheres.

Luminal progenitor cell are suggested to act as the tumor initiating cells in the basal breast cancer subtype (Lim et al. 2009, Shehata et al. 2012), and MMTV-Wnt1 tumors exhibit the basal phenotype (Herschkowitz et al. 2007). Given the increase in tumor initiating cells in the mammary gland after 8 weeks of alcohol consumption, we were interested in determining if tumor latency was also affected. Our data indicate that while overall tumor latency was not affected by alcohol consumption, the animals in the alcohol group exhibited a biphasic curve for this parameter, with a subset of animals developing tumors earlier in the alcohol group. The increased rate of tumor development in the alcohol group slowed after 43 weeks of age, after which time it was similar to the control group. When we analyzed only the animals that presented a tumor by 43 weeks of age, we found a decrease in tumor latency in the alcohol group ($p<0.05$). MMTV-Wnt1 tumors have also been found to be influenced by other mutations, such as Ha-Ras (Podsypanina, Li and Varmus 2004), Pten (Li et al. 2001), and p53 (Donehower et al. 1995). p53 has been shown to play a critical role in alcohol-induced cell damage in breast cancer cells, with loss of p53 inhibiting cell cycle arrest after alcohol exposure (Zhao et al. 2017). c-myc, a target of p53 in mammary stem cells, mammary cancer stem cells, and a downstream target of Wnt signaling, has been shown to be constitutively active in the absence of p53 in ErbB2 tumors (Santoro et al. 2019). It would be interesting to determine if different mutations are present in the early tumors versus the late tumors in animals exposed to alcohol.

Given that we saw an early change in mamamary epithelial cell composition in terms of luminal progenitor cells, we were interested to see whether alcohol consumption ultimately alters the tumor epithelial cell composition. Our data indicate that alcohol did not change the luminal, basal, or luminal progenitor composition of the mammary tumors, nor did it affect tumorsphere forming efficiency. Therefore, while alcohol decreased tumor latency, overall composition of the resulting tumors was not affected. In terms of tumor composition, our data showed an approximately equal number of luminal and basal cells within the tumors, confirming what has been reported for MMTV-Wnt1 mammary tumors (Cho et al. 2008). In contrast, another study found an approximate ratio of luminal to basal cells of 1.9 in this model (Rota et al. 2014), while another study reported a relatively small basal population (Kim et al. 2011). Tumorsphere number has been shown to increases with serial passaging, specifically in ErbB2 mammary tumors, indicating the persistence and immortality of mammary stem cells within the tumors of this model (Cicalese et al. 2009). Our data did not match this finding, with tumorsphere forming efficiency decreasing from the secondary to tertiary passage in both groups, suggesting that the stem-like cells within the tumorspheres may have been more differentiated and further along the hierarchy.

EMT is a characteristic of cancer cells that is necessary for metastasis. To determine if this pathway was affected by alcohol treatment, we analyzed 3 EMT genes. Snail expression decreased in both the mammary gland and tumor mRNA, ($p<0.05$), and there was a decrease in Twist expression in the mammary glands ($p=0.064$). Snail has been implicated in embryonic development, more specifically neural crest development and cell division and apoptosis (Nieto 2002), and is also necessary for mesoderm

development in mice (Carver et al. 2001). In breast cancer development, Snail has been found to play an antagonistic role with E-cadherin, which maintains cell-cell junctions in epithelial cells (Batlle et al. 2000, Cano et al. 2000). Twist is another developmental gene that plays a pivotal role in neural tube development, and has been implicated in breast cancer. Specifically, Twist transactivates AKT2 which promotes survival and proliferation (Cheng et al. 2007), and Twist has been shown to bypass Ras activated cell senescence (Ansieau et al. 2008). Human mammary epithelial cells that undergo EMT and express EMT genes have flow cytometry profiles matching those of mammary stem cells, and those that are induced to undergo EMT by transfection of EMT-inducing genes generate more mammospheres (Mani et al. 2008). This may explain the decrease in tertiary mammosphere forming efficiency in the alcohol group, which as stated above may reflect a lower stem cell population. There was also a decrease in Ki67 expression in the mammary glands ($p=0.053$), indicating lower proliferation in the mammary epithelial cells. These data also support the decrease in mammosphere forming efficiency in the alcohol group, as well as the findings of another study which found that consumption of 18% alcohol decreased metastasis to the lungs and other mammary glands compared to the control group (Vorderstrasse et al. 2012). We also stained a small subset of lungs with H&E to grossly look for micro metastases, however we did not find any as others have also found no metastases with this model.

Alcohol consumption has been shown to increase circulating levels of estrogen, which plays a role in breast cancer risk (Wong et al. 2012). To begin to examine the E2 system, we determined whether alcohol consumption affected ERα positivity. Tumors in each group varied greatly in terms of ERα positivity, with some tumors in the alcohol

and control groups staining very positive, and some very negative. However, overall, there was no difference between ERα expression in the tumors, consistent with what was found with gene expression analysis. The MMTV-Wnt1 model may be an acceptable model for the study of positive tumors, as others have demonstrated positive ERα staining (Zhang et al. 2005). This is interesting as very few rodent models of ERα breast cancer cells exist.

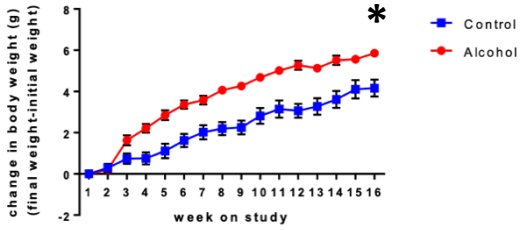
In the present study, females that consumed alcohol were significantly heavier and tended to have an increased body fat composition compared to the control animals, likely due to an increase in overall calorie consumption. Obesity is a risk factor for breast cancer, especially in post menopausal women (Bhaskaran et al. 2014, Calle et al. 2003, Robinson, Bell and Davis 2014). Recently it was shown that high fat diet-induced obesity in mice alters the mammary epithelial cell composition, with an increase in the luminal to basal cell ratio, a decrease in the basal cell population, and reduced ductal brancing during puberty (Chamberlin, D'Amato and Arendt 2017). Therefore, we cannot rule out that the changes in body weight and fat composition observed with alcohol consumption may act indirectly on the mammary gland to alter the mammary epithelial cell architecture. It will be important to determine if alcohol affects mammary epithelial cell composition when caloric intake is comparable between the alcohol and control groups.

To summarize, this study suggests that alcohol may decrease the mammary stem cell population but promote tumorigenesis by increasing the luminal progenitor population, which has been found to be the cell of origin in basal breast tumors. While the tumor epithelial cell populations were unaffected, animals in the alcohol group had a

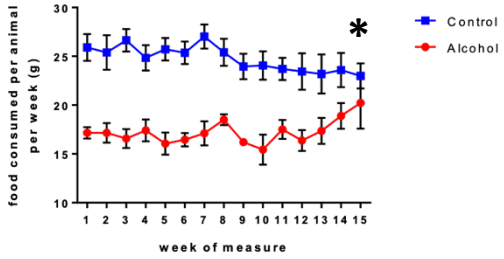
decrease in tumor latency in early forming tumors, perhaps promoted by the increase in luminal progenitor cells in the mammary gland which may be targeted for oncogenesis in this model. Alcohol consumption decreased EMT gene expression in both mammary glands and tumors, further supporting the possibility that alcohol decreases the mammary stem cell population.

Figures

a)



b)



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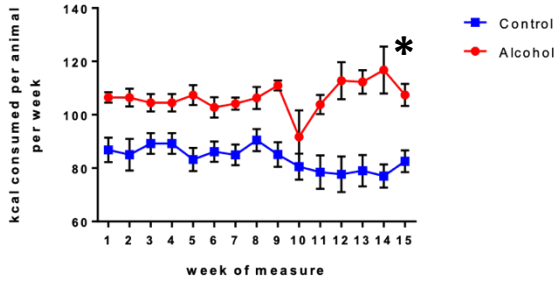

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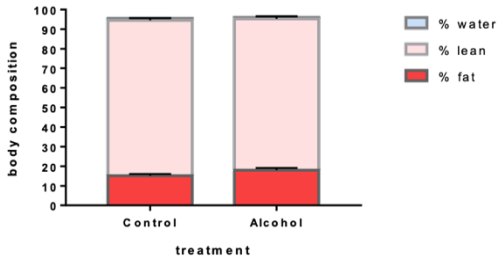


Figure 3 a) Body weight gain over 16 weeks of treatment. MMTV-Wnt1 females were assigned to alcohol (n=23) or control (n=23) treatment groups at 7 weeks of age. The alcohol group was acclimated to 20% alcohol over a 9 day period while the control group received a saccharin 0.12% solution for the duration of the study. Animals were weighed once per week until sacrifice. Alcohol animals gained more weight after 16 weeks on treatment than control animls (Two-way ANOVA with repeated measures, * indicates $p<0.05$). b) Food intake per animal per week was determined by weighing cage tops with food each week/number of animals in the cage. Food consumption analysis started in March of 2018 once a significant difference in weight was observed, and contintued for 15 weeks (Two-way ANOVA with repeated measures, * indicates $p<0.05$). c) Kilocalories (kcal) consumed per animal per day was determined by calculating the number of kcal of mouse chow consumed per animal per day (3.35 kcal/gram) plus the number of kcal of alcohol consumed per animal per day (approximately 1 gram of alcohol/day/animal, 7 kcal/gram alcohol). Alcohol animals consumed more calories per day compared to the control animals (Two-way ANOVA with repeated measures, * indicates $p<0.05$). d) Body composition was analyzed using the EchoMRI 3-in-1 analyzer after 16 weeks on treatment. Alcohol animals tended to have greater body fat percentage (Student's t-test, $p=0.06$).

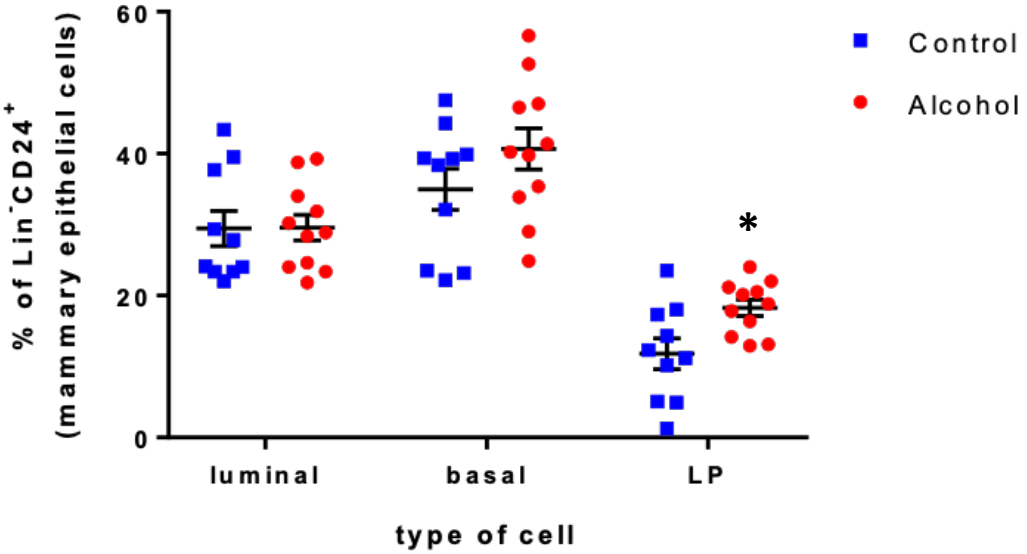


Figure 4: Composition of the mammary epithelial cell populations after 8 weeks of treatment. All cells that were lineage negative (Lin^-) and stained positive for CD24 were included for analysis. Mouse mammary epithelial cells were isolated from mammary glands of alcohol (n=11) and control (n=10) groups. Approximately 500,000 cells were analyzed by flow cytometry. The luminal population was identified as $Lin^-CD24^+CD49f^{lo}$. The basal population was identified as $Lin^-CD24^+CD49f^{hi}$. The luminal progenitor (LP) population was identified as $Lin^-CD24^+CD49f^{lo}CD61^+$. Alcohol treatment significantly increased the percentage of luminal progenitor cells (Student's t test, * indicates $p<0.05$).

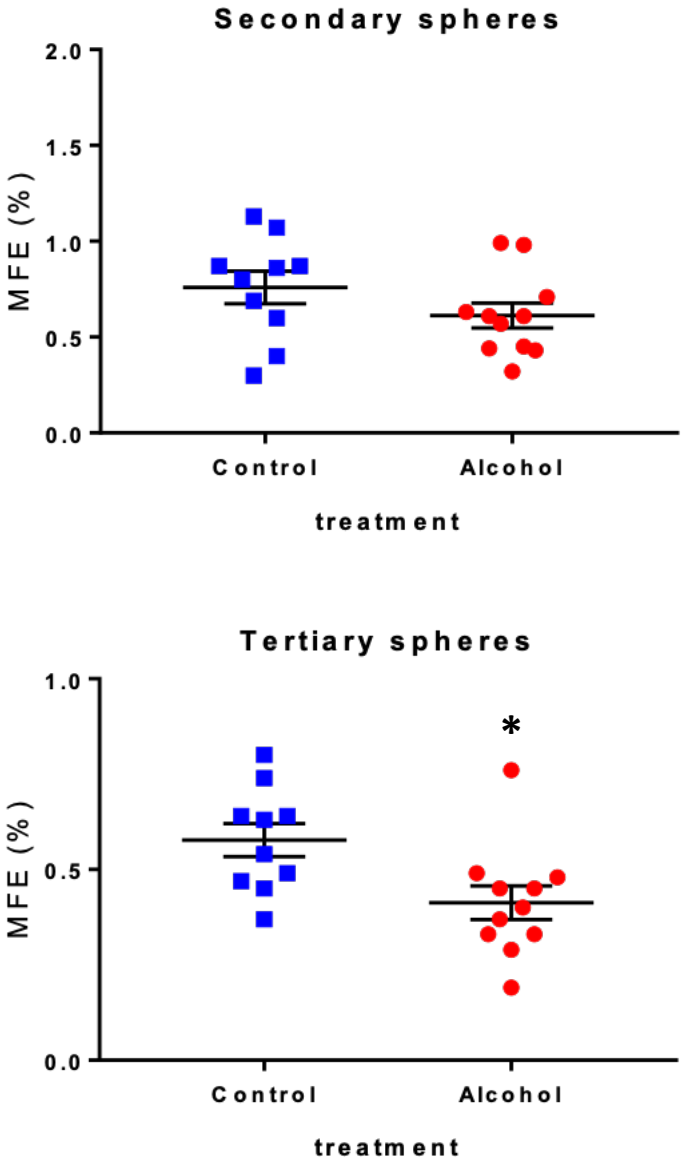
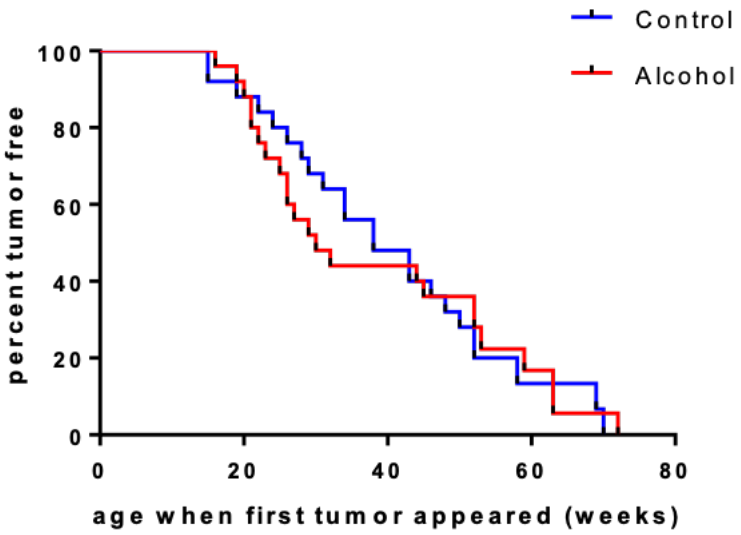


Figure 5: Mammosphere forming efficiency (MFE) of mammary epithelial cells after 8 weeks of treatment. Isolated mamamary epithelial cells from alcohol (n=11) and control (n=10) animals were plated as primary spheres at 10,000 cells/well in triplicate in MS media on ultra low attachment plates. Cells were fed with MS media every 3 days, and passaged after 1 week. Secondary and tertiary mammospheres were plated at 5,000 cells/well in triplicate. All spheres were counted and tertiary spheres were trypsinized and frozen after counting. Alcohol treatment significantly decreased mammosphere forming efficiency (Student's t test, * indicates $p<0.05$).

a)



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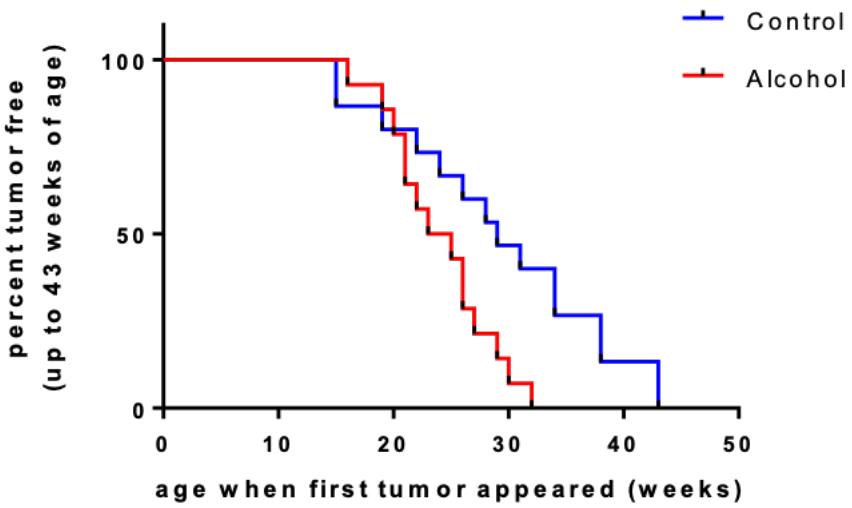

Figure 6: Effect of alcohol treatment on tumor latency. Females were palpated twice per week to detect tumor formation. a) Graph depicts age (in weeks) when a tumor was first found (n=23 for alcohol and control groups). b) Alcohol consumption decreased tumor latency in females that develop tumors by 43 weeks of age, though overall tumor latency was not affected (Mantle-Cox test, $p<0.05$).

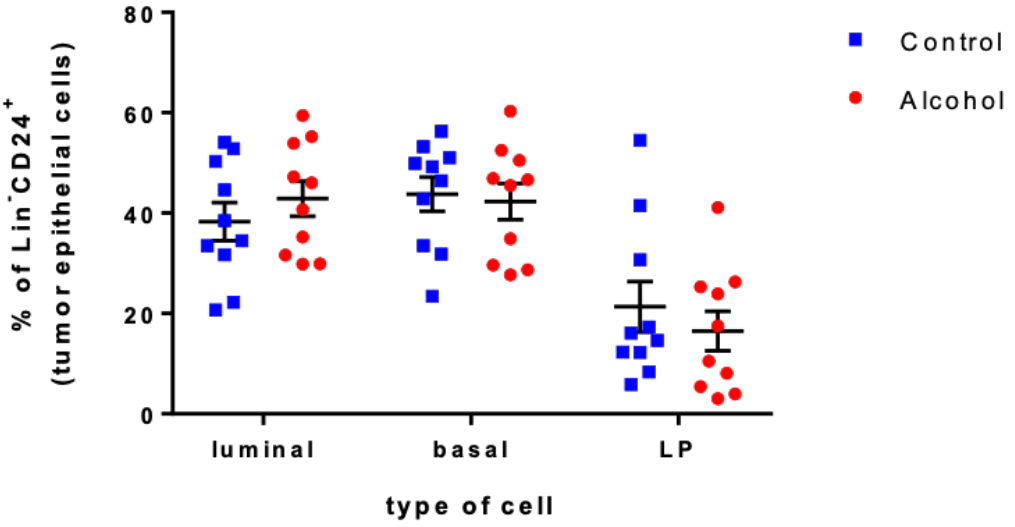

Figure 7: Composition of tumor epithelial cell populations. All cells that were lineage negative (Lin^-) and stained positive for CD24 were included for analysis. Mouse mammary tumor epithelial cells were isolated from tumors that had reached an average diameter of 1.5 cm (n=10 for alcohol and control). Approximately 500,000 cells were analyzed by flow cytometry. Luminal cells were identified as $Lin^-CD24^+CD29^{lo}$. Basal cells were identified as $Lin^-CD24^+CD29^{hi}$. Luminal progenitor (LP) cells were identified as $Lin^-CD24^+CD29^{lo}$ $CD61^+$ (Student's t test, $p>0.1$).

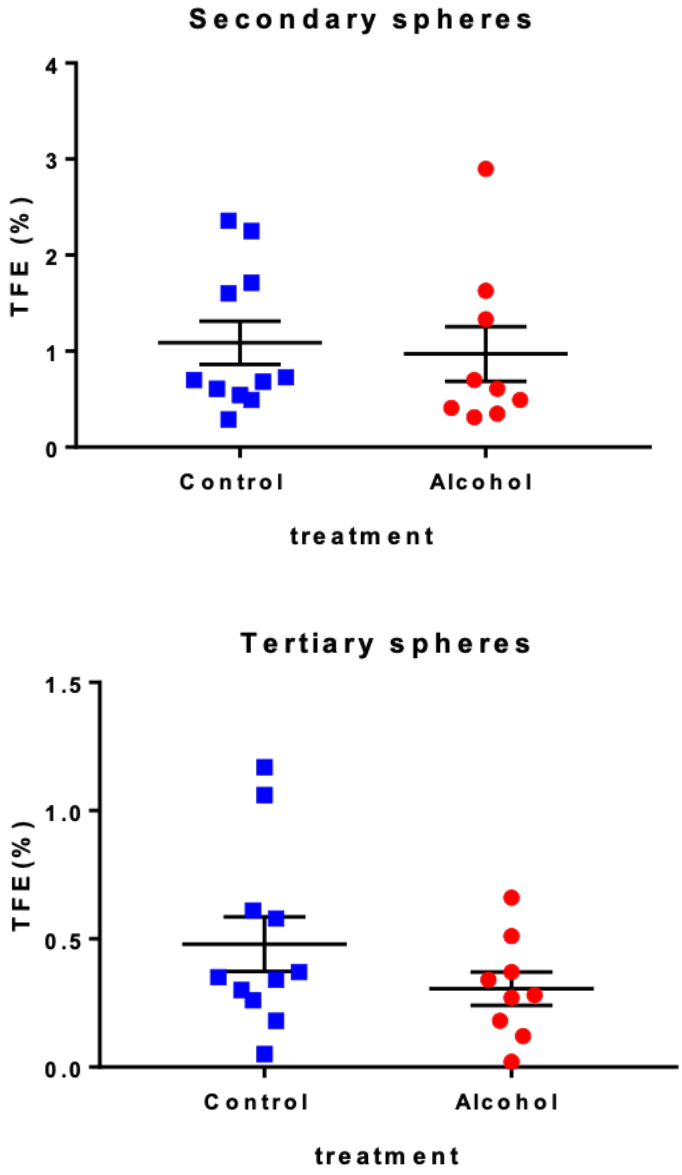

Figure 8: Tumorsphere forming efficiency (TFE) of tumor epithelial cells isolated from tumors that had reached an average diameter of 1.5 cm. Isolated tumor epithelial cells from alcohol (n=9) and control (n=11) animals were plated for primary spheres at 100,000 cells/well in triplicate in MS media on ultra low attachment plates. Cells were fed with MS media every 3 days, and passaged after 1 week. Secondary and tertiary tumorspheres were plated at 5,000 cells/well in triplicate. All spheres were counted and passaged after one week and tertiary spheres were trypsinized and frozen after counting (Student's t test, $p>0.1$).

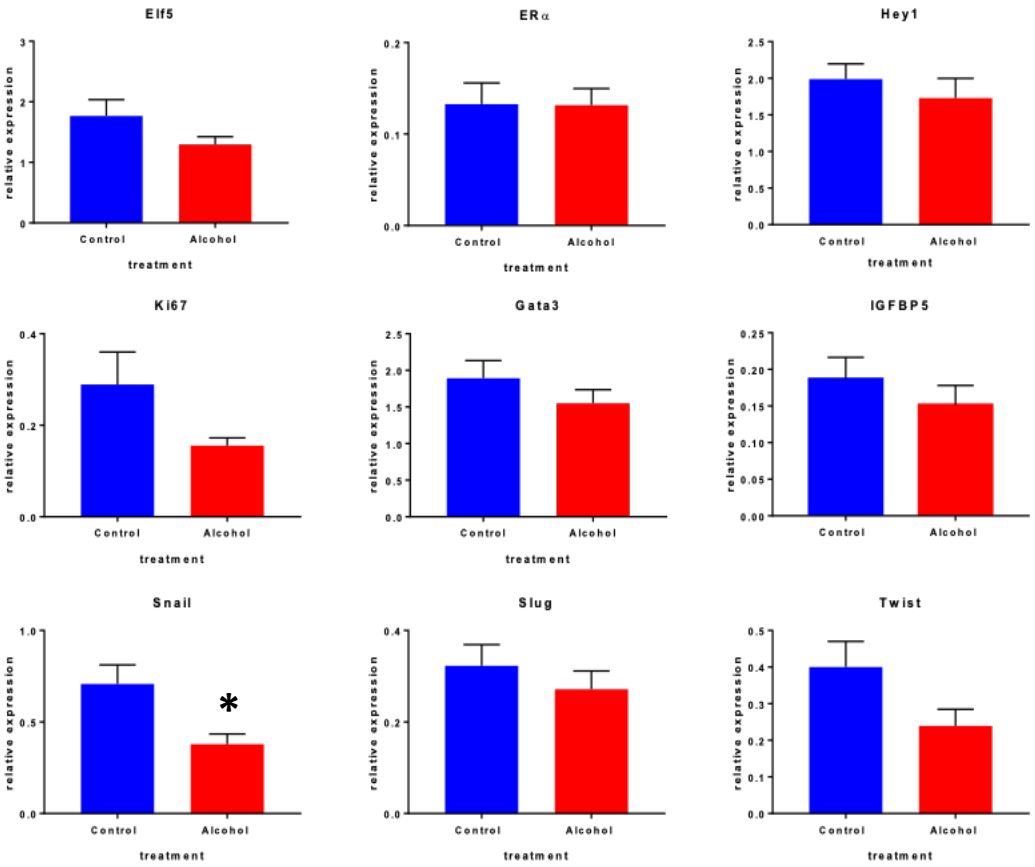

Figure 9: Gene expression analysis of mammary gland mRNA found a significant decrease in Snail expression in alcohol mammary glands and a decrease in Ki67 and Twist expression in alcohol mammary glands. mRNA was isolated from mammary epithelial cells (n =10 for alcohol and control). Isolated mRNA was reverse transcribed to cDNA which was used for gene expression analysis. Relative expression was based on expression compared to a calibrator which was composed of either a wild type untreated mammary gland pool or a tumor pool. Calibrators were set at 1, and data were analyzed as $2^{-\Delta\Delta Ct}$. (Student's t test, * indicates $p<0.05$).

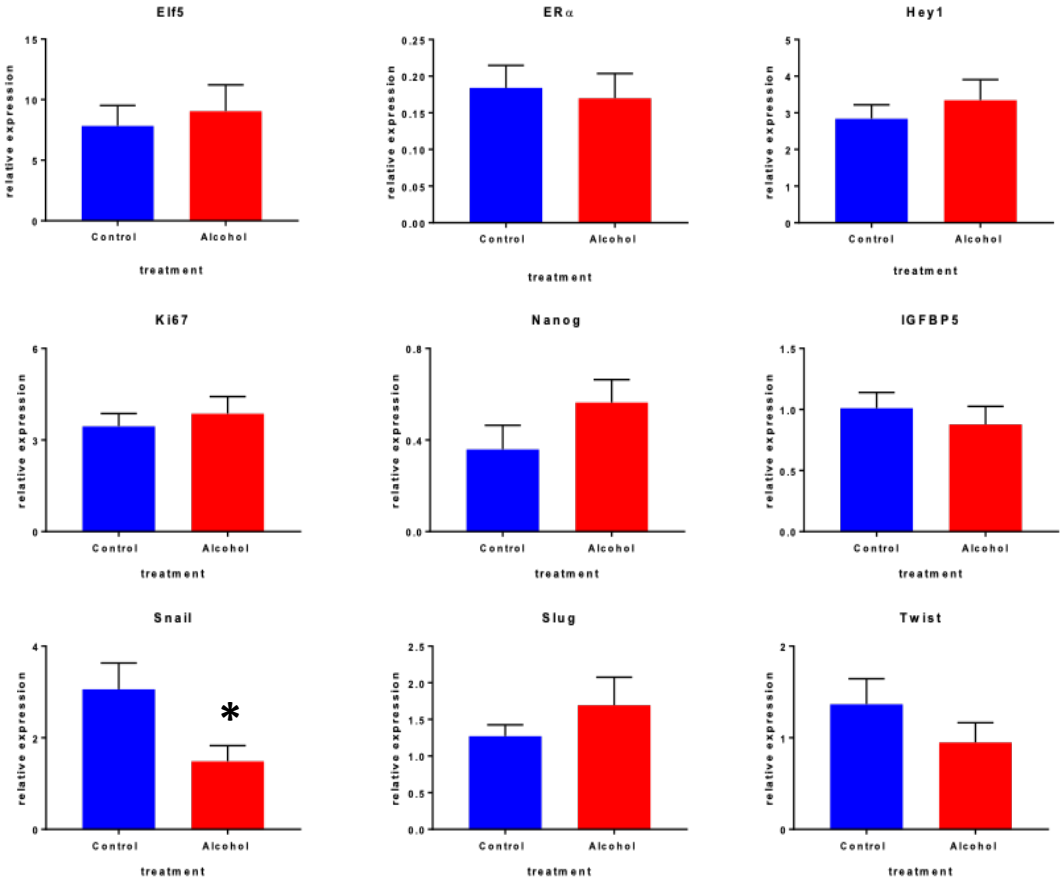

Figure 10: Gene expression analysis of mammary tumor mRNA found a significant decrease in Snail expression in alcohol mammary tumors. mRNA was isolated from mammary tumor epithelial cells and tumor tissue (n=16 for alcohol and control). Isolated mRNA was reverse transcribed to cDNA which was used for gene expression analysis. Relative expression was based on expression compared to calibrator which was composed of either a wild type untreated mammary gland pool or a tumor pool. Calibrators were set at 1 and data were analyzed as $2^{-\Delta\Delta Ct}$. (Student's t test, * indicates $p<0.05$).

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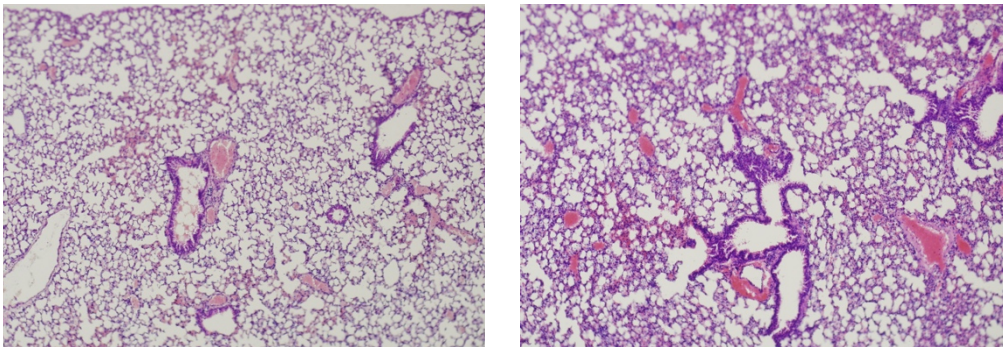

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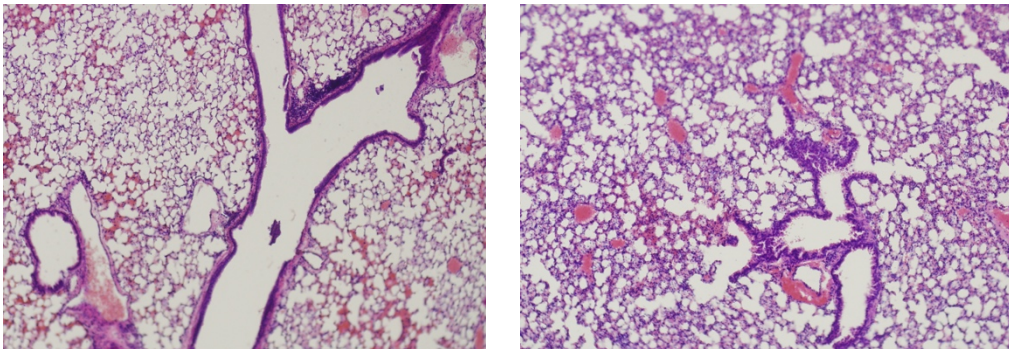
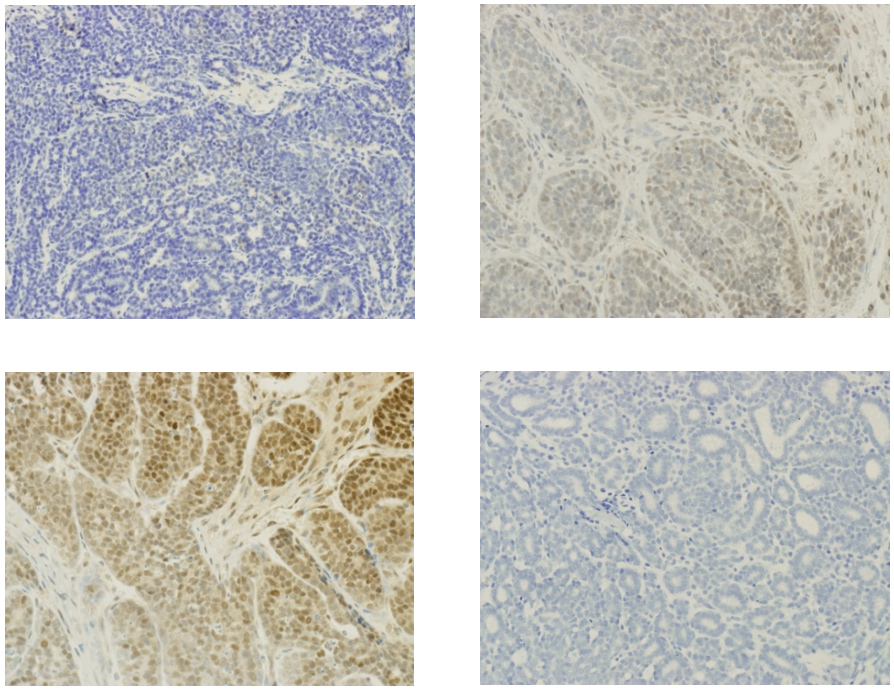


Figure 11: Representitive images of alcohol (a) and control (b) lungs from animals that presented with tumors. Photographs are 4.2 x magnification. Lungs were fixed in 10% NBF on the day of sacrifice. 5 μm tissue sections were stained with H&E to determine the presence of micrometastases.

a)



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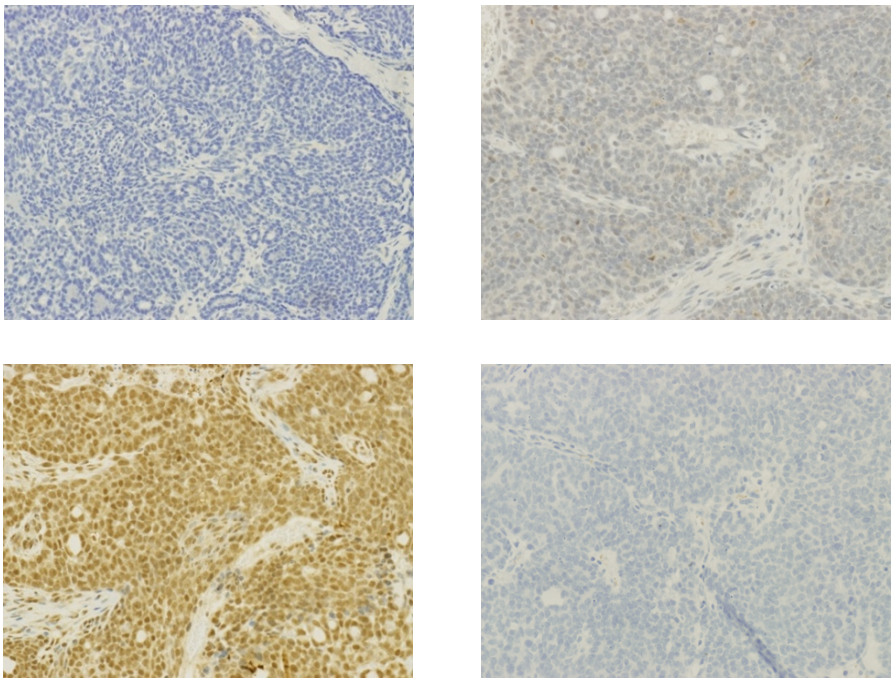

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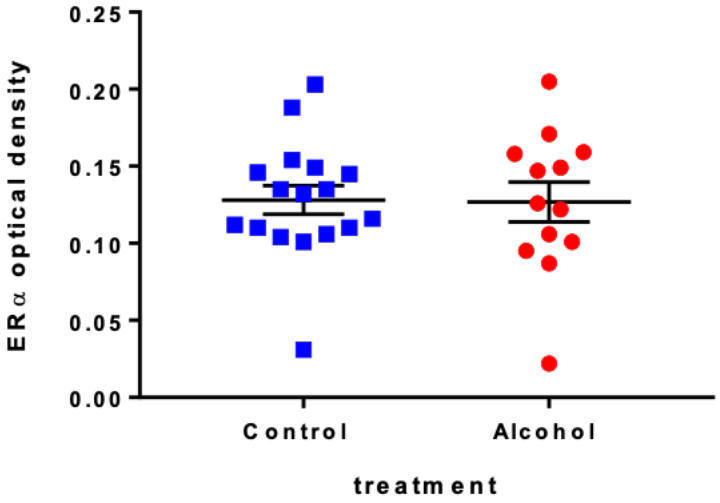

Figure 12: a and b) Representitve images of alcohol (a) and control (b) mammary tumors that were stained for ERα protein expression by IHC. Photographs are 20 x magnification. Tumors stained for ERα expression exhibited a wide range of expression from not positive (top left a and b) to medium positive (top right a and b) to very positive (bottom left a and b). The bottom right photographs (a and b) are negative controls. c) Optical density for ERα expression in mammary tumors. A portion of each tumor was fixed in 10% NBF on day of sacrifice. 5 μm sections of mammary tumors were stained with ERα using a DAB staining protocol to determine ERα positivity of tumors in both treatment groups (n=13 for alcohol and n=17 for control). 5 photographs of each sample were taken at 20 x magnification and used for analysis. ERα positivity was determined by calculating optical density using Fiji software (Student's t test, $p>0.1$).

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