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Alcohol Promotes Mammary Tumor Development through Regulation of Estrogen Signaling

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Alcohol Promotes Mammary Tumor Development through Regulation of Estrogen Signaling

by

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Dedication

To my parents and my brother, for their unconditional love and support.

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Alcohol Promotes Mammary Tumorigenesis through Regulation of Estrogen Signaling

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Breast cancer is the most common malignancy affecting women and the second leading cause of death among women in the United States. Alcohol consumption is one of the few modifiable risk factors for breast cancer development but the mechanism by which it contributes to mammary cancer development and progression remains unclear, although it has been suggested that estrogen is critical for this process. To determine if alcohol promotes mammary tumor development via the estrogen pathway, estrogen receptor alpha-negative (ERα-negative) MMTV-neu mice were treated with various doses of ethanol and activation of estrogen signaling was measured. Our results showed that alcohol consumption increased estrogen signaling activation, serum estrogen levels and, most interestingly, expression of ERα in tumor tissue in the ERα-negative mice. Several lines of evidence in literature suggest that ERα expression in ERα-negative cancer cells is inhibited through epigenetic regulation. Epigenetics is the study of heritable changes in gene expression caused by mechanisms other than DNA sequence changes. Thus, to determine whether alcohol may regulate ERα re-expression in ERα-negative breast cancer cells through epigenetic mechanisms, we examined the effects of ethanol on CpG methylation and histone modifications (acetylation and methylation) of two ERα-negative breast cancer cell lines, MDA-MB-231 (human) and MMTV-neu

(mouse). We also examined whether the epigenetic modifications subsequently affect the recruitment of transcriptional regulation complexes to the ERα promoter to regulate ERα transcription. Results showed that alcohol promotes ERα re-expression in these ERα-negative cell lines and that this effect was associated with decreased CpG methylation, an overall increase of histone acetylation and decrease of histone methylation, and an alteration in the enrichment of the ERα transcriptional regulation complexes (pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1) at the ERα promoter, which may contribute to cancer cell progression. In addition, we found that the inhibition of ERα by tamoxifen specifically blocks the effects of alcohol on ERα reactivation. To determine how alcohol promotes cell invasive ability, a critical process for cancer progression, we examined the role of two genes, metastasis suppressor Nm23 and integrin alpha-5 ITGA5, which we identified to be important for alcohol–induced breast cancer cell invasion. It has previously been shown that estrogen may regulate Nm23 expression and that estrogen regulation may be important for ITGA5-mediated cancer progression. Our results showed that alcohol promotes cancer cell invasion through the down-regulation of Nm23, which led to the subsequent increase of ITGA5 and increase of cell invasion. Collectively, data from my research strongly supports and provides evidence that alcohol promotes breast cancer development and progression through the regulation of estrogen signaling.

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Chapter 1: Introduction

1.1 Breast Cancer Background

According to the National Cancer Institute, cancer is a disease caused by the accumulation of various genetic mutations that result in abnormal cell growth [1]. Most cancers are named according to the tissue in which the cancer originates – for example, cancer that begins in the breast is called breast cancer. Breast cancer is the second leading cause of all cancer-related deaths among women in the United States after lung cancer. Approximately 200,000 women were expected to be diagnosed with malignant breast cancer in 2010 and 40,000 will die from the disease [2].

Breast cancer is defined as a malignant tumor progressing from the lobules and ducts of the breast and have the ability to invade surrounding tissues and spread to other parts of the body, which can increase the likelihood of cancer reoccurrence after treatment [2]. The spread of cancer cells from its original site to secondary sites, such as the lungs, is called metastasis. Often, it is not the primary tumor that leads to cancer patient mortality but, rather, the metastases of the cancerous cells [3].

Based on rates from 2004-06, women have a 12% lifetime risk for developing breast cancer [4]. Although breast cancer incidence rates have declined in recent years due to improved early screening and reduction of risk factors, it is important to note that the actual number of Americans who die from the disease is projected to rise in the coming years as the population increases and baby boomers reach the high-risk age for cancer [5].

Risk factors for breast cancer can be divided into two groups, non-modifiable and modifiable. Non-modifiable risk factors include: 1) *gender*, as women are 100 times more

likely to have breast cancer than men, and 2) *age*, as approximately 80% of breast cancers occur in women older than 50. Modifiable risk factors include: 1) *alcohol consumption*, as women who consume 4-5 drinks/day have an approximately 50% higher risk of developing breast cancer compared to non-drinkers, and 2) *excess weight*, as obesity increases the risk of developing breast cancer by about 50% [6]. In this dissertation, we focus on the effects of alcohol consumption and its role in promoting breast cancer risk.

1.2 ALCOHOL AND BREAST CANCER

1.2.1 REVIEW OF EPIDEMIOLOGICAL STUDIES

Alcohol consumption is a modifiable risk factor of breast cancer [7]. A total of 389,100 cases of cancer in 2002 were attributable to alcohol consumption worldwide, which represented 3.6% of all cancers that year [8]. Epidemiological studies have consistently reported a positive association between alcohol consumption and breast cancer risk. In women, breast cancer accounts for about 60% of alcohol-related cancers [8]. Alcohol is estimated to be responsible for approximately 4% of breast cancer cases in developed countries [9]. Alcohol consumption increases breast cancer risk in a dose-dependent manner; risk is increased by approximately 10% for every alcoholic drink consumed daily. For example, the risk increases by about 20% for two daily drinks, 30% for three drinks, and 50% for four or more drinks consumed [9, 10]. As evidence, Weiss et al. reported a 2.5 fold increase in breast cancer diagnosis in women consuming 14 or more alcohol drinks per week when compared to non-drinkers [11]. A drink is defined as 12 ounces of beer or 5 ounces of wine. The increase in risk remains the same regardless of the type of alcoholic beverage consumed [7].

Alcohol intake is positively related to both ductal and lobular tumors and to hormone receptor-positive tumors [12]. The most common types of breast cancer expresses both estrogen and progesterone receptors. A statistically significant positive association with alcohol was found for ER+/PR+ tumors but not for ER-/PR- tumors [12]. Interestingly, there seems to be a stronger association between alcohol use and lobular tumors than with ductal tumors [13].

While there are numerous studies addressing the linkage between alcohol use and breast cancer risk, there is limited epidemiological data concerning patient survival and prognosis after diagnosis. Recently, research conducted at the Kaiser Permanente suggested moderate to heavy consumption of alcohol (at least 3-4 drinks per week) is associated with a 1.3-fold increased risk of breast cancer recurrence [14]. The data was obtained from the Life After Cancer Epidemiology (LACE) study, which was conducted with 1,897 women successfully treated with early-stage breast cancer survivors diagnosed with early-stage invasive breast cancer and data was collected over the next eight years. A total of 349 breast cancer recurrences and 332 deaths were found among the women [14]. Elevated risk of both cancer recurrence and mortality was found predominantly among those who consumed moderate to heavy amounts of alcohol [14].

1.2.2 REVIEW OF ANIMAL STUDIES

Animal models have been used to study the effects of alcohol consumption on breast cancer progression. Available mouse models of breast cancer include: 1) chemically induced cancer models (e.g., methylnitrosourea-induced model of mammary cancer), 2) tumor xenograft models in which breast cancer cells are injected into immunodefincient or syngeneic mice, and 3) transgenic mice in which tumors develop due to a genetic disruption or mutations of key genes associated with cell growth or

differentiation. Each of these models has their strengths and weaknesses. For example, breast cancers can be divided into estrogen-responsive and estrogen-unresponsive cancers; however, to the best of our knowledge, there are no chemicals capable of inducing both estrogen-responsive and estrogen-unresponsive breast cancers. Thus, the chemically induced approach would not be a good model to determine if the estrogen responsiveness of the cancer cells influences their response to alcohol exposure. In the tumor xenograft model, the cancer is already present; therefore, if alcohol affects cancer initiation and/or promotion, it would be overlooked, as cancer is already established. One of the best models available is the transgenic model. Certain transgenic mice can be used to determine the effects of alcohol on the entire carcinogenesis process (e.g. initiation, promotion) in the absence of ovarian hormones, which mimics the postmenopausal phase in women. In addition, some transgenic mouse models of breast cancer can be used to determine if the estrogen responsiveness of the tumors cells influences their response to alcohol exposure.

Using the above models, numerous studies have shown the deleterious effects of alcohol on mammary cancer. For example, to determine the effects of alcohol on mammary tumor metastasis, Yirmiya et al. implanted mammary cancer cells into the mammary glands of rats and then fed them water or alcohol; his results showed that alcohol consumption promoted mammary adenocarcinoma metastasis [15]. Furthermore, using DMBA (7, 12-dimethylbenz(a)antracene) to induce mammary cancer, Singletary et al. reported that alcohol consumption promotes mammary tumor development by affecting both the initiation and promotion stages of mammary cancer development [16, 17]. Moreover, using DMBA in their studies, Hilakivi-Clarke et al. reported that alcohol may promote mammary tumorigenesis in rats by modulating mammary gland morphology and ERα expression [18]. However, not all studies have reported deleterious

effects of alcohol on mammary tumor development. Some animal studies report no effect [19] while others show a negative effect [20] and still others show a positive effect [21]. One of the reasons for these inconsistencies may relate to the study design or the manner in which alcohol was provided to the animals. In many cases, alcohol was provided to the animals in a liquid diet containing high levels of lipids (e.g., Lieber DeCarli diet). It is clear that consumption of high-fat diets can lead to obesity, which increases breast cancer risk in post-menopausal women [22]. Therefore, high levels of lipids in the diet can become a confounding factor in the study of the effects of alcohol on mammary cancer development. In addition, 75-80% of breast cancers occur in women after menopause [6], yet most animal studies focusing on the effects of alcohol on breast cancer were performed in animals with intact ovaries (pre-menopausal phase).

1.3 ALCOHOL METABOLISM AND BREAST CANCER

Figure 1.1 shows an overview of alcohol metabolism in the human body. Following consumption, alcohol is absorbed through the gastrointestinal tract; 20% of alcohol is absorbed in the stomach and 80% is absorbed as it passes into the small intestines [23]. Approximately 2-10% of alcohol is lost through respiration, perspiration and urination, while approximately 90% is metabolized by the liver into acetaldehyde before being converted into acetate and subsequently acetyl-CoA to enter the citric acid cycle.

In the chemical reaction in which alcohol is metabolized in the liver into acetaldehyde, NAD^+ is reduced to NADH. Acetaldehyde is toxic and can lead to oxidative stress and DNA damage [17]. Accumulated acetaldehyde and NADH may increase the activities of the enzymes xanthine oxidoreductase (XOR) and aldehyde

oxidase (AOX), which have the ability to produce high levels of reactive oxygen species (ROS) [24]. In the second step of alcohol metabolism, acetaldehyde is converted to acetate by aldehyde dehydrogenase (ALDH); this reaction does not generate ROS [24]. Another alcohol-metabolizing enzyme is cytochrome P450 2E1 (CYP2E1), which is found in liver microsomes. Metabolism of alcohol by CYP2E1 can produce high levels of ROS, causing oxidative stress and inflammation [25]. Moreover, alcohol-activated CYP2E1 can lead to the generation of lipid peroxidation products, possibly leading to DNA damage [6].

Several hypotheses have been proposed to explain the positive relationship between alcohol consumption and breast cancer risk, including: 1) alcohol induces DNA damage through the production of ROS and acetaldehyde [26] and 2) alcohol increases the production of the growth hormones (e.g., estrogen, insulin, leptin) which can promote tumor growth and metastases [26]. However, these hypotheses have not been proven. In our view, the most likely hypothesis is that alcohol affects breast cancer through growth factors such as estrogen, insulin, and leptin. Many studies have shown that factors such as estrogen and leptin can positively affect breast cancer cells [27, 28]; thus, it is conceivable for alcohol to impact breast cancer via these growth factors.

1.4 ALCOHOL, GROWTH FACTORS AND BREAST CANCER

1.4.1 GROWTH FACTORS INSULIN AND LEPTIN

As previously stated, alcohol increases the production of growth hormones (e.g., insulin, leptin, estrogen) which may promote tumor growth and metastases [26]. Insulin is a hormone secreted by the pancreas and regulates glucose uptake in skeletal muscle and adipose tissue. Insulin binds the insulin receptor (IR) and activates a signaling

cascade leading to the translocation of glucose transporter-4 (GLUT-4) to the cell membrane, where GLUT-4 mediates the diffusion of glucose molecules into the cell [29]. Binding of insulin and activation of the IR leads to increased levels of PI3K/Akt, as shown in **Figure 1.2**, which may increase breast cancer progression. Insulin resistance is the state of reduced sensitivity and responsiveness of cells to the effects of insulin, leading to increased blood insulin levels or hyperinsulinemia, and is positively associated with type 2 diabetes. Interestingly, alcohol can overcome insulin resistance [30], decreasing type 2 diabetes risk, although increasing breast cancer risk [31]. We have shown that alcohol increases insulin sensitivity [32]. However, if alcohol sensitizes breast cancer cells to insulin or other growth factors, then the tumors of alcohol-consuming mice may grow at a faster rate or may obtain a more aggressive metastatic phenotype [7]. In fact, we have shown that alcohol-consuming mice developed larger tumors than water-consuming mice [32]. Alternatively, the tumor-enhancing effects of alcohol may be independent of the insulin pathway. This could include alcohol increasing the production of growth factors (e.g. leptin and estrogen) and simultaneously increasing the sensitivity of breast cancer cells to such factors [24], as shown in **Figure 1.2**.

Leptin, which is synthesized by fat cells, acts on the hypothalamus to decrease food intake and increase energy expenditure [33]. With respect to breast cancer, evidence suggests that leptin may positively affect breast cancer development. Cell culture studies have shown that leptin stimulates the growth of cancer cells and increases their invasive phenotype [27]. Because leptin increases aromatase activity, it is suggested that leptin may promote breast cancer development indirectly through increased estrogen production [27]. Interestingly, alcohol consumption is associated with high circulating leptin levels [34]; women who consume alcohol have significantly higher serum leptin levels compared to abstainers [35].

1.4.2 GROWTH FACTOR ESTROGEN

Estrogen is a steroid hormone in the body that exists in three forms, estrone (E1), estradiol (E2), and estriol (E3). E2 is the predominant form and is produced mainly by the ovaries in premenopausal women. Following menopause, however, estrogen is produced in the peripheral tissues (e.g. adipose tissue) by the aromatase enzyme. Alcohol consumption may increase blood estrogen levels by as much as 22% in women [36]. Post-menopausal women who consume alcohol have higher circulating estrogen levels compared to non-drinkers [17]. Estrogen signaling can promote proliferation and survival of cancer cells; thus, the increased estrogen levels as a result of alcohol consumption can be considered a risk factor for breast cancer [28], as shown in **Figure 1.2**.

Estrogen replacement therapy (ERT) is often used to protect post-menopausal women from hypo-estrogenic problems such as loss of bone mineral density (BMD). Interestingly, alcohol has a larger effect on breast cancer risk in post-menopausal women on ERT compared to post-menopausal women not on ERT [36]. One study reported that, compared to the 1.0 relative risk of breast cancer development in women abstaining from alcohol, the relative risk for those consuming alcohol was 1.28, those on ERT 1.45, and those consuming alcohol and on ERT was 2.08 [37]. It is possible that alcohol sensitizes cells to estrogen. Experimental studies have shown that alcohol increases the levels of estrogen metabolites, and chronic exposure to alcohol and estrogen metabolites significantly increases the proliferation of ERα-positive tumor cells [12]. In fact, we have reported that both alcohol and estrogen can increase the invasive phenotype of breast cancer cells; however, the combination of alcohol and estrogen magnified the invasive ability of breast cancer cells at a higher level than either treatment alone [32].

1.4.3 ESTROGEN SIGNALING AND BREAST CANCER

Figure 1.3 briefly summarizes the estrogen signaling pathway involving ERα. The estrogen receptor exists in two forms, α and β. We focus exclusively on ERα as our preliminary data support the notion that ERα plays an essential role in the alcohol-breast cancer relationship. The ERα ligand, estrogen, is produced in the ovaries and peripheral tissues (e.g. adipose tissue, tumor cells) [38]. The rate-limiting enzyme in the synthesis of estrogen is aromatase [39]. Estrogen binds to ERα located in the cell membrane and in the cytosol. Activation of ERα by estrogen can lead to non-genomic and genomic signaling [40]. Non-genomic signaling occurs independent of gene expression and usually involves activation of the PI3K-Akt signaling pathway [41]. Genomic signaling involves binding of estrogen to ERα and this complex migrates to the nucleus where it can activate the expression of estrogen-responsive genes, such as ERα and pS2 [42].

As stated previously, estrogen signaling can promote cell proliferation and survival of cancer cells and that high levels of estrogen are considered a risk factor for breast cancer [28]. It is possible that alcohol increases systemic estrogen levels by increasing the levels of aromatase, an enzyme which converts androgens to estrogens [43]. High levels of estrogen may promote the development of breast cancer by activating the estrogen pathway to stimulate cancer cell proliferation.

1.4.4 ALCOHOL AND ESTROGEN SIGNALING IN BREAST CANCER

In this dissertation, we examine the role of estrogen activation (focusing on estrogen and the estrogen receptor α, ERα) as a mechanism by which alcohol consumption promotes breast cancer. To do so, we utilize the mouse mammary tumor virus (MMTV)-neu mouse model of human breast cancer and various cell lines expressing and not expressing ERα. The course of mammary tumorigenesis in MMTV-

neu mice is similar to that of humans, which proceeds from hyperplasia, to ductal carcinoma in situ, to invasive breast cancer [44].

In women, the transition from the pre-menopausal to the post-menopausal phase is linked to a drastic reduction in circulating estrogen levels [45, 46]. With respect to alcohol and breast cancer, it is not clear if the risk of breast cancer due to alcohol consumption is altered by the different levels of estrogen found in these two phases [17, 46, 47]. In this work, we determine if the effects of alcohol on mammary tumor development are affected by the presence/absence of ovarian hormones. We exposed non-ovariectomized (NOVX, representing the pre-menopausal state) and ovariectomized (OVX, representing the post-menopausal state) MMTV-neu mice to alcohol and assessed the effects of various doses of alcohol on mammary tumor development and progression. In short, we found that alcohol promoted breast cancer progression and estrogen activation in our MMTV-neu mice in the presence of ovarian estrogen (NOVX animals only), despite previous reports indicating MMTV-neu mice lacks tumor ERα expression. In other words, alcohol exposure increased tumor expression of ERα in a reportedly ERα-negative mouse model.

As mentioned previously, alcohol intake is positively related to the development of hormone receptor-positive tumors [12]. The most common types of breast cancer express the estrogen receptor. A statistically significant positive association with alcohol was found for ERα-positive tumors but not for ERα-negative tumors [12]. In a case-controlled study by Nasca *et al.* using 1,152 breast cancer patients and 1,617 controls to evaluate the relationship between alcohol consumption and estrogen receptor status, the risk of ERα-positive breast cancer was shown to increase with increasing amounts of alcohol intake in grams per day; odds ratio (OR)=1.18 for <1.5 g/day, OR=1.28 for 1.5-4.9 g/day, OR=1.28 for 5.0-14.9 g/day, and OR=1.35 for ≥15.0 g/day [48]. More

importantly, there was no relation found between alcohol consumption and ERα-negative tumors [48]. In another case-control study, conducted by Deandrea *et al*., 989 breast cancer patients and 1,350 controls were used to investigate the role of alcohol according to hormone receptor status. Findings from this study also showed that alcohol intake was associated with ERα-positive tumors; OR=2.16 for intake of ≥13.8 g/day and OR=1.13 for a 10-gram increase in daily intake [49]. For ERα-negative tumors, there was no significant relation with alcohol consumption [49]. Collectively, findings by Nasca *et al.* and Deandrea *et al*. suggest that alcohol only increases the risk of ERα-positive breast cancers [48, 49]. Other studies have also been conducted and reported similar results [28, 50, 51]. Altogether, these studies show an association between alcohol consumption and the development of ERα-positive breast cancer. However, the major weakness of these studies is that the authors had not identified the status of ERα expression in the tumor cells when the women first began to consume alcohol. It is possible that the cancer had been ERα-negative prior to alcohol exposure. As such, it is critical to determine the temporality or "time-order" of events before one can make conclusions regarding causality: Did alcohol exposure cause the development of ERα-positive breast cancer or did alcohol exposure cause the transition of ERα-negative breast cancer cells to ERα-positive? Time-order can be uncertain when a disease such as breast cancer has a long latent period and when the alcohol exposure may also represent a long duration of effect.

In the MMTV-neu mouse model, the tumors developed are reportedly estrogen-driven (ERα-positive) initially but, interestingly, become estrogen-independent (ERα-negative) later as the cancer progresses to adenocarcinoma [52]. Consistent with others [53], we show that mammary epithelial cells of MMTV-neu mice are ERα-positive; however, as the cells progress to cancer cells they become ERα-negative, as shown in **Figure 1.4**. Interestingly, we found that alcohol exposure in the mice resulted in

increased ERα expression at the mRNA level (determined by qRT-PCR) and at the protein level (determined by immunohistochemistry and immunoblot); this data is described in Chapter 2. Therefore, the expression of ERα in the tumor tissues of a reportedly ERα-negative mouse model as a result of alcohol exposure suggests that alcohol may either: 1) prevent the loss or extend the expression and function of ERα, or 2) cause re-expression of ERα, in an otherwise ERα-negative breast cancer subtype. Our study described in Chapter 3 seeks to examine the latter hypothesis. Results from our study reveal that alcohol-mediated ERα regulation is more important in the development of breast cancer than previously believed.

Chapter 3 explores the role of ERα in alcohol-induced breast cancer. Evidence suggests that the loss of ERα gene expression in ERα-negative breast cancers is not the result of DNA mutations in the ERα gene [54, 55]. Therefore, it is possible that the loss of ERα in ERα-negative breast cancer cells may be due to the loss of ERα transcription. Epigenetic dysregulation has been implicated to be critical in ERα expression [56, 57]. In fact, previous studies have shown that aberrant methylation of the ERα promoter appears in more than 25% of ERα-negative breast cancer cells [55, 56, 58]. A recent study examining altered methylation of CpG loci associated with over 700 cancer-related genes has unveiled a strong trend for patients with increasing alcohol intake to have overall decreased DNA methylation [59]. Histone modifications such as histone acetylation/deacetylation have also been linked to ERα transcriptional repression [57]. In addition, *in vivo* occupancy of the ERα promoter by the multimolecular complexes pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 has been suggested to regulate ERα transcription [60]. The role of ERα in the activation of the estrogen pathway makes it an important target for many breast cancer therapeutic strategies. Therefore, the lack of ERα at initial diagnosis in 30%

of all breast cancer cases and the loss of ERα expression from ERα-positive breast cancer cells during the course of tumor progression calls for an investigation to examine how ERα is lost and methods for ERα re-expression. In our studies, we show that alcohol exposure results in the re-expression and reactivation of ERα in ERα-negative MMTV-neu mice and ERα-negative breast cancer cells and that this may be due to epigenetic changes at the ERα promoter, namely alterations in promoter DNA methylation, histone modifications and recruitment of transcriptional regulation complexes caused by alcohol exposure.

1.5 THE LINK BETWEEN ALCOHOL AND METASTATIC BREAST CANCER

1.5.1 ALCOHOL AND METASTATIC BREAST CANCER

As mentioned above, more often than not, it is not the primary tumor that leads to the death of breast cancer patients. Often, it is the metastases, where breast cancer cells spread from the primary tumor site to secondary sites such as the lungs by invading through the basement membranes and connective tissues, which kill cancer patients [3]. Epidemiological and animal studies suggest that alcohol increases the invasive and metastatic phenotype of breast cancer cells [21, 61]. For example, Vaeth et al. showed that frequent alcohol drinkers were 1.45 times more likely than infrequent drinkers to be diagnosed with later stage breast cancer [61]. On the same note, animal studies suggest that alcohol consumption (9-12g/kg of body weight/day) may increase the incidence of lung metastasis [15]. Some researchers have suggested that alcohol may decrease tumor immune surveillance by decreasing the amount of cells capable of killing cancer cells, such as natural killer cells (NK cells). Studies have proposed that alcohol decreases the number of NK cells and T-lymphocytes in the body, mainly through destruction in the

spleen, resulting in increased tumor growth and metastasis [15, 62]. Alternatively, alcohol may influence metastasis by increasing the production of inflammation factors known to affect breast cancer progression, such as interleukin-1 (IL-1), interleukin-6 (IL-6), matrix metalloproteases (MMPs), and vascular endothelial growth factors [63-65].

1.5.2 NM23 AND ITGA5

Studies show that alcohol increases the invasive and metastatic ability of breast cancer cells [21, 61] but the mechanism is unclear. Metastases suppressing genes encode proteins that hinder the establishment of metastases without blocking the growth of the primary tumor [66]. Two such genes are the human *Nm23* genes (*Nm23-H1* and *Nm23-H2*) which have been localized to chromosome 17q21 and encode 17 kDa proteins that use its nucleoside diphosphate (NDP) kinase [67], histidine kinase [68], and exonuclease activities [69] to inhibit multiple metastatic-related processes. In this work, we focus only on *Nm23-H1*. Overexpression of *Nm23-H1* in tumor cells reduces tumor cell motility and invasion, promotes cellular differentiation, and inhibits anchorage-independent growth and adhesion to fibronectin, laminin, and vascular endothelial cells [70, 71].

While *Nm23* works to prevent the spread of breast cancer, *ITGA5* produces an integral membrane protein that increases the metastasis of breast cancer cells [72]. *ITGA5* is found on chromosome 12q11-q13 and encodes integrin alpha-5, a fibronectin receptor protein [73]. Through binding to fibronectin, an extracellular glycoprotein, ITGA5 facilitates cellular growth and migration [72, 74]. Integrins associate with adaptor proteins, cytoplasmic kinases and transmembrane growth factor receptors to trigger biochemical signaling pathways [75]. Overexpression of *ITGA5* leads to increased cellular adhesion and interaction with fibronectin, resulting in promoted tumor metastasis [72].

We report, for the first time, the effects of alcohol on the Nm23-ITGA5 pathway and show that regulation of this pathway is important for *in vitro* cellular invasion of T47D human breast cancer cells.

1.6 SUMMARY AND PERSPECTIVES

Alcohol consumption is a modifiable risk factor for breast cancer. In this dissertation, we note possible mechanisms by which alcohol may promote breast cancer development and progression. However, many questions remain to be answered; for example: Does the estrogen responsiveness of breast cancer cells influence their response to alcohol exposure? Additionally, the data suggest that alcohol affects breast cancer risk through estrogen; thus, we ask: Is adipose tissue, which is the main source of estrogen after menopause, necessary for alcohol to impact mammary cancer development? Continued research in this area will provide greater insight into the relationship between alcohol consumption and breast cancer promotion.

Chapter 2 will determine if the effects of alcohol on mammary tumor development are dependent on the presence of ovarian estrogen.

Chapter 3 will examine the role of alcohol on ERα re-expression through changes in DNA methylation, histone modifications (histone acetylation and histone methylation) and recruitment of transcriptional regulation complexes on the ERα promoter *in vitro*.

Chapter 4 will describe the identification of two genes, Nm23 and ITGA5, and examine their role in alcohol-induced breast cancer cell invasion.

Chapter 5 will provide a summary and directions for future work.

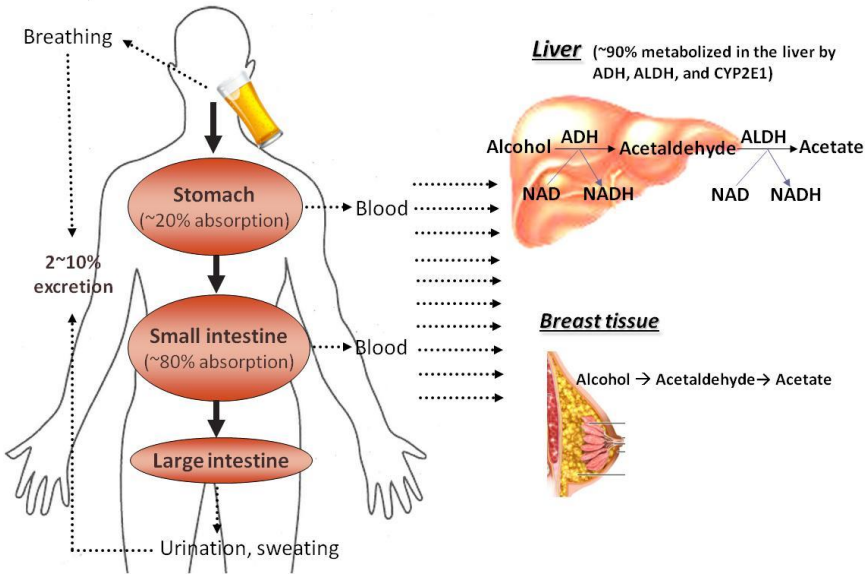

Figure 1.1: Alcohol metabolism. Approximately 20% of the alcohol consumed is absorbed in the stomach and about 80% in the small intestine. Most of the alcohol is metabolized by the liver (~90%), while 2-10% is excreted through breathing, urinating, and sweating. In the liver, alcohol is metabolized by the enzymes alcohol dehydrogenase (ADH), cytochrome P4502E1 (CYP2E1), and acetaldehyde dehydrogenase (ALDH). Alcohol can also be metabolized in the mammary gland since ADH and ALDH are expressed in mammary epithelial cells as well.

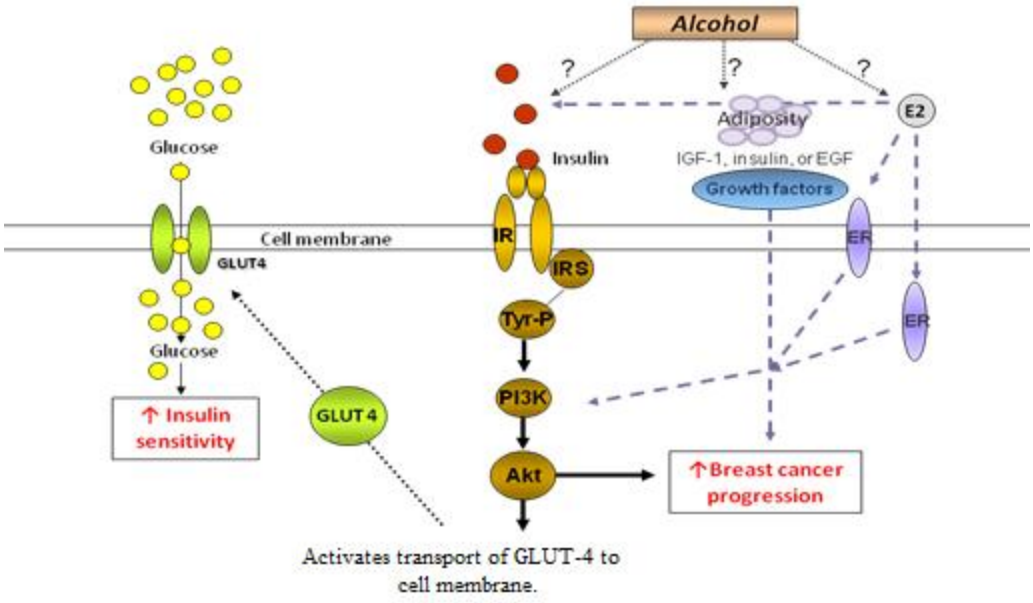

Figure 1.2: PI3K/Akt signaling pathway in insulin sensitivity and breast cancer. In the fed state, insulin is secreted by the pancreas and binds the insulin receptor to activate insulin signaling and to enhance the uptake of blood glucose by GLUT4. The activated PI3K/Akt signaling may induce breast cancer progression. Alcohol consumption may sensitize insulin and estrogen signaling pathways to enhance insulin sensitivity and promote breast cancer progression.

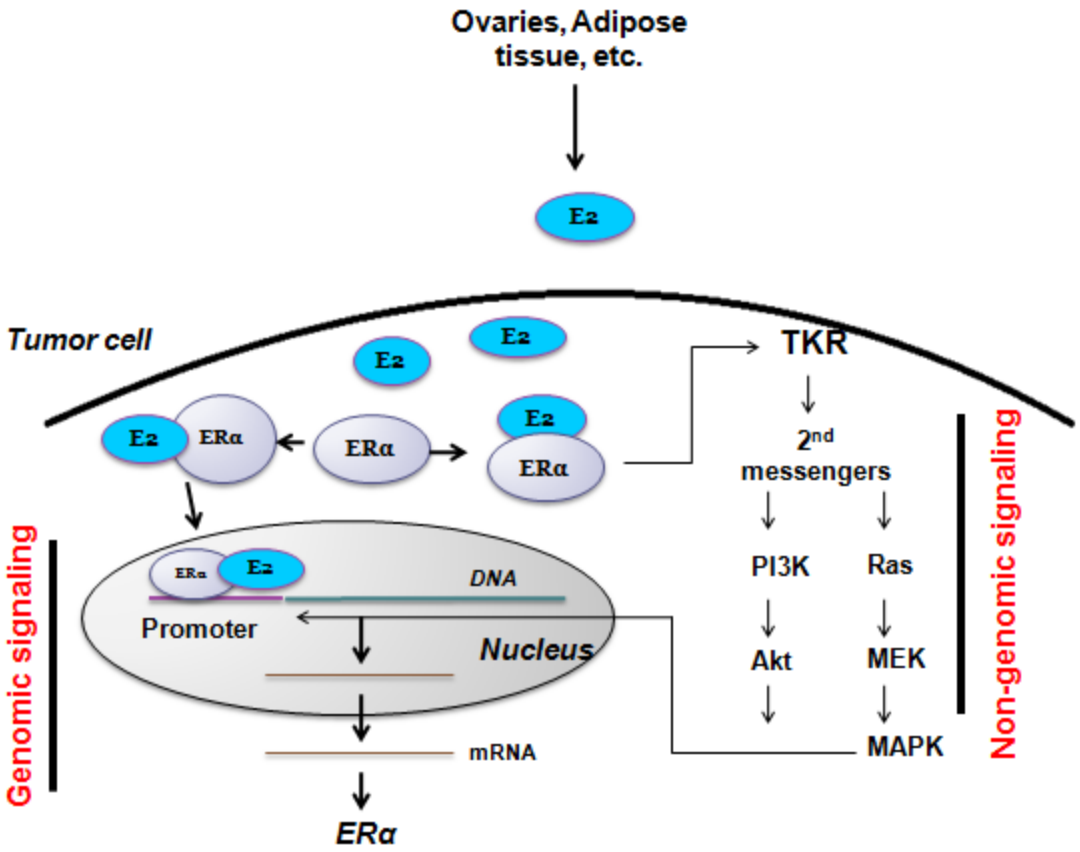

Figure 1.3: Estrogen signaling. Estrogen is produced from the ovaries, adipose tissue and tumor cells and binds to activate the estrogen receptor α (ERα). Activation of ERα leads to genomic and non-genomic signaling. Genomic signaling involves the binding of estrogen to ERα and this complex migrating to the nucleus where it can activate the expression of estrogen-responsive genes (e.g., ERα and pS2). Non-genomic signaling occur independent of gene transcription or protein synthesis and usually involves the activation of the PI3K-Akt pathway.

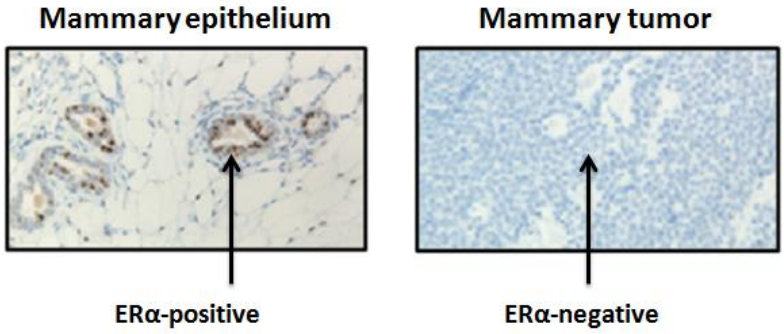

Figure 1.4: Mammary ERα expression in MMTV-neu mice. Immunohistochemical analysis shows MMTV-neu mice exhibit ERα-positive expression in mammary epithelial cells. However, as the cells progress to tumor cells, ERα expression is lost.

Chapter 2: Alcohol promotes mammary tumor development via the estrogen pathway in estrogen receptor alpha-negative HER2/neu mice

2.1 ABSTRACT

Background: Alcohol consumption is an established risk factor for breast cancer. Yet, the mechanism by which alcohol affects breast cancer development remains unresolved. The transition from the pre-menopausal to the post-menopausal phase is associated with a drastic reduction in systemic estrogen levels. It is not clear if the risk of breast cancer due to alcohol consumption is modified by the different levels of estrogen found in pre- and post-menopausal women. The objective of the present studies is to determine if the effects of alcohol on mammary tumor development are dependent on the presence of ovarian estrogen.

Methods: As a model of breast cancer, we used mouse mammary tumor virus (MMTV)-neu transgenic mice which over-express the Human Epidermal growth factor Receptor 2 (HER2/neu) in the mammary epithelium, resulting in the development of estrogen receptor α - negative mammary tumors. The mammary tumorigenesis process in these mice is similar to that of HER2 breast cancer patients. Non-ovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice were exposed to 0%, 5%, and 20% ethanol in the drinking water. Breast cancer development and progression were determined alongside the effects of alcohol on estrogen availability and signaling.

Results: Our data shows that 20% alcohol consumption promoted tumor development in MMTV-neu mice only in the presence of ovarian hormones. Tumor promotion was associated with increased systemic estrogen levels, increased expression of aromatase (the rate-limiting enzyme in estrogen synthesis), and increased expression

of estrogen receptor α (ERα) in the tumors of 20% alcohol-consuming MMTV-neu mice. Additionally, we show that ovariectomy (removal of the ovaries and ovarian hormone production) blocked the effects of 20% alcohol on tumor development.

Conclusions: Our results support the notion that alcohol consumption promotes HER2 breast cancer development via the estrogen signaling pathway. Additionally, they suggest that the effects of alcohol on breast cancer may be prevented by blocking estrogen signaling.

2.2 INTRODUCTION

Approximately 200,000 women were expected to be diagnosed with malignant breast cancer in 2010 and 40,000 will die from the disease [2]. Alcohol consumption is an established risk factor for breast cancer and increases breast cancer risk in a dose-dependent manner: risk increases by ~10% for each drink consumed per day (i.e. risk increases ~20% for two daily drinks) [7]. A drink is defined as 12 ounces of beer or 5 ounces of wine [7]. Not only does alcohol increase the risk of developing breast cancer but it may also increase the risk of breast cancer recurrence and death following breast cancer [14]. Several hypotheses have been proposed to explain how alcohol increases breast cancer risk, including: 1) alcohol increases breast cancer risk via reactive oxygen species (ROS) and acetaldehyde [76] and 2) alcohol increases breast cancer risk via hormones, such as estrogen and insulin like growth factor 1 (IGF-1) [77]. However, none of these hypotheses have been proven. In this study, we examined the role of the estrogen pathway as a mechanism by which alcohol consumption promotes breast cancer using the mouse mammary tumor virus (MMTV)-neu mouse as a model of HER2 breast cancer.

Human Epidermal growth factor Receptor 2 (HER2)-positive breast cancers account for 25% of all breast cancer cases [78]. Breast cancer patients over-expressing the HER2 protein (encoded by the neu proto-oncogene also called ErbB2) have poor prognosis [78]. In addition, HER2 cancer cells are considered more aggressive (faster growth and invasion) and result in higher rates of reoccurrence in patients [79]. To determine the effects of alcohol on HER2 breast cancer, we use MMTV-neu mice. These mice over-express the neu proto-oncogene in the mammary epithelium, which leads to the development of mammary tumors comparable to those found in HER2 patients [80]. During the initial stages of tumor development in these mice, tumor progression can be prevented or blocked with the use of tamoxifen, which blocks the binding of estrogen to ERα [52]. However, the use of tamoxifen becomes ineffective in the treatment of these tumors as tumors become estrogen-independent or ERα-negative [52]. Thus, mammary tumors in MMTV-neu mice are initially estrogen-driven (ERα-positive) but become estrogen-independent (ERα-negative) as cancer progresses to adenocarcinoma [52, 53].

In women, the transition from the pre-menopausal to the post-menopausal phase is linked with a drastic reduction in circulating estrogen levels [45, 46]. With respect to alcohol and breast cancer, it is not clear if the risk of breast cancer due to alcohol consumption is altered by the different levels of estrogen found in these two phases [17, 46, 47]. In these studies we determine if the effects of alcohol on mammary tumor development are affected by the presence/absence of ovarian hormones. For this purpose we exposed nonovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice to 0%, 5% and 20% alcohol in the drinking water and assessed the effects of the different doses of alcohol on mammary tumor development and progression.

Results show that 20% alcohol promotes mammary tumor development in MMTV-neu mice but only in the presence of ovarian estrogens; ovariectomy, resulting in

the absence of ovarian estrogen, blocked the effects of 20% alcohol on tumor development in MMTV-neu mice. The acceleration of tumor development due to alcohol consumption is associated with increased systemic estrogen levels, increased tumor expression of aromatase and increased expression of the estrogen receptor alpha (ERα) in tumor tissues. Our data supports the hypothesis that alcohol consumption promotes mammary tumor development via the estrogen pathway.

2.3 MATERIALS AND METHODS

MOUSE HUSBANDRY AND DIETS

A total of 180 pathogen-free female mouse mammary tumor virus (MMTV)-neu mice in the FVB/N genetic background were purchased from The Jackson Laboratory (Bar Harbor, MA, USA). These mice were obtained at 6-weeks of age and housed in the Animal Resources Center at the University of Texas at Austin (UT-Austin) in accordance with NIH guidelines. All animal procedures were approved by UT-Austin's Institutional Animal Care and Use Committee. Mice were singly housed in a 22-24ºC room and kept on a 12-h light/dark cycle. Following an acclimation period of one week, mice were randomized into six groups: 90 NOVX mice and 90 OVX mice; each of these groups was divided into three subgroups: 30 mice not consuming alcohol (control groups), 30 mice consuming 5% v/v ethanol, and 30 mice consuming 20% v/v ethanol *ad libitum* throughout the study. Alcohol treatment began at 9-weeks of age. Mice were fed Diet #D12450B (Research Diets Inc., New Brunswick, NJ, USA), composed of 19% protein, 67% carbohydrate and 4% fat, *ad libitum* throughout the study. Body weight, food and liquid consumption, and tumor volume were measured weekly. Serum was collected at the endpoint (week 52) for analysis.

BODY COMPOSITION

Final body weight was determined at the end of the study. All mice were used for final body weight analysis (n=30 per group). Percent body fat was determined using Dual energy X-ray Absorptiometry (DXA) with a GE Lunar Piximus II densitometer (Waukesha, WI, USA). A total of 8 mice per group were used for percent body fat analysis (n=8 per group).

OVARIECTOMY

Mice in the OVX groups were ovariectomized at 8-weeks of age. Mice were anesthetized and a small midline incision (~1.0 cm) was made in the skin halfway between the middle of the back and the base of the tail, starting at the last rib. A small incision was then made through the peritoneal lining on each side and the ovaries were removed with a single cut between the fallopian tube and the uterine horn.

TUMOR DEVELOPMENT

To detect the appearance of tumors, mice were palpated weekly starting at the age of 13-weeks until the first tumors appeared. Following the appearance of the first tumor, mice were palpated twice weekly, and tumor volume was determined by measuring the length, width, and depth of the tumor using Fisherbrand digital calipers (Thermo Fisher Scientific, Rockford, IL). Mice bearing tumors were sacrificed either at 52-weeks or once tumors reached 1.5 cm^3, whichever occurred first. At the end of the 52-week study, tumors were collected and fixed in 10% buffered formalin and embedded in paraffin. The z-test for proportions statistical analysis was used to determine significance in tumor incidence. All mice were used for tumor incidence analysis (n=30 per group). Tumor growth rate was determined by measuring the difference in tumor volume per day. All mice bearing tumors were used for tumor growth rate analysis ($n \geq 3$ per group).

SERUM ANALYSIS

At necropsy, blood from mice was collected via cardiac puncture, allowed to clot for 20 min, and centrifuged at 10000 rpm for 5 minutes. Serum was separated and stored at -80^{o}C until analysis. Serum estrogen levels were measured using an ELISA for 17β-estradiol (E2; IBL-America, Minneapolis, MN, USA). Each sample was assayed in duplicate on a single plate. A total of eight mice per group were used for analysis (n=8 per group). The reported range of this assay is 9.7–2000 pg/ml. Blood alcohol levels were determined by measuring alcohol dehydrogenase activity using the Sigma-Aldrich NADH assay (St. Louis, MO, USA). A total of nine mice per group were used for this analysis (n=9 per group).

IMMUNOHISTOCHEMISTRY

Mouse tumor tissues were harvested and fixed in 10% formalin for 24 hours before transferring to 70% ethanol. Samples were paraffin embedded, sectioned to obtain unstained tissue slides and stained to detect c-erbB2 (sc-284, 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Ki67 (#M7249, 1:200, Dako, Denmark) and ERα (sc-542, 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, USA) by the Histology and Tissue Core at the University of Texas M. D. Anderson Cancer Center (Smithville, TX, USA). Results were quantified using NIS Elements Imaging Software (Nikon Precision Inc., Belmont, CA, USA). A total of four tissue samples per group were used for analysis for each protein (n=4 per group).

WHOLE MOUNTS

Mouse mammary gland #9 was harvested and fixed onto slides with 10% formalin for 24 hours before transferring to 70% ethanol. Samples were then processed by the Histology and Tissue Core at the University of Texas M. D. Anderson Cancer Center

(Smithville, TX, USA). Visual examination of the mammary whole mounts was carried out by pathologists at the University of Texas. No quantitation was performed.

IMMUNOBLOTS

Mouse tumor tissues were harvested at necropsy, snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis. Whole-cell lysates were prepared from tumor tissue. Frozen tissues were ground into powder and suspended in RIPA buffer (Sigma, St. Louis, MO, USA) containing protease and phosphatase inhibitors (Pierce, Rockford, IL, USA). Samples were then homogenized and extracts were clarified by centrifugation at 10000 rpm for 10 min. A Bradford protein assay (Bio-Rad, Hercules, CA, USA) was used to determine protein concentrations. Lysates (50 µg) were resolved by 10% SDS-PAGE and proteins were detected by immunoblot. Expression of tumor aromatase, ERα, p-MAPK, MAPK, p-Akt and Akt were analyzed by immunoblotting using antibodies for the following proteins: aromatase (ab18995, 1:1000, Abcam, Cambridge, MA, USA), ERα (sc-542, 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), p-MAPK (#9101, 1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA), MAPK (#9102, 1:1000, Cell Signaling Technology, Inc.), p-Akt (#9275, 1:1000, Cell Signaling Technology, Inc.) and Akt (#9272, 1:1000, Cell Signaling Technology, Inc.) followed by incubation with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.) and ECL substrate (Thermo Fisher Scientific, Rockford, IL, USA). Quantification of the band intensity was determined by densitometry using ImageJ software (NIH website: http://rsbweb.nih.gov/ij/index.html). A total of 3 tumor tissue samples were used per group (n=3) for analysis.

STATISTICAL ANALYSIS

Data are expressed as means ± SEM. Significant differences among control and treatment groups were determined using two-way analysis of variance (ANOVA) followed by Bonferroni adjustment unless otherwise noted. SPSS v16 for Windows (IBM Corporation, Chicago, IL, USA) was used for all statistical comparisons. To detect statistical significance, *p* value was set to 0.05.

2.4 RESULTS

BODY WEIGHT AND BODY FAT

High body weight and high body fat levels are risk factors for breast cancer [81]. Results show that the body weight and body fat levels in alcohol and non-alcohol consuming mice were similar (**Figure 2.1A-B,** $p>0.05$). The only noticeable effect was that of ovariectomy, which increased the susceptibility of gaining body weight and body fat levels **(Figure 2.1B)**. Although ovariectomy resulted in an increase in both body weight and body fat levels, interestingly, these effects did not translate to increased tumor incidence or growth (**Figures 2.1 and 2.3**). Thus, results suggest that alcohol does not affect tumor development via body weight or body fat levels.

BLOOD ALCOHOL CONTENT (BAC) IN MMTV-NEU MICE

Consumption of 5% and 20% v/v ethanol led to low (~0.01%) and medium high (~0.05%) blood alcohol levels, respectively, in the mice (**Figure 2.2**). At present, there is limited knowledge in literature describing the relationship between blood alcohol levels, drinks/day, and breast cancer risk and linking this information from animal to human studies. From what we can currently extrapolate from literature, consumption of 1-2 drinks and 3-4 drinks in women can lead to similar (~0.02% and ~0.05%, respectively)

blood alcohol levels, depending on body weight [7, 82]. According to a study which evaluated data from over 300,000 women for up to 11 years, consumption of alcohol at these levels significantly increased breast cancer risk approximately 16% and 41%, respectively [7].

ALCOHOL PROMOTES TUMOR DEVELOPMENT IN MMTV-NEU MICE

Results show that alcohol consumption increased tumor incidence in a dose-dependent manner in NOVX mice but not in OVX mice (**Figure 2.3A**). Tumor incidence was defined as the appearance of a single palpable tumor on an animal. (In this study, multiple tumors on the mammary gland of a single animal were not observed.) Tumor incidence in the 5% and 20% alcohol consuming NOVX mice was 53.33% and 66.67%, respectively, compared to 40% in 0% alcohol consuming mice, at the endpoint of study (**Figure 2.3A**). Among NOVX mice, statistical significance in tumor incidence was seen only between the 0% and 20% alcohol consuming mice ($p<0.05$). In addition, alcohol decreased the tumor latency period in NOVX mice (**Figure 2.3A**). Conversely, no statistically significant change in mammary tumor incidence or tumor latency was observed among the OVX groups as a result of alcohol consumption ($p>0.05$). Thus, it is likely that alcohol promotes mammary tumor development only in the presence of normal systemic estrogen levels, which the OVX animals lack due to the surgical removal of the ovaries.

Results also show that 20% ethanol treatment led to a significant increase in tumor growth rate ($p<0.05$) in NOVX mice (**Figure 2.3B**). Tumor growth rate was determined using the following equation: [(Final tumor volume) - (Tumor volume at first detection)] / (Number of days tumor was present). This suggested that alcohol may promote cancer cell proliferation in NOVX mice. To determine if this was the case,

protein levels of the proliferation marker Ki67 in tumor tissues was measured (Schluter et al., 1993). Findings show alcohol significantly increased the level of Ki67 in the tumors of NOVX mice consuming 20% alcohol (2.6-fold change, $p<0.05$) but not in any other groups of mice (**Figure 2.3C**).

ALCOHOL CONSUMPTION AFFECTS THE MAMMARY GLAND STRUCTURE

Mammary gland morphology may be used as a tool for assessing breast cancer risk in women [83-85]. High mammographic density, which is associated with a high number of terminal ductal lobular units (TDLUs), can increase the risk of breast cancer 4-6 fold [84]. In the mouse, the TDLU is more often referred to as the lobualveolar (LA) structure [86]. We examined the effects of alcohol on the structure of the mammary gland of alcohol-consuming mice. **Figure 2.4** shows that alcohol consumption increased the number of LA (TDLU in humans) which is the site where many epithelial hyperplasias and carcinomas of the breast arise [84-87]. This suggests that alcohol may increase the number of cells that can potentially become cancer cells in the mammary gland.

EFFECTS OF ALCOHOL ON SYSTEMIC ESTROGEN AND TUMOR AROMATASE LEVELS

Alcohol may exert its effects on breast cancer by increasing estrogen (17β-estradiol, E2) availability [51, 88, 89]. To determine if alcohol affected systemic estrogen levels, we measured the levels of E2 in the serum of our mice. Results in **Figure 2.5A** shows that 20% alcohol significantly increased E2 levels in NOVX mice compared to NOVX mice consuming 0% and 5% alcohol ($p<0.05$). Moreover, results show ovariectomy decreased systemic E2 levels in OVX mice ($p<0.05$) to levels below the sensitivity of the ELISA, thereby verifying that the ovariectomy surgeries were successful. While 20% alcohol consumption increased estrogen levels in OVX mice, the estrogen levels were still significantly lower than those of NOVX control mice. Since

OVX mice lack ovaries, any estrogen found in their blood may come from peripheral tissues such as adipose or tumor tissue [38].

The rate-limiting enzyme in the synthesis of estrogen in these tissues is known as cytochrome p450 aromatase [38, 39]. Alcohol may increase systemic estrogen levels by affecting the expression of aromatase in tissues [39, 90]. Thus, we measured aromatase levels in the tumor tissues of the NOVX and OVX animals to determine if alcohol affected the expression of this enzyme. We found that the pattern of aromatase expression was similar to systemic E2, with NOVX mice expressing higher levels in tumor tissues compared to OVX mice (**Figures 2.5A and 2.5B**, $p<0.05$). In addition, **Figure 2.5B** shows 20% alcohol consumption in MMTV-neu mice resulted in higher tumor aromatase expression compared to 0% alcohol consuming mice in both NOVX and OVX groups ($p<0.05$). Thus, our data show that the tumor promoting effects of alcohol on NOVX mice are associated with an increase in systemic estrogen levels and an increase in tumor aromatase expression.

ALCOHOL INCREASES EXPRESSION OF ERA IN MMTV-NEU MICE

Estrogen stimulates tumor growth and proliferation of breast cancer cells by binding to the receptor ERα [77]. Tumor ERα expression in alcohol and non-alcohol consuming mice were measured to determine whether the tumor promoting effects of alcohol were associated with increased ERα expression. Immunohistochemistry (IHC) results show that alcohol increased the expression of ERα in both NOVX and OVX tumors in MMTV-neu mice which are reported to develop ERα-negative tumors (**Figure 2.6A**) [44]. These results were verified by immunoblot with breast cancer cell lines MCF-7 and MDA-MB-231 as the positive and negative controls, respectively, for ERα expression (**Figure 2.6B**). Although alcohol consumption increased the expression of

ERα in OVX mice, this increase in ERα by alcohol was not associated with an increase in tumor incidence, tumor growth, or cellular proliferation in the OVX 5% or 20% alcohol consuming mice, suggesting that ERα expression by itself is not sufficient to promote tumor development. Thus, it is feasible that alcohol-mediated HER2 breast cancer development requires the presence of normal circulating estrogen levels, which OVX animals lacked.

ALCOHOL ACTIVATES THE ESTROGEN SIGNALING PATHWAY

The above data suggest alcohol may promote HER2 tumor development through the estrogen pathway. Thus, we determined the activation of MAPK and Akt, two downstream targets of the estrogen signaling cascade, which may signify the activation of the estrogen pathway [91]. Expression of total MAPK and Akt, and their active, phosphorylated forms, p-MAPK and p-Akt were determined by immunoblot using tumor tissues. Consumption of 5% and 20% alcohol significantly increased activation of these downstream targets in the estrogen pathway in NOVX MMTV-neu mice ($p<0.05$) but not in OVX mice ($p>0.05$), as shown in **Figure 2.7**.

ALCOHOL DOES NOT AFFECT ERBB2 TRANSGENE EXPRESSION OR IGFR LEVELS

To verify that alcohol did not affect tumor development by increasing the expression of the erbB2/HER2 transgene, we measured HER2 levels in the tumors by immunohistochemistry. HER2 levels were similar among all the groups consuming alcohol and non-alcohol (data not shown). Moreover, ovariectomy did not affect HER2 levels (data not shown). We also measured systemic levels of IGF-1, a hormone that may promote mammary tumor development [92]; systemic IGF-1 levels were not affected by alcohol consumption (data not shown). In addition, we measured the expression of the IGF-1 receptor (IGF-1R) in the tumors and found the levels similar among all groups

(data not shown) [93]. Thus, these findings suggest that alcohol does not affect mammary tumor development by affecting the expression of the HER2 transgene or by affecting the IGF-1 signaling pathway.

2.5 DISCUSSION

Previous studies have reported an association between alcohol abuse or excessive drinking (≥ 3 drinks/day) and an increase in mammary tumor incidence in women (studies summarized by [17]). Smith-Warner et al. also reported a linear relationship between alcohol and breast cancer, for ethanol intake of up to 60 grams per day (approximately 4.5 bottles of beer, 5.6 glasses of wine or 4 shots of liquor) [7]. However, the mechanism by which alcohol consumption contributes to mammary carcinogenesis remains unresolved. Our studies provide direct evidence suggesting alcohol increases mammary tumor development via the estrogen pathway. Our data show that alcohol consumption promotes HER2 mammary tumor development in MMTV-neu mice but only in the presence of ovarian hormones. The acceleration of tumor development due to alcohol consumption is associated with increased systemic estrogen levels, and increased expression of aromatase and ERα in tumor tissues. Furthermore, we show that the effects of alcohol on tumor development in HER2 mice can be blocked by ovariectomy. Since alcohol only promoted tumor development in the presence of physiologically normal systemic levels of estrogen, results suggest that the effects of alcohol on HER2 mammary tumor development might be mediated via estrogen. However, one caveat in this study is that ovariectomized mice failed to develop tumors in numbers comparable to non-ovariectomized mice. It is, therefore, important to keep in mind that the presence of

steroidal hormones such as estrogen may be important for the tumor model in general and that failure to see tumor promotion with alcohol is a secondary effect.

Nevertheless, existing evidence suggests alcohol may affect breast cancer via the estrogen pathway [37, 51, 88, 89, 94] and elevated estrogen levels are considered a risk factor for breast cancer [37], which our data supports. Epidemiological studies show that women who consume alcohol have higher systemic estrogen levels [37, 43, 95]. It is possible that alcohol increases systemic estrogen levels by increasing the level of aromatase, an enzyme which converts androgens to estrogens [37, 43, 77, 95]. According to Gordan et al., since aromatization involves a series of hydroxylation reactions, it is not unexpected that alcohol, a known hydroxylase inducer, increases the level of activity of the aromatase enzyme [96]. High levels of estrogen may promote the development of breast cancer by activating the estrogen pathway to stimulate cellular proliferation [97]. In addition, previous studies using cell culture conditions show that alcohol exposure increases the expression of ERα in cancer cells [98]. Thus, not only does alcohol increase systemic estrogen levels but it also increases the expression of its receptor ERα, which may explain how breast cancer cells are more sensitive to estrogen in the presence of alcohol [37, 43, 95]. This may suggest that alcohol consumption in conjunction with estrogen replacement therapy may increase breast cancer risk to a higher degree than either treatment alone. In fact, alcohol in conjunction with estrogen-containing hormone replacement therapy (HRT) increases breast cancer risk to a greater extent than HRT or alcohol alone [37]. For example, alcohol alone increases breast cancer risk by 28%, estrogen alone by 45%, however, the combination of estrogen and alcohol elevates the risk by 108% [37]. Although studies show alcohol exposure increases systemic estrogen levels in women, and in cell culture conditions alcohol increases the expression levels of aromatase and ERα, it is not known how the effects of alcohol in the estrogen signaling

pathway relate to breast cancer development (e.g. tumor incidence, tumor growth). Results of this study support the role of the estrogen pathway as a mechanism by which alcohol consumption promotes the development of HER2 mammary cancer.

The MMTV-neu mouse model of HER2 breast cancer over-express the wild-type erbB2 gene in the mammary gland and develop ERα-negative mammary carcinomas [44, 99]. Initially, the tumors are estrogen-driven (ERα-positive) but become estrogen independent as cancer progresses to adenocarcinoma. The course of mammary tumorigenesis in these mice is similar to that of humans, which proceeds from hyperplasia, to ductal carcinoma in situ, to invasive breast cancer [44]. Interestingly, alcohol consumption increased mammary tumor incidence and growth, decreased tumor latency, and led to the development of ERα-positive tumors in MMTV-neu mice. The expression of ERα in the tumor tissues suggest that alcohol may either: 1) prevent the loss or extend the expression and function of ERα, or 2) cause re-expression of ERα, in an otherwise ERα-negative breast cancer subtype.

Evidence suggests that the loss of ERα gene expression in ERα-negative breast cancers is not the result of DNA mutations in the ERα gene [54, 55]. Therefore, it is possible that the loss of ERα expression may be due to decreased transcription. In fact, previous studies show that aberrant methylation status of the ERα promoter appears in more than 25% of ERα-negative breast cancer cells [56, 58, 100]. Histone acetylation/deacetylation has also been implicated as a possible mechanism by which ERα transcription is repressed [57]. Thus, epigenetic regulation of ERα may play an important role in the loss of ERα expression in ERα-negative breast cancer subtypes such as the HER2 subtype. Our objective in future studies will be to determine the mechanism by which alcohol affects the expression of ERα gene.

With respect to systemic estrogen levels, numerous studies have shown that alcohol consumption increases systemic estrogen levels in both pre- and post-menopausal women [37, 43, 95]. In our own studies we show that alcohol increased systemic estrogen levels in both NOVX and OVX female mice. Even though alcohol increased estrogen levels in OVX mice, the levels were still lower than those of NOVX mice not consuming alcohol. Since OVX mice lack ovaries, the estrogen found in their blood may originate from peripheral tissues (i.e. adipose or tumors tissue) [38]. The rate-limiting enzyme in the synthesis of estrogen in these tissues is cytochrome p450 aromatase [38, 39]. It is possible that alcohol increases systemic estrogen levels by affecting aromatase expression in these tissues. We show that alcohol increased the expression of aromatase in the tumors of alcohol consuming mice and this effect was more noticeable in NOVX mice than in OVX female mice.

Overall, we show that the promotion of tumor development by alcohol in MMTV-neu mice is associated with increased systemic estrogen levels, and increased expression of aromatase and ERα in the tumors of NOVX mice. However, even though alcohol increased the expression of ERα in the OVX mice, alcohol did not increase in tumor incidence and tumor growth in these OVX mice. It is feasible that alcohol only affects HER2 tumor development in the presence of normal circulating estrogen levels, which our OVX mice lacked. On the other hand, systemic estrogen levels were elevated in NOVX mice, and this was associated with a higher tumor incidence and tumor growth rate. Therefore, it is plausible that alcohol consumption requires a certain threshold of systemic estrogen levels in order to promote mammary tumor development. This is supported by data showing that ovariectomy, which reduced systemic estrogen levels, inhibited the tumor promoting effects of alcohol on MMTV-neu mice.

In conclusion, alcohol may promote HER2 mammary tumor development via the estrogen pathway. Interestingly, alcohol mediates the expression of ERα in HER2 tumors which are reported to be ERα-negative. Thus, drug therapies targeting the estrogen pathway may be able to block the effects of alcohol on an otherwise ERα-negative breast cancer. Given that many women drink some form of alcoholic beverage frequently, it is important to understand how alcohol consumption promotes breast cancer development in pre- and post-menopausal women. Determining the mechanism by which alcohol affects breast cancer may provide a therapeutic strategy to prevent alcohol-related breast cancers in women. In terms of the impact on breast cancer prevention, our findings may help define better recommendations on alcohol consumption for women in order to decrease their risk of developing this disease.

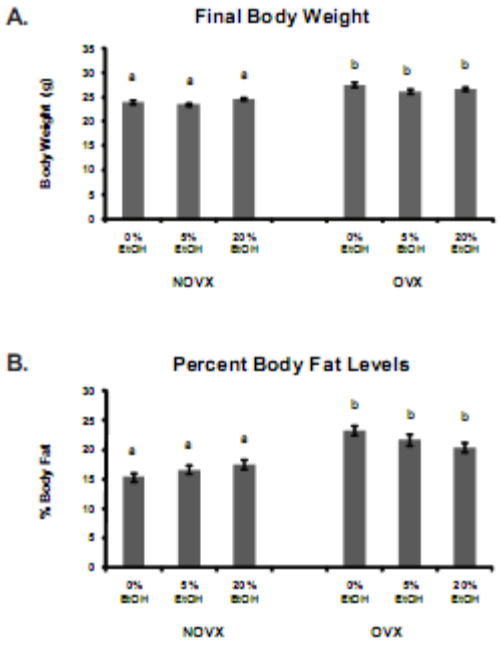

Figure 2.1: Final body weight and percent body fat in MMTV-neu mice.
A. Body weight of non-ovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice after 52 weeks of alcohol exposure. The results are expressed as the mean ± SEM of 30 mice per group (n=30 per group). **B.** Percent body fat levels of NOVX and OVX MMTV-neu mice as determined by Dual energy X-ray Absorptiometry (DXA) following 52 weeks of alcohol exposure. The results are expressed as the mean ± SEM of 8 mice per group (n=8 per group). For each graph, different lower-case letters represent statistical significance ($p<0.05$).

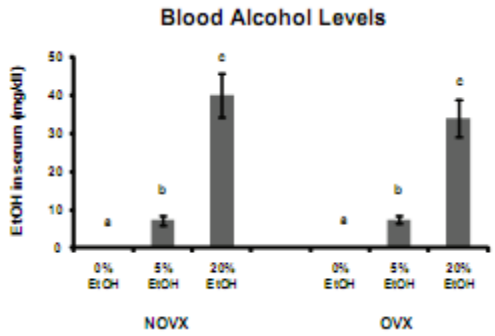

Figure 2.2. Blood alcohol content in MMTV-neu mice.
Blood alcohol levels were determined in all groups of mice by assaying alcohol dehydrogenase activity in mouse sera. The results are expressed as the mean ± SEM of 9 mice per group (n=9 per group). Different lower-case letters represent statistical significance ($p<0.05$).

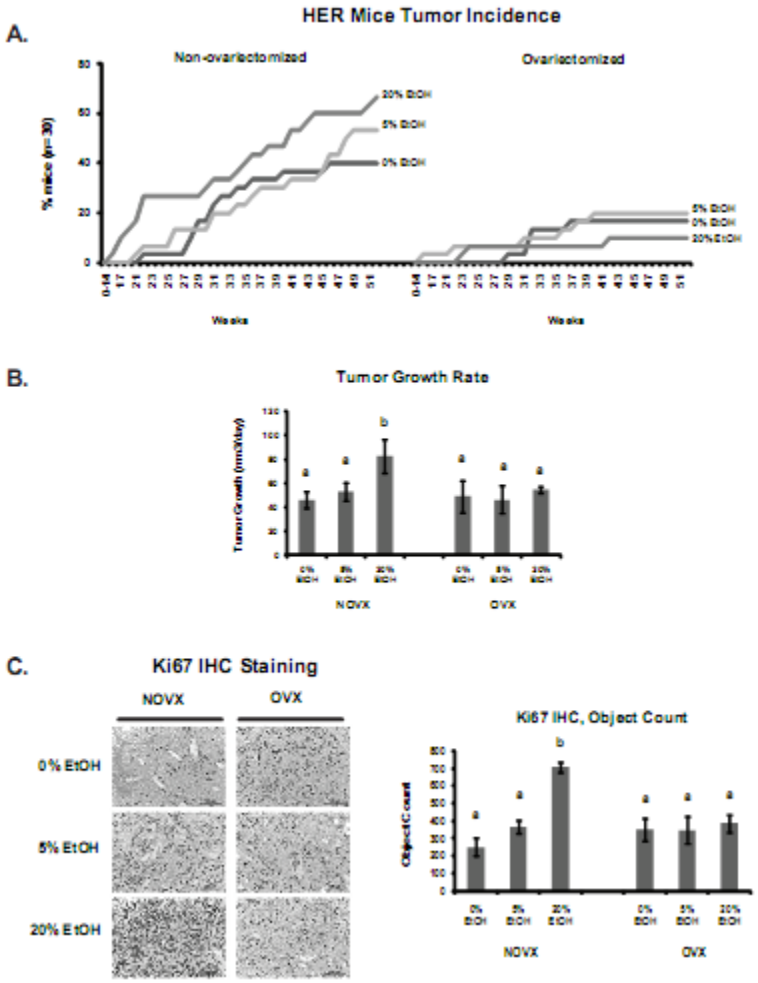

Figure 2.3. Effects of alcohol on HER2 tumor development and progression in MMTV-neu mice.

A. Alcohol consumption of 5% and 20% v/v ethanol increased mammary tumor incidence of non-ovariectomized (NOVX) MMTV-neu mice but not ovariectomized (OVX) mice. Tumor incidence was determined by palpating mice at least once per week throughout the study. The results represent the percentage of mice (n=30 per group) exhibiting palpable tumors. The z-test for proportions was used to determine statistical significance. Asterisks represents significance (p<0.05). * represents significance between 0% and 20% alcohol consuming NOVX mice. ** represents significance between NOVX and OVX mice on their respective liquid diet groups (ex. 0% EtOH NOVX vs. 0% EtOH OVX, 5% EtOH NOVX vs. 5% EtOH OVX, etc.). **B.** Consumption of 20% v/v ethanol significantly increased tumor growth rate within NOVX MMTV-neu mice but not OVX mice. Tumor growth rate was determined by: [(Final tumor volume) - (Tumor volume at first detection)] / (Number of days tumor was present). The results are expressed as the mean ± SEM of all tumor-bearing mice per group (n≥3 per group).

Different lower-case letters represent statistical significance ($p<0.05$). **C.** Consumption of 20% v/v ethanol significantly increased cellular proliferation in NOVX MMTV-neu mice but not OVX mice. Proliferation was determined by immunohistochemical (IHC) staining of cellular proliferation marker Ki67. Quantification of results using imaging software is shown alongside a representative of stained slides. The results are expressed as the mean ± SEM of 4 quantified slides per group (n=4 per group). Different lower-case letters represent statistical significance ($p<0.05$).

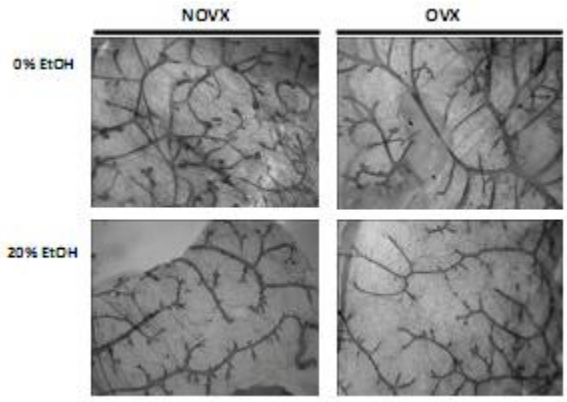

Figure 2.4. Effects of alcohol on the structure of the mammary gland.
Alcohol consumption alters mammary gland morphology in non-ovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice. Whole-mounts were obtained from mouse mammary gland #9. Most pronounced is the effect of 20% v/v ethanol consumption on mammary gland structure compared to non-alcohol groups, as can be seen by the increased lobualveolar (LA) structures (indicated by arrows). The images above are representatives of whole-mounts taken from at least 3 slides per group ($n \geq 3$ per group).

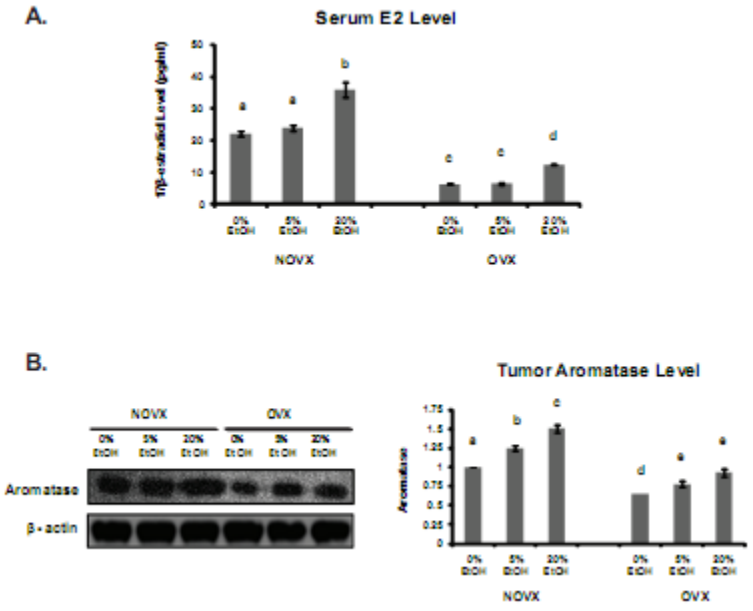

Figure 2.5. Alcohol promotes estrogen availability in MMTV-neu mice.
A. Systemic 17β-estradiol (E2) levels were significantly increased in non-ovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice consuming 20% v/v ethanol compared to their 0% and 5% alcohol consuming counterparts at 52 weeks following initial alcohol exposure. E2 levels were determined by ELISA. (Note: OVX mice consuming 0% and 5% v/v ethanol exhibited average estradiol levels which fell below the sensitivity level of the ELISA and may be considered as non-detectible.) The results are expressed as the mean ± SEM of 8 mice per group (n=8 per group). Different lower-case letters represent statistical significance (p<0.05). **B.** Tumor aromatase expression was significantly increased in alcohol-consuming NOVX and OVX mice compared to non-alcohol consuming groups. Aromatase levels were determined by immunoblot. Bands migrated at approximately 55 kDa. Quantification of results by densitometry is shown alongside a representative immunoblot. The results are expressed as the mean ± SEM of 3 quantified blots per group (n=3 per group). Different lower-case letters represent statistical significance (p<0.05).

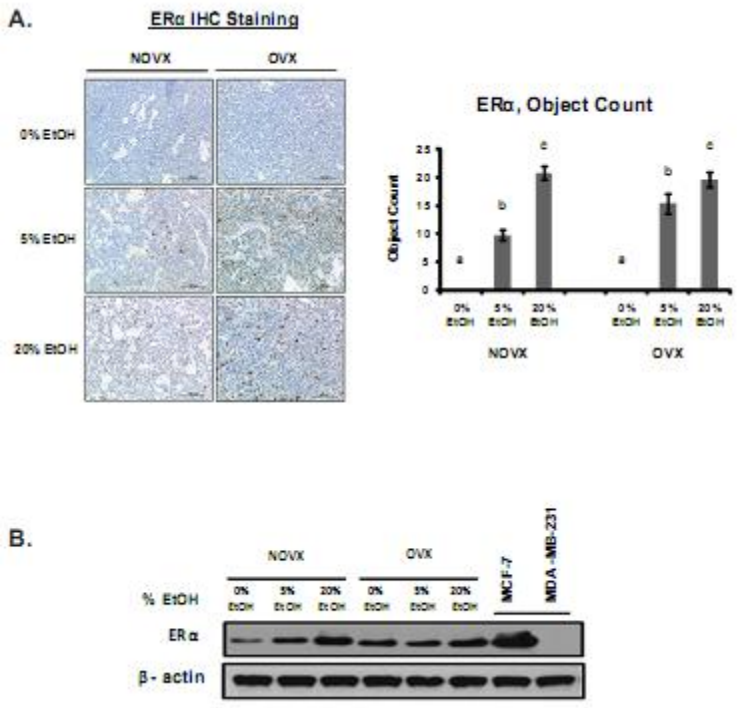

Figure 2.6. Alcohol promotes the expression of ERα in MMTV-neu mice.
Tumor estrogen receptor α (ERα) expression levels were significantly increased in non-ovariectomized (NOVX) and ovariectomized (OVX) MMTV-neu mice consuming alcohol compared to the non-alcohol groups following 52 weeks of alcohol exposure. Tumor ERα expression was determined by **(A)** immunohistochemical (IHC) staining and **(B)** immunoblot. Quantification of IHC results using imaging software is shown alongside a representative of stained slides. The results are expressed as the mean ± SEM of 4 quantified slides per group (n=4 per group). Quantification of immunoblots by densitometry is shown alongside a representative immunoblot. MCF-7 and MDA-MB-231 breast cancer cells were used as positive and negative controls, respectively, for ERα expression. The results are expressed as the mean ± SEM of 3 quantified blots per group (n=3 per group). For each graph, different lower-case letters represent statistical significance ($p<0.05$).

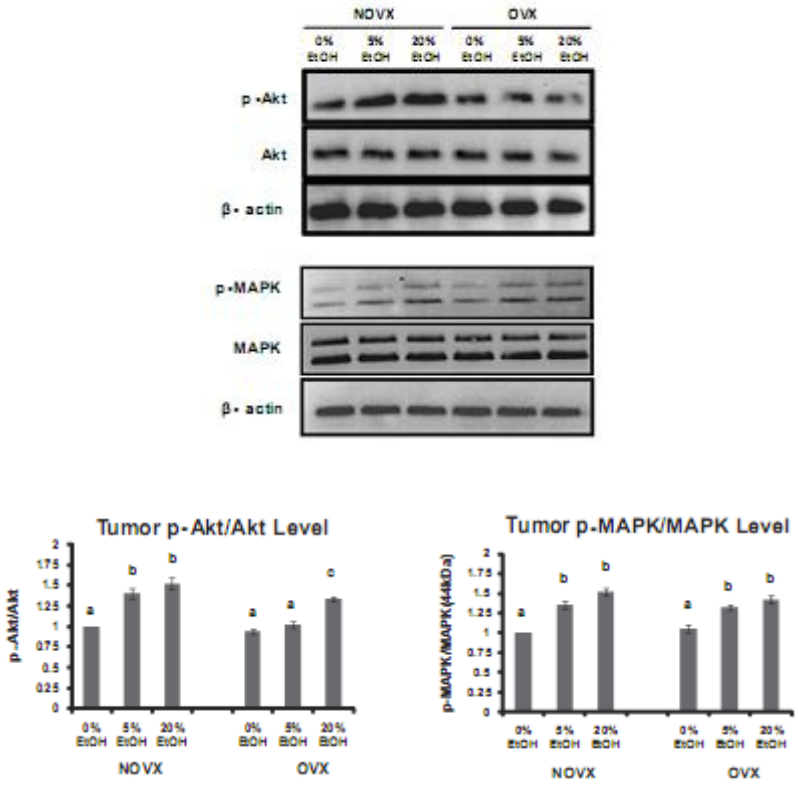

Figure 2.7. Alcohol consumption in NOVX mice activates downstream targets of the estrogen signaling pathway.

Alcohol consumption increased activation of Akt and MAPK, downstream targets of the estrogen signaling pathway, in non-ovariectomized (NOVX) but not ovariectomized (OVX) MMTV-neu mice. Expression of Akt, p-Akt, MAPK and p-MAPK in tumor tissues were determined by immunoblot. Quantification of results by densitometry is shown alongside a representative immunoblot. The results are expressed as the mean ± SEM of 3 quantified blots per group (n=3 per group). Different lower-case letters represent statistical significance ($p<0.05$).

Chapter 3: Epigenetic regulation by alcohol reactivates estrogen receptor alpha (ERα) in ERα-negative human MDA-MB-231 and mouse MMTV-neu breast cancercells

3.1 ABSTRACT

Background: Alcohol consumption is an established risk factor for breast cancer development and contributes to mammary tumorigenesis through the regulation of estrogen receptor alpha (ERα) expression. Previously, we reported that alcohol consumption in the ERα-negative MMTV-neu mouse model of human HER2+ breast cancer resulted in increased tumor ERα expression compared to non-alcohol consuming mice. Several lines of evidence suggest that ERα expression in ERα-negative cancer cells is inhibited through epigenetic mechanisms. Here we report the role of alcohol on ERα re-expression through changes in DNA methylation, histone modification (histone acetylation and histone methylation) and recruitment of transcriptional regulation complexes to the ERα promoter region in ERα-negative breast cancer cells.

Methods: ERα-negative MMTV-neu-42 cells (tumor cells extracted from the MMTV-neu mouse model of human HER2+ breast cancer) and ERα-negative MDA-MB-231 human breast cancer cells were treated with ethanol at various doses for 24 h. DNA methylation at three CpG sites on the ERα promoter was assessed using a qRT-PCR-based method amplifying regions containing methyl-specific restriction enzyme (MSRE) recognition sites. Chromatin immunoprecipitation (ChIP) assays were used to examine the enrichment of active and inactive markers of chromatin at the ERα promoter. ChIP was also used to determine the enrichment of transcriptional activation and suppression complexes on the ERα promoter. Cells were treated with tamoxifen and the reactivation of ERα by alcohol was assessed by examining downstream Akt.

Results: Our results show that, 1) alcohol significantly decreased DNA methylation at three CpG sites on the ERα promoter in a dose-dependent manner, 2) increased the enrichment of H3K4-acetyl, H3K14-acetyl and H4K5-acetyl, markers of active chromatin, and decreased levels of H3K9-methyl and H3K27-methyl, markers of inactive chromatin, 3) alcohol may alter the recruitment and/or binding of the pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 complexes to regulate the ERα promoter region, and 4) the inhibition of ERα by tamoxifen specifically blocks the effects of alcohol on ERα reactivation and function in ERα-negative cell lines.

Conclusions: Alcohol promotes ERα gene transcription epigenetically by affecting DNA methylation, histone modification and recruitment of multimolecular complexes important for ERα transcriptional regulation, on the ERα promoter. Together, these consequences may lead to a functional change in the cell. This study helps to identify the specific epigenetic mechanisms and targets at the ERα promoter and the transcriptional regulation complexes which may be important for ERα reactivation. Identification of these targets may contribute to the development of novel therapeutic strategies for ERα -negative breast cancer treatment.

3.2 INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths among women in the US [6]. In 2010, approximately 200,000 women were diagnosed with breast cancer and 40,000 women were expected to die from this disease in the US [2]. Alcohol is an established risk factor for breast cancer development and increases breast cancer risk in a dose-dependent manner: risk increases by 10% for each alcoholic drink (12 oz of beer or

5 oz of wine) consumed daily [7]. Existing evidence supports the hypothesis that alcohol may affect breast cancer via the estrogen pathway. Elevated estrogen levels are considered a risk factor for breast cancer [37] and epidemiological studies have shown that women who regularly consume alcohol have higher systemic estrogen levels [37, 43, 95]. Alcohol may increase systemic estrogen levels by increasing the level of aromatase, an enzyme critical for the conversion of androgens to estrogens [37, 43, 77, 95]. High levels of estrogen stimulate breast cancer cell proliferation by binding to the estrogen receptor α (ERα) to activate the estrogen pathway [77]. *In vitro* and *in vivo* studies have shown that alcohol increases ERα expression in cancer cells, thus, not only does alcohol increase levels of systemic estrogen but it also increases the expression of ERα, which may explain how breast cancer cells are more sensitive to estrogen in the presence of alcohol [37, 98, 101].

Previously, we reported that alcohol exposure increased ERα expression in MMTV-neu mice and that this effect was associated with increased systemic estradiol, increased tumor aromatase and increased activation of the estrogen pathway [101]. More importantly, the increase in ERα expression we observed may contribute to higher tumor incidence and accelerated tumor progression in the mice [101]. The MMTV-neu mouse model of HER2+ (ErbB2/neu) breast cancer over-expresses the wild-type erbB2 gene in the mammary gland and develops ERα-negative mammary carcinomas [44, 99]. The course of HER2+ mammary tumorigenesis in these mice is similar to that of humans, which proceeds from hyperplasia, to ductal carcinoma in situ, to invasive breast cancer [44]. Initially, the tumors are estrogen-driven (ERα-positive) but, interestingly, become

estrogen-independent (ERα-negative) as cancer progresses to adenocarcinoma. Therefore, the expression of ERα in the tumor tissues of a reportedly ERα-negative mouse model suggests that alcohol exposure may either: 1) prevent the loss or extend the expression and function of ERα, or 2) cause re-expression of ERα, in an otherwise ERα-negative breast cancer subtype. This study seeks to examine the latter hypothesis using the ERα-negative human breast cancer cell line MDA-MB-231 and the ERα-negative MMTV-neu cell line derived from the tumors of the MMTV-neu mouse model mentioned above.

Evidence suggests that the loss of ERα gene expression in ERα-negative breast cancers is not the result of DNA mutations in the ERα gene [54, 55]. Therefore, it is possible that the loss of ERα in ERα-negative breast cancer cells may be due to the loss of ERα transcription. Epigenetic dysregulation has been implicated to be critical in ERα expression [56, 57]. In fact, previous studies have shown that aberrant methylation of the ERα promoter appears in more than 25% of ERα-negative breast cancer cells [55, 56, 58]. Interestingly, a recent study examining altered methylation of CpG loci associated with over 700 cancer-related genes has unveiled a strong trend for patients with increasing alcohol intake to have overall decreased DNA methylation [59]. Histone modifications such as histone acetylation/deacetylation have also been linked to ERα transcriptional repression [57]. In addition, *in vivo* occupancy of the ERα promoter by the multimolecular complexes pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 has been suggested to regulate ERα transcription [60]. The role of ERα in the activation of the estrogen pathway makes it an important target for many breast cancer therapeutic strategies. Therefore, the lack of ERα at initial

diagnosis in 30% of all breast cancer cases and the loss of ERα expression from ERα-positive breast cancer cells during the course of tumor progression calls for an investigation to examine how ERα is lost and methods for ERα re-expression. Here we show that re-expression and reactivation of ERα in ERα-negative MMTV-neu mouse and MDA-MB-231 human breast cancer cells may be due to epigenetic changes at the ERα promoter, namely alterations in promoter DNA methylation, histone modifications and recruitment of transcriptional regulation complexes.

3.3 Materials and Methods

CELL LINES AND GROWTH CONDITIONS

Mouse MMTV-neu-42 tumor cells were kindly provided by Dr. Powel Brown of the Baylor College of Medicine in Houston, TX. Human breast cancer MDA-MB-231 and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Non-treated MCF-7 human breast cancer cells served as an ERα-positive control for MDA-MB-231 cells. All cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) media supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin and 1% L-glutamine (Invitrogen). Cultures were maintained at 37˚C with 5% CO_2 and harvested when confluency reached 70-80%.

TREATMENT CONDITIONS

Cells were washed 2X with PBS (Invitrogen) and culture media was switched to phenol-red-free DMEM and maintained in 10% dextran-charcoal-stripped FBS (Invitrogen) at least 24 h before treatment. *Ethanol treatment:* Once MMTV-neu-42 and

MDA-MB-231 cells reached 60-70% confluency, 0.1%, 0.2% and 0.5% v/v ethanol was added to culture media for 24 h. *Tamoxifen treatment:* Once MMTV-neu-42 and MDA-MB-231 cells reached 60-70% confluency, 4-OH-Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was added to culture media to a final concentration of 100 nM for 24 h. Control cells were left untreated.

QUANTITATIVE REAL-TIME PCR

Total cellular RNA was isolated from MMTV-neu-42, MDA-MB-231 and MCF-7 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), using 2 mg of RNA for each reaction. Primer pairs were designed using Primer3 software [102] for estrogen receptor alpha (ERα) (5'-GACCAGATGGTCAGTGCCTT-3' and 5'-ACTCGAGAAGGTGGACCTGA-3') and GAPDH (5'-ATGGTGAAGGTCGGTGTGAAC-3' and 5'-GCCTTGACTGTGCCGTTGAAT-3'). Real-time PCR was performed with the SYBR GreenER qPCR kit (Invitrogen, Carlsbad, CA, USA) in the Mastercycler ep Realplex PCR thermocycler (Eppendorf, Wesseling-Berzdorf, Germany). The relative expression level of ERα was normalized to the housekeeping gene GAPDH. The annealing temperature used for the primers was 65^{o}C. Triplicate wells were used per group in each independent experiment.

IMMUNOBLOT

Cells were lysed using RIPA buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 20 mM MgCl2, 1% Nonidet P40 containing protease inhibitors (1 ug/ml PMSF, 1 ug/ml aprotinin and 1 ug/ml pepstatin), incubated on ice for 1 h with agitation and centrifuged at 12,000 x g for 20 min. Protein samples were subjected to

electrophoresis on 4-12% SDS-polyacrylamide gradient gels and transferred to PVDF membranes. Membranes were probed with antibodies for the following proteins: ERα (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), p-Akt (Cell Signaling Technology, Inc., Danvers, MA, USA), Akt (Cell Signaling Technology, Inc.) and beta actin (Oncogene, Cambridge, MA, USA). Protein-antibody complexes were detected with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) followed by enhanced chemiluminescence reaction. Quantification of the band intensity was determined by densitometry using ImageJ software (NIH website: http://rsbweb.nih.gov/ij/index.html). Each blot shown is a representative of three independent experiments.

METHYLATION ASSAY: DNA DIGESTION AND REAL-TIME PCR

DNA methylation was analyzed using the OneStep qMethyl Kit (Zymo Research Corp., Irvine, CA, USA) following manufacturer instructions. DNA used for this assay included the following: 1) human methylated DNA standard as a positive control (Zymo Research Corp.), 2) human non-methylated DNA standard as a negative control (Zymo Research Corp.), 3) untreated MCF-7, 4) untreated MMTV-neu-42 and MDA-MB-231, and 5-7) MMTV-neu-42 and MDA-MB-231 treated with 0.1%, 0.2% and 0.5% v/v ethanol. Briefly, 20 ng DNA was digested with a reaction mix containing the methylation sensitive restriction enzymes AccII (recognition site 5'-CG^{m}CG-3'), HpaII (recognition site 5'-C^{m}CGG-3') and HpyCH4IV (recognition site 5'-A^{m}CGT-3'), and then amplified by real-time PCR using primers specific to a CpG-rich region on the ERα promoter containing the three restriction enzyme sites. Primers were designed using Primer3 software [102]; ERα promoter sense 5'-ATT-CTA-TCT-GCC-CTA-TCT-CG-3' and antisense 5'-AGG-GAA-GAC-TGG-GCT-TAA-AA-3'. Real-time PCR was performed

using a Mastercycler Realplex real-time PCR thermocycler (Eppendorf). Percent methylation was determined by the formula: 100 x $2^{-\Delta Ct}$, where ΔCt = [average of Ct value from duplicate wells of digested reactions] – [average of Ct value from duplicate wells of undigested reactions].

CHIP ASSAY

ChIP assays were performed using EZ-ChIP™ (Millipore, Billerica, MA, USA) following manufacturer instructions. To assess the enrichment of markers of active transcription, the following antibodies were used: H3K4-Ac (cat #17-10050, Millipore), H3K14-Ac (cat #17-10051, Millipore) and H4K5-Ac (cat #17-10045, Millipore). To assess the enrichment of markers of inactive transcription, the following antibodies were used: H3K9-me (cat #17-625, Millipore) and H3K27-me (cat #17-10108, Millipore). The following ERα promoter primers were used: sense 5'-GAA-CCG-TCC-GCA-GCT-CAA-GAT-C-3' and antisense 5'-GTC-TGA-CCG-TAG-ACC-TGC-GCG-TTG-3' and have previously been described [55].

To assess the enrichment of each protein involved in the multimolecular complexes pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1, at the ERα promoter, the following antibodies were used: pRb2/p130 (sc-317, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), E2F4/5 (sc-511, Santa Cruz Biotechnology Inc.), HDAC1 (sc-6298, Santa Cruz Biotechnology Inc.), SUV39H1 (sc-13608, Santa Cruz Biotechnology Inc.), p300 (sc-585, Santa Cruz Biotechnology Inc.) and DNMT1 (ab92453, Abcam, Cambridge, MA, USA). The ERα promoter primers used are described above.

STATISTICAL ANALYSIS

Data are expressed as means ± SEM where appropriate. Three independent experiments were performed for each assay for data analysis. Significant differences among groups were determined using two-way analysis of variance (ANOVA) with Bonferroni adjustment. SPSS v16 for Windows (IBM Corporation, Chicago, IL, USA) was used for all statistical comparisons. To detect statistical significance, *p* value was set to 0.05.

3.4 RESULTS

ALCOHOL PROMOTES ER ALPHA RE-EXPRESSION IN ER ALPHA-NEGATIVE CELLS

ERα-negative MMTV-neu mouse and MDA-MB-231 human breast cancer cells were treated with 0.1%, 0.2% and 0.5% v/v ethanol. Following 24 h incubation, mRNA levels of ERα were determined by qRT-PCR. Results show that ethanol treatment significantly increased ERα expression 96%, 153% and 222%, respectively, in MMTV-neu cells and 82%, 114% and 193%, respectively, in MDA-MB-231 cells (**Figures 3.1A and 3.1B**, $p<0.05$). ERα protein expression is shown in **Figures 3.1C and 3.1D**; 0.1%, 0.2% and 0.5% v/v ethanol significantly increased expression of ERα by 137%, 151% and 171%, respectively, in MMTV-neu cells and 125%, 142% and 170%, respectively, in MDA-MB-231 cells ($p<0.05$).

ALCOHOL EXPOSURE MODIFIES METHYLATION STATUS AT ER PROMOTER CPG SITES

It has recently been reported that alcohol intake is associated with an overall decline in CpG methylation in over 700 cancer-related genes characterized from 162 samples of human primary breast tumors [59]. The methylation of cytosines by DNA methyltransferase to form 5'-methylcytosine occurs exclusively where a cytosine appears

immediately 5' to a guanosine, forming a CpG dinucleotide [103]. CpG dinucleotides are not evenly distributed throughout the human genome; rather, they are clustered in regions of large repetitive DNA sequences known as "CpG islands" [104]. These islands are sites of transcriptional initiation, as shown by the 50% proportion of CpG islands associated with annotated with transcriptional start sites [105]. In addition, 60% of human gene promoters contain CpG islands [106]. Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells [107].

To determine whether ERα re-expression by alcohol may be due to changes in CpG methylation of the ERα promoter, we performed a qRT-PCR-based method amplifying a promoter region (-270 to -445 bp) containing three methyl-specific restriction enzyme (MSRE) recognition sites. Methyl-sensitive AccII, HpaIII and HpyCH4IV restriction enzymes were used for digestion. Results show that 0.1%, 0.2% and 0.5% v/v ethanol significantly decreased ERα promoter CpG methylation by 21%, 32% and 50%, respectively, in MMTV-neu cells and 9%, 20% and 46%, respectively, in MDA-MB-231 cells (**Figures 3.2A and 3.2B**, $p<0.05$). Human methylated DNA and non-methylated DNA standards were used for validation of this assay, according to manufacturer instructions (Zymo Research Corp., Irvine, CA, USA).

ALCOHOL INDUCES HISTONE MODIFICATIONS AT THE ER PROMOTER

Histone proteins contain a large proportion of positively charged residues (i.e. Lys and Arg) and can, therefore, bind to the negatively charged phosphate groups on DNA through electrostatic interactions [108]. DNA packaging is important for the storage of

genetic information into a small portion of cell volume; however, it poses the issue of how genetic information can be accessed by the transcriptional machinery. The histone code hypothesis postulates that the appearance of a given modification on histone tails may dictate or prevent the presence of subsequent modifications elsewhere on the same histone and that these modifications, together, may lead to the opening of chromatin and recruitment of different regulatory protein or protein complexes important for gene expression [60].

We analyzed the ERα promoter for changes in the levels of active and inactive markers of chromatin in response to alcohol treatment in MMTV-neu and MDA-MB-231 cells using ChIP assays. **Figures 3.3A and 3.3B** shows that 0.1%, 0.2% and 0.5% v/v ethanol increased active chromatin markers H3K4-Ac, H3K14-Ac and H4K5-Ac, and decreased inactive chromatin markers H3K9-Me and H3K27-Me, in a dose-dependent manner in both ERα-negative cell lines. Our data suggest that ethanol promotes overall histone acetylation and decreases histone methylation to promote ERα re-expression. In fact, it is known that histone acetylation promotes the open state of chromatin [109], which allows for the recruitment of regulatory complexes to induce ERα transcription, and that histone methylation promotes the closed state of chromatin [110, 111], which prevents ERα transcription.

ALCOHOL MODULATES RECRUITMENT OF ER TRANSCRIPTIONAL REGULATION COMPLEXES

Figures 3.2 and 3.3 show that ethanol treatment results in changes in ERα promoter CpG methylation and histone modification patterns. As mentioned above,

methylation patterns of CpG dinucleotides and histone modifications may dictate transcription initiation or repression by either promoting or preventing the recruitment of regulatory proteins to DNA. Previous studies have reported that the *in vivo* occupancy of pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 at the ERα promoter is associated with ERα transcriptional repression in ERα-negative MDA-MB-231 cells and that the switching of DNMT1 (a DNA methylatransferase) with p300 (a histone acetyltransferase) in this complex induces ERα transcription [55, 60]. Therefore, we sought to determine whether ethanol influences the recruitment of these transcriptional regulatory complexes to the ERα promoter using ChIP assays. **Figures 3.4A and 3.4B** show that the pRb2/p130 complex is recruited to both ERα-negative MMTV-neu and MDA-MB-231 cells as well as to ERα-positive MCF-7 cells. Interestingly, we show that 0.1%, 0.2% and 0.5% v/v ethanol suppresses the recruitment of DNMT1 while increasing the recruitment of p300 in a dose-dependent manner in the MMTV-neu and MDA-MB-231 cell lines (**Figures 3.4A and 3.4B**). Our data therefore suggests that alcohol promotes ERα re-expression by promoting the recruitment of pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and inhibiting the recruitment of pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1.

ALCOHOL PROMOTES ER RE-ACTIVATION

Activation of ERα can lead to non-genomic and genomic signaling [40]. Non-genomic signaling occur independent of gene transcription or protein synthesis and usually involves activation of the PI3K-Akt signaling pathway [41]. On the other hand, genomic signaling involves binding of estrogen to ERα and this complex migrates to the nucleus where it can activate the expression of estrogen-responsive genes, such as ERα [42]. We wanted to determine if alcohol could affect non-genomic and genomic estrogen

signaling and if these effects of alcohol could be inhibited by tamoxifen. Tamoxifen blocks the binding of estrogen to ERα; currently, tamoxifen is being used for the treatment of ERα-positive breast cancers [112]. To determine if alcohol affected non-genomic signaling, we assess the activation/phosphorylation of Akt. In **Figures 3.5A and 3.5B**, we show that 0.5% v/v ethanol significantly increases Akt phosphorylation in both MMTV-neu and MDA-MB-231 cell lines ($p<0.05$).

One contributing factor leading to the poor prognosis for ERα-negative breast cancers is that endocrine therapies, such as tamoxifen, are mostly ineffective for ERα-negative tumors [113]. Thus, in cells lacking ERα expression, tamoxifen is mostly ineffective. This is demonstrated in **Figures 3.5A and 3.5B**; in the absence of alcohol, tamoxifen treatment results in no significant change to Akt phosphorylation ($p>0.05$). Interestingly, however, in the presence of alcohol, tamoxifen specifically blocks the effects of ethanol on ERα reactivation, leading to reduced Akt phosphorylation (**Figures 3.5A and 3.5B**, $p<0.05$). In other words, tamoxifen decreases alcohol-mediated non-genomic ERα reactivation. Since tamoxifen acts specifically on the ERα, this reduction indicates that Akt activation is a direct measure of ERα expression.

We also assess the effects of tamoxifen on the genomic activation of the estrogen receptor. Estrogen-bound ERα induces activation of the receptor, triggering dimerization of the receptor and co-regulatory proteins recruitment. Subsequently, this facilitates the classical genomic estrogen pathway, in which the receptor binds to the estrogen response element (ERE)-containing gene promoters to enhance transcription [114, 115]. The gene encoding the estrogen receptor alpha gene, ESR1, includes an ERE-containing promoter

[116]. In other words, ERα is able to regulate its own promoter to increase its own transcription. **Figures 3.5C and 3.5D** show the effects of alcohol and tamoxifen, alone and in combination, on ERα at the transcript level. In **Figures 3.5C and 3.5D**, exposure of ethanol significantly increased ERα transcription ($p<0.05$) and tamoxifen treatment had no significant effect on ERα transcription ($p>0.05$). Interestingly, in the presence of alcohol, tamoxifen is able to specifically block the effects of alcohol on ERα transcription (**Figures 3.5C and 3.5D**, $p<0.05$). Thus, tamoxifen treatment in ERα-negative cells is able to decrease the genomic effects of estrogen activation.

3.5 DISCUSSION

Previously, we showed that alcohol consumption promoted ERα expression and activation in the primary mammary tumors of MMTV-neu mice, a mouse model which is reported to develop ERα-negative tumors [101]. In this study, we examine the methods underlying ERα re-expression and reactivation in the ERα-negative MMTV-neu cell line derived from the tumors of MMTV-neu mice and in the ERα-negative MDA-MB-231 human breast cancer cell line. ERα is an important prognostic biomarker and therapeutic target in primary breast cancer. ERα-negative tumors appear to be more malignant and have poorer prognosis compared with ERα-positive tumors [117, 118]. Epigenetic mechanisms have previously been used to explain the repression of ERα expression in ERα-negative breast cancer cells. The present study supports this notion and shows that ERα re-expression and reactivation in ERα-negative breast cancer cells is associated with

changes in ERα promoter CpG methylation status, enrichment of active chromatin markers and the increased recruitment the p300 histone acetyltransferase within the pRb2/p130-associated ERα transcription regulation complex.

CpG methylation is known to induce overall gene silencing [119]. The ERα gene contains a CpG island in its promoter and first exon region and is marked by the clustering of sites for methylation-sensitive restriction endonucleases [56, 120, 121]. Therefore, it is possible that transcription of ERα may be regulated by CpG methylation. Our results show that there is a strong association between ERα re-expression and a decrease of methylation at three CpG sites on the ERα promoter. In accordance with this finding, Ottaviano et al. reported a tight relationship between the absence of ERα gene transcripts and increased cellular capacity for overall DNA methylation [56]. On the other hand, some previous studies have found no association between ERα expression and ERα gene methylation [122, 123]; however, it is likely that this may be due to examining the methylation pattern in the internal ERα gene rather than the CpG sites of the ERα promoter.

Methylation patterns on CpG islands are important for the regulation of gene transcription at least partially because of their ability to regulate histone modifications [124]. As mentioned earlier, the histone code hypothesis postulates that modifications to histone tails alter DNA-chromatin structure, influencing the ability of transcriptional regulation complexes to access its target gene. The acetylation of histone is associated with the loosening of chromatin structure and is generally correlated with increased transcriptional activity [125]. Alternatively, the methylation of histones is generally

associated with heterochromatin assembly and may be critical for the maintenance of DNA methylation-related gene silencing [126, 127]. Our results indicated that ERα re-expression is associated with the enrichment of acetylated histone and decrease of methylated histone. Therefore, ERα re-expression and reactivation may require the loosening of DNA-chromatin interactions, allowing for the access of transcriptional regulation protein complexes to the ERα promoter.

Macaluso et al. reported previously that the pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 protein complex represses ERα transcription and that the switching of DNMT1 with p300 in this complex promotes ERα transcription [60]. As a DNA methyltransferase, DNMT1 may, as mentioned above, methylate CpG dinucleutide-associated cytosines to promote gene silencing. DNMT1 has been shown to physically interact with histone deacetylase 1 (HDAC1) or histone deacetylase 2 (HDAC2) to form a transcriptional repression complex important for the regulation of gene expression [100, 128, 129]. Therefore, the association of DNMT1 within the pRb2/p130 complex may function to maintain the transcriptionally repressed state of ERα in ERα-negative breast cancer cells. On the other hand, Hanstein et al. showed in 1996 that p300 significantly increases ERα activation and suggested that p300 associates with an agonist bound ERα complex to potentiate the estrogen signal [130]. It was later discovered that p300 is a histone acetyltransferase, which, as mentioned above, may facilitate the transcriptionally active state of ERα by promoting the open state of chromatin.

In conclusion, we previously showed that alcohol intake in MMTV-neu mice, a HER2+ breast cancer mouse model reported to develop ERα-negative tumors, resulted in

an increase of ERα expression and activation. In this study, we found that ERα re-expression and reactivation may be due to epigenetic mechanisms such as changes in DNA methylation, histone modifications (histone acetylation and histone methylation) and recruitment of transcriptional regulation complexes on the ERα promoter *in vitro*. Collectively, our results revealed the specific epigenetic mechanisms and targets at the ERα promoter and the transcriptional regulation complexes which may be important for ERα reactivation. Identification of these targets may contribute to the development of novel therapeutic strategies for ERα-negative breast cancer treatment

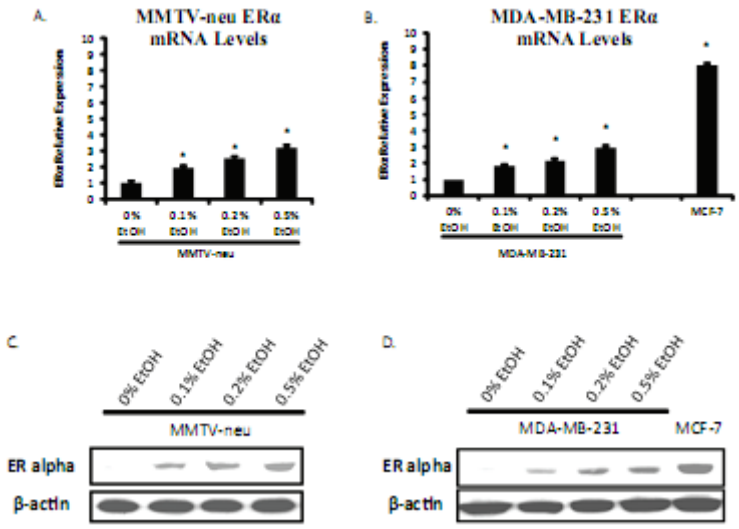

Figure 3.1. Effects of alcohol on ERα re-expression in two ERα-negative cell lines. Alcohol treatments of 0.1, 0.2 and 0.5% v/v ethanol significantly increased ERα mRNA levels in both the MMTV-neu mouse (**A.**) and MDA-MB-231 human (**B.**) breast cancer cell lines, as determined by qRT-PCR ($p<0.05$). The results are expressed as the mean ± SEM of 3 samples per group (n=3 per group). The same ethanol treatments significantly increased ERα protein expression in both MMTV-neu (**C.**) and MDA-MB-231 cells (**D.**), as determined by immunoblot ($p<0.05$). MCF-7 human breast cancer cells served as an ERα-positive control. One representative blot is shown from three independent experiments for each cell line.

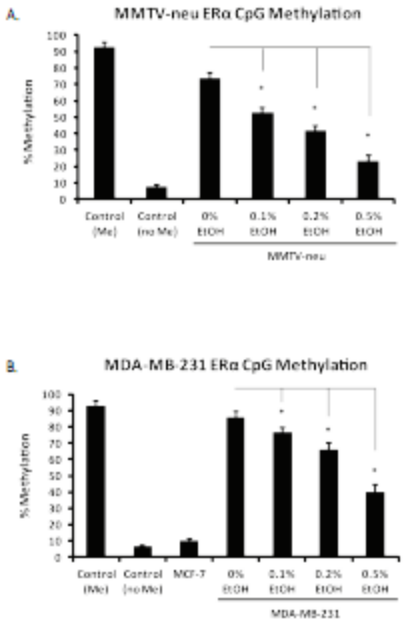

Figure 3.2. Effects of alcohol on ERα promoter CpG methylation. A qRT-PCR-based method was used to amplify the -270 to -445 bp region of the ERα promoter to assess the methylation levels at three known CpG sites. Alcohol treatment significantly decreased ERα promoter CpG methylation levels in a dose-dependent manner in MMTV-neu (**A.**) and MDA-MB-231 (**B.**) cells ($p<0.05$). Methylated and non-methylated human DNA standards were used to validate the assay. The results are expressed as the mean ± SEM of 3 samples per group (n=3 per group).

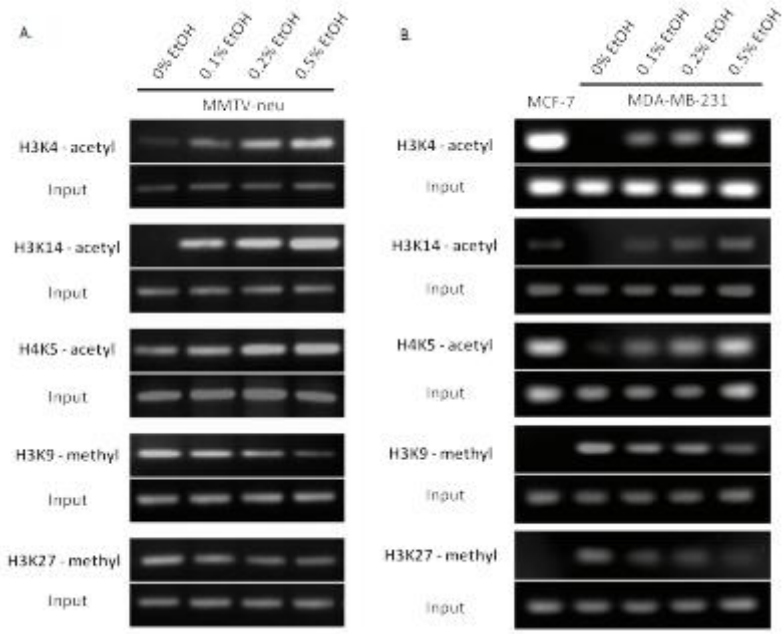

Figure 3.3. Effects of alcohol on markers of active and inactive chromatin at the ERα promoter. The ERα promoter was analyzed for changes in levels of active and inactive markers of chromatin as a result of alcohol treatment using ChIP assays. Alcohol treatment increased the enrichment of H3K4-Ac, H3K14-Ac and H4K5-Ac, markers of active chromatin in a dose-dependent manner in the MMTV-neu (**A.**) and MDA-MB-231 (**B.**) cell lines. Meanwhile, the same doses of ethanol significantly decreased the levels of H3K9-Me and H3K27-Me, markers of inactive chromatin, in both cell lines in a dose-dependent manner. One representative ChIP assay result is shown from three independent experiments for each cell line.

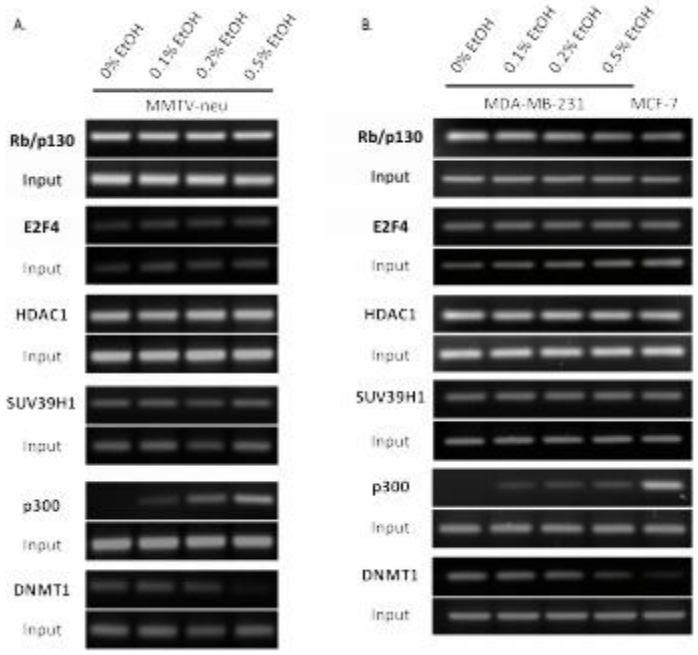

Figure 3.4. Effects of alcohol on the recruitment of ERα transcriptional regulation complexes. The *in vivo* occupancy of pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 on the ERα promoter had been reported to inhibit ERα transcription in MDA-MB-231 cells, while the switching of DNMT1 with p300 induces active ERα transcription. The recruitment of the pRb2/p130-associated regulation complex was analyzed by ChIP. Alcohol treatment decreased the recruitment of DNMT1 to the ERα promoter and increased the enrichment of p300 in a dose-dependent manner in the MMTV-neu (**A.**) and MDA-MB-231 (**B.**) cell lines. One representative ChIP assay result is shown from three independent experiments for each cell line.

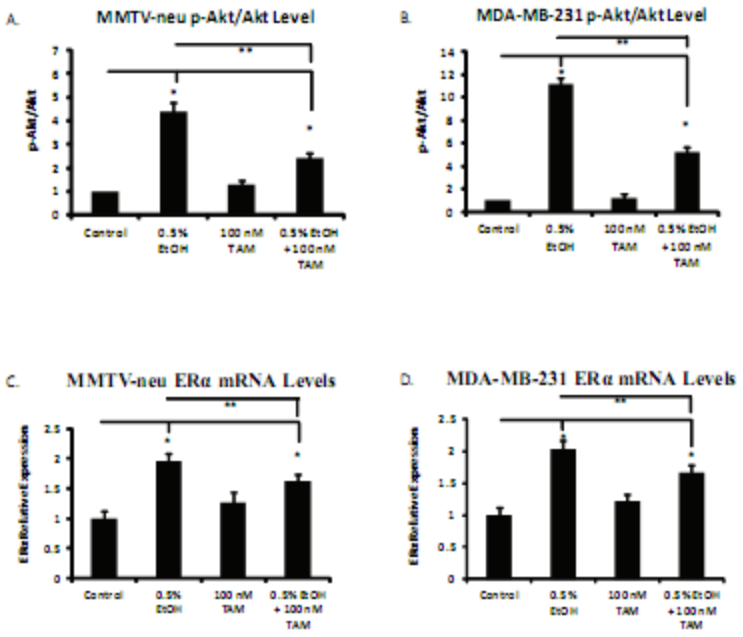

Figure 3.5. Effects of alcohol on ERα reactivation. Activation of ERα was determined by the phosphorylation of Akt, a downstream target in the estrogen pathway. Alcohol treatment significantly increased Akt phosphorylation ($p<0.05$) and tamoxifen treatment had no significant affect on Akt phosphorylation ($p>0.05$) in the MMTV-neu (**A.**) and MDA-MB-231 (**B.**) cell lines. In both cell lines (**A and B**), tamoxifen treatment in the presence of alcohol decreases Akt phosphorylation when compared to the alcohol alone treatment ($p<0.05$). The results are expressed as the mean ± SEM of 3 samples per group (n=3 per group). The effects of alcohol and tamoxifen on ERα transcript levels were determined by qRT-PCR. Alcohol treatment significantly increased transcription of ERα ($p<0.05$) and tamoxifen treatment had no effect ($p>0.05$) in the MMTV-neu (**C.**) and MDA-MB-231 (**D.**) cell lines. In the presence of alcohol, tamoxifen decreases ERα transcript levels when compared to the alcohol alone treatment ($p<0.05$). The results are expressed as the mean ± SEM of 3 samples per group (n=3 per group).

Chapter 4: Alcohol promotes breast cancer cell invasion by regulating the Nm23-ITGA5 pathway

4.1 ABSTRACT

Background: Alcohol consumption is an established risk factor for breast cancer metastasis. Yet, the mechanism by which alcohol promotes breast cancer metastases is unknown. The ability of cancer cells to invade through tissue barriers (such as basement membrane and interstitial stroma) is an essential step towards establishing cancer metastasis. In the present study, we identify and examine the roles of two genes, *Nm23* and *ITGA5*, in alcohol-induced breast cancer cell invasion.

Methods: Human breast cancer T47D cells were treated with ethanol at various concentrations. Boyden chamber invasion assays were used to measure cellular invasive ability. The mRNA expression level of metastasis suppressor genes including *Nm23* was determined by qRT-PCR. *ITGA5* was identified using a qRT-PCR array of 84 genes important for cell-cell and cell-extracellular matrix interactions. *Nm23* overexpression in addition to *Nm23*- and *ITGA5* knock-down were used to determine the role of the Nm23-ITGA5 pathway on cellular invasive ability of T47D cells. Protein expression levels were verified by Western blot.

Results: Alcohol increased the invasive ability of human breast cancer T47D cells in a dose-dependent manner through the suppression of the *Nm23* metastatic suppressor gene. In turn, *Nm23* down-regulation increased expression of fibronectin receptor subunit *ITGA5*, which subsequently led to increased cellular invasion. Moreover, *Nm23* overexpression was effective in suppressing the effects of alcohol on cell invasion.

In addition, we show that the effects of alcohol on invasion were also inhibited by knock-down of *ITGA5*.

Conclusion: Our results suggest that the Nm23-ITGA5 pathway plays a critical role in alcohol-induced breast cancer cell invasion. Thus, regulation of this pathway may potentially be used to prevent the establishment of alcohol-promoted metastases in human breast cancers.

4.2 INTRODUCTION

In 2010, approximately 200,000 women were diagnosed with breast cancer and 40,000 women were expected to die from this disease in the US [2]. Breast cancer is the second leading cause of cancer-related deaths among women in the US, after lung cancer [6]. Often, it is not the primary tumor that leads to the death of cancer patients but, rather, the metastases of the cancerous cells [3, 131]. Breast cancer cells typically spread from the primary tumor site (the breast) to secondary sites (i.e. lungs, liver, bones, etc.) resulting in an increased likelihood of mortality [132]. The invasion of cancer cells into surrounding tissues is an initial step in tumor metastasis and requires the migration of cancer cells and their attachment to the extracellular matrix [133].

Cell culture and animal studies have previously shown that alcohol consumption increases the risk of developing breast cancer by increasing the ability of breast cancer cells to invade and metastasize [7, 10]. Alcohol consumption increases breast cancer risk in a dose-dependent manner; the risk increases by 10% for each alcoholic drink consumed daily [7, 9, 10]. Thus, consumption of two daily alcoholic drinks may lead to a 20% increase in breast cancer risk [7]. A drink is defined as 12 oz of beer or 5 oz of wine [7]. Studies also show that alcohol may increase the risk of breast cancer recurrence in

previously diagnosed women, which may affect their survival [14]. Therefore, in order to develop strategies for the prevention and treatment of alcohol-related breast cancers, it is essential to understand the molecular mechanisms by which alcohol promotes the invasive phenotype of the cancer cells. In this study, we show that alcohol promotes the invasive ability of human breast cancer T47D cells *in vitro* in a dose-dependent manner and show that the Nm23-ITGA5 pathway plays a critical role in the promotion of cancer cell invasion by alcohol.

Metastases suppressing genes encode proteins that hinder the establishment of metastases without blocking the growth of the primary tumor [66]. Two such genes are the human *Nm23* genes (*Nm23-H1* and *Nm23-H2*) which have been localized to chromosome 17q21 and encode 17 kDa proteins that use its nucleoside diphosphate (NDP) kinase [67], histidine kinase [68], and exonuclease activities [69] to inhibit multiple metastatic-related processes. Mutants that disrupt the NDP kinase and exonuclease functions of Nm23 still suppress metastasis to varying degrees, suggesting complex and overlapping roles in metastasis regulation [134]. In this report, we focus only on *Nm23-H1*. Overexpression of *Nm23-H1* in tumor cells reduces tumor cell motility and invasion, promotes cellular differentiation, and inhibits anchorage-independent growth and adhesion to fibronectin, laminin, and vascular endothelial cells [70, 71].

While *Nm23* works to prevent the spread of breast cancer, *ITGA5* produces an integral membrane protein that increases the metastasis of breast cancer cells [72]. *ITGA5* is found on chromosome 12q11-q13 and encodes integrin alpha-5, a fibronectin receptor protein [73]. Through binding to fibronectin, an extracellular glycoprotein, ITGA5 facilitates cellular growth and migration [72, 74]. Integrins associate with adaptor proteins, cytoplasmic kinases and transmembrane growth factor receptors to trigger

biochemical signaling pathways [75]. Overexpression of *ITGA5* leads to increased cellular adhesion and interaction with fibronectin, resulting in promoted tumor metastasis [72].

In the present study, we report, for the first time, the effects of alcohol on the Nm23-ITGA5 pathway and show that regulation of this pathway is important for *in vitro* cellular invasion of T47D human breast cancer cells.

4.3 MATERIALS AND METHODS

CELL CULTURE, TRANSFECTION, AND SIRNA

T47D, MCF-7 and MDA-MB-231 breast cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C, 5% CO2, on 75-cm^3 tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, St Louis, MO, USA). The *Nm23* siRNA, *ITGA5* siRNA, and negative controls were purchased from Invitrogen (Carlsbad, CA, USA). *pcDNA3-Nm23-H1* cDNA and the control vector were kindly provided by Dr. Patricia Steeg (National Cancer Institute, Bethesda, MD, USA). T47D cells were transfected with the above vectors and siRNAs using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Neomycin-resistant clones were isolated by growth in media containing 800 ug/ml G418 (Gibco, St Louis, MO, USA). Alcohol was added to the medium at concentrations of 0.1%, 0.2%, and 0.5% v/v ethanol. RNA and proteins were collected from the cells 48-hours post alcohol treatment.

INVASION ASSAY

The *in vitro* invasion studies were performed using the BD Bio-Coat Matrigel invasion assay system (Becton Dickinson Labware, Franklin Lakes, NJ, USA). To determine the ability of alcohol to affect the invasive ability of breast cancer cells, 2×10^5 T47D cells were suspended in serum-free DMEM medium containing 0.1% bovine serum albumin (BSA) and placed in the upper chamber. The bottom chamber was filled with DMEM containing 10% FBS. The FBS attracted the cancer cells and triggered their migration to the underside of the membrane. Breast cancer cells that have the ability to invade secrete factors which allow them to degrade the Matrigel (e.g., matrix metalloproteinases) and migrate through the 8 μm pores to the lower chamber of the membrane. After 24-hour incubation, the membrane of the upper chamber was cleaned with cotton swabs to remove the Matrigel and the cells that did not migrate. The membrane was fixed and stained using Diff-Quik solutions (Dade-Behring, Newark, DE). Staining of cells allows their visualization and quantification using a light microscope. Five fields of adherent cells were randomly counted in each well with a Nikon Diaphot-TMD (Atlantic Lab Equipment, Salem, MA, USA) inverted microscope at 20X magnification.

REAL-TIME REVERSE TRANSCRIPTION PCR ANALYSIS

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), using 2 mg of RNA for each reaction. Primer pairs were designed using Primer3 software [22] and are shown in **Table 4.1**. Real-time PCR was performed with the SYBR GreenER qPCR kit (Invitrogen, Carlsbad, CA, USA) in the Mastercycler ep Realplex Real-time PCR thermocycler (Eppendorf, Wesseling-Berzdorf, Germany). The

relative expression levels of target genes were normalized to the housekeeping gene 18S rRNA. Amplification specificity was confirmed by melting curve analysis.

WESTERN BLOT ANALYSIS

Cells were lysed using RIPA buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 20 mM MgCl2, 1% Nonidet P40 containing protease inhibitors (1 µg/ml PMSF, 1 µg/ml aprotinin and 1 µg/ml pepstatin). Samples were incubated for 1-hour on ice with agitation and centrifuged at 12,000 x g for 20 min. Protein samples were subjected to electrophoresis on 4-12% SDS-polyacrylamide gradient gels and transferred to a PVDF membrane. Membranes were probed with anti-Nm23-H1 (BD Biosciences, San Jose, CA, USA) and anti-actin (Oncogene, Cambridge, MA, USA) antibodies. Protein-antibody complexes were detected with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) followed by enhanced chemiluminescence reaction. Immunoblots were quantified using ImageJ software (NIH website: http://rsbweb.nih.gov/ij/index.html).

REAL-TIME QUANTITATIVE PCR ARRAY OF 84 HUMAN EXTRACELLULAR MATRIX AND ADHESION MOLECULES

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA was prepared by reverse transcription using the RT^2 PCR Array First Strand kit (SA Biosciences, Frederick, MD) as recommended by the manufacturer's instructions. PCR array analysis of 84 genes related to cell-cell and cell-matrix interactions as well as human extracellular matrix and adhesion molecules (RT^2 Profiler™ PCR array, PAHS-013A-1, SA Biosciences, Frederick, MD) was performed using the Mastercycler ep Realplex real-time PCR thermocycler (Eppendorf, Wesseling-Berzdorf, Germany). Briefly, 25 µl of PCR mixture, which contained cDNA equivalent to 1 µg RNA in

SuperArray RT^2 qPCR Master Mix solution, was loaded in each well of the PCR array plate. PCR amplification of cDNA was performed under the following conditions: 10 min at 95°C for one cycle, 15 sec at 95°C, followed by 1 min at 60°C for 40 cycles. All mRNA Ct values for each sample [Ct (sample)] were normalized to glyceraldehyde-3-phosphate dehydrogenase [Ct (GAPDH)] in the same sample. The relative mRNA level was expressed as the value of $2^{-\Delta\Delta Ct}$ (sample).

STATISTICS

One-way analysis of variance (ANOVA) was used to test the statistical significance of the qRT-PCR and invasion assay results (SPSS 12.0 student edition, SPSS Inc. Chicago, IL, USA). To detect statistical significance, p value was set at 0.05, and data are presented as the mean ± standard error of the mean (SEM).

4.4 RESULTS

ALCOHOL INCREASES THE INVASIVE ABILITY OF BREAST CANCER CELLS IN A DOSE-DEPENDENT MANNER

To investigate the role of alcohol in cell invasive ability, human breast cancer T47D cells were treated with 0.1%, 0.2%, and 0.5% v/v ethanol for 24 h. Previous studies have shown that alcohol exposure at these concentrations and length of time *in vitro* yielded biological effects seen in breast cancer patients [98, 135]. We show that alcohol treatment *in vitro* increased the ability of T47D cells to invade in a dose-dependent manner (**Figure 4.1A**). Treatment with 0.1%, 0.2%, and 0.5% v/v alcohol increased cell invasion by approximately two-, four-, and six-fold, respectively (**Figure 4.1A**, $p<0.05$). Similar results were seen with MCF-7 and MDA-MB-231, human breast cancer cell lines with low and high, respectively, invasive potential (**Figure 4.1B**).

ALCOHOL INCREASES BREAST CANCER CELL INVASIVENESS BY SUPPRESSING NM23 EXPRESSION

To investigate the possibility that alcohol may increase cellular invasive ability by inhibiting the expression of specific metastasis suppressing genes, we determined the effects of alcohol on known metastasis suppressor genes. We examined the effects of 0.5% v/v ethanol on the expression levels of *Nm23*, *KISS1*, *Mkk4*, *RRM1*, *KAI1*, and *BRMS1* metastasis suppressor genes *in vitro* by qRT-PCR (**Figure 4.2**). Our results show that alcohol significantly suppressed the expression of *Nm23* by approximately 50% (**Figure 4.2**, $p<0.05$), suggesting that the *Nm23* metastasis suppressor gene may be involved in alcohol-induced cell invasion.

To determine whether the effects of alcohol on the invasive ability of T47D cells can be blocked via *Nm23*, we transfected T47D cells with the *pcDNA3-Nm23-H1* vector (kindly provided by Dr. Patricia Steeg at the National Cancer Institute, Bethesda, MD, USA) to overexpress *Nm23*. As expected, *Nm23* overexpression resulted in a significant decrease in T47D cell invasion (**Figure 4.3A**, $p<0.05$) while treatment of T47D control cells (transfected with an empty vector) with 0.5% v/v alcohol significantly increased cell invasive ability (**Figure 4.3A**, $p<0.05$). (Note: Results from **Figure 4.1A** and **Figure 4.3A** indicate that 0.5% v/v ethanol increased cell invasion by 600% and 50%, respectively. This difference may be attributed to the addition of G418 (Gibco, St Louis, MO, USA) in the media used for the invasion assay shown in **Figure 4.3A**. As an inhibitor of protein synthesis, addition of G418 may have led to a decline in cell proliferation over the 24-hour invasion period.) However, 0.5% v/v alcohol was unable to increase the invasive ability of T47D cells overexpressing *Nm23* (**Figure 4.3A**, $p>0.05$), suggesting that *Nm23* expression is critical in alcohol-induced T47D breast cancer cell invasion. Nm23 protein levels are shown in **Figure 4.3B**.

DOWN-REGULATION OF NM23 INCREASES ITGA5 EXPRESSION TO PROMOTE BREAST CANCER CELL INVASION

To examine the downstream targets of Nm23 involved in alcohol induced cell invasion, we determined the effects of *Nm23* overexpression and 0.5% v/v ethanol treatment on 84 genes associated with extracellular matrix regulation and adhesion molecules in the following groups of breast cancer cells: 1) T47D controls cells (empty vector), 2) T47D cells treated with 0.5% v/v alcohol (empty vector), 3) T47D cells overexpressing *Nm23*, and 4) T47D cells overexpressing *Nm23* and treated with 0.5% alcohol. Results are presented in **Table 4.2**, with only the most significantly affected genes shown. Interestingly, one gene observed to be affected by alcohol and *Nm23* in the opposite manner was fibronectin receptor subunit integrin alpha 5 (*ITGA5*). In cells overexpressing *Nm23*, alcohol treatment was no longer able to increase *ITGA5* expression (**Table 4.2**). Additionally, alcohol exposure increased the expression of *ITGA5* nine-fold; however, this effect was eliminated by the overexpression of *Nm23* (**Figure 4.4A** and **Table 4.2**), suggesting that *Nm23* blocked the effects of alcohol. Thus, our data suggests that the effects of alcohol on *ITGA5* are *Nm23*-dependent.

To determine the relationship between Nm23 and ITGA5 in alcohol-treated T47D breast cancer cells, we knocked down each gene separately and in combination, using small interfering RNA (siRNA), and subsequently measured cell invasion. If alcohol increases the invasive ability of T47D cells through the down-regulation of *Nm23*, as suggested earlier, then down-regulation of *Nm23* should increase the invasiveness of T47D cells. Indeed, results show that knock-down of *Nm23* by siRNA increased the invasiveness of T47D cells and alcohol was unable to further increase the invasive ability of T47D cells significantly when *Nm23* was suppressed (**Figure 4.5A**). This work is in

agreement with our results in **Figure 4.2** and provides further evidence that alcohol increases the invasiveness of T47D cells through *Nm23*.

To establish the relationship between alcohol, Nm23, ITGA5 and cell invasion, we knocked down *ITGA5* with siRNA in T47D cancer cells and measured the ability of alcohol to affect the invasive ability of these cells. Results show that down-regulating *ITGA5* significantly inhibited the ability of T47D breast cancer cells to invade (**Figure 4.5A**, $p<0.05$). In agreement that decreased *ITGA5* expression reduces cell invasive ability, we show that both the *Nm23* overexpressing cells and the alcohol-treated *Nm23* overexpressing cells have significantly reduced *ITGA5* expression (**Figure 4.4A**) as well as have an overall lower cell invasive ability (**Figure 4.3A**) compared to controls. We also show that alcohol-treated *Nm23* overexpressing cells have slightly higher *ITGA5* levels compared to non-alcohol-treated *Nm23* overexpressing cells (**Figure 4.4A**) and this translated to a slightly higher, although not statistically significant, number of invaded cells (**Figure 4.3A)**. Nm23 and ITGA5 protein expression in T47D cells is shown in **Figure 4.4B**. To examine whether the Nm23-ITGA5 effects on invasion were specific to T47D cells, we exposed MCF-7 and MDA-MB-231 cells to various doses of ethanol. We show that alcohol is able to increase Nm23 and decrease ITGA5 in a dose-dependent manner (**Figure 4.4C**) and this correlated with increasing cell invasive ability (**Figure 4.1B**). Moreover, when *ITGA5* was knocked down with siRNA, alcohol was unable to increase the invasion of T47D cancer cells, suggesting that *ITGA5* is necessary for alcohol to increase the invasive ability of T47D cancer cells. Furthermore, in *ITGA5* knocked-down cells, suppression of *Nm23* by siRNA did not rescue their invasive ability (**Figure 4.5A**). Results also show that *Nm23* knock-down increased *ITGA5* expression; however, knockdown of *ITGA5* did not affect Nm23 expression (**Figure 4.5B**), suggesting that Nm23 is an upstream factor of ITGA5. Depletion of Nm23 and ITGA5 in

T47D cells following siRNA transfection is shown in **Figure 4.5C**. In summary, the above findings suggest that alcohol increases the invasive ability of breast cancer cells by down-regulating *Nm23*, which increases *ITGA5* expression, and this elevation in *ITGA5* increases the ability of breast cancer cells to invade.

4.5 DISCUSSION

We show that alcohol increases the invasive ability of breast cancer cells in a dose-dependent manner. This suggests that alcohol may increase the ability of the cancer to metastasize. In fact, both animal and epidemiological findings suggest that alcohol increase the metastatic ability of breast cancers [131]. Vaeth et al. showed that frequent alcohol drinkers were 1.45-times more likely to be diagnosed with later stage breast cancer than infrequent drinkers [61]. Additionally, animal studies suggest that alcohol consumption increases the incidence of lung metastasis [15]. Thus, it is critical to understand the mechanism by which alcohol promotes the invasive ability of breast cancer cells in order to develop prevention and treatment options for cancer metastasis. Our data suggest that alcohol increases the invasive ability of breast cancer cells via the *Nm23* metastasis suppressor gene. More importantly, we show that the invasive ability associated with alcohol can be blocked by regulating *Nm23* levels.

The expression of integrins (e.g., ITGA5) in cancer cells is essential as they allow the cells to attach to the endothelium found within the blood vessels of organs such as the lungs (a secondary site for tumor metastasis) [136]. Thus, the levels of integrins such as ITGA5 determine how aggressively the cancer cells may spread to secondary tissues. Our data shows that alcohol exposure increases the expression of the fibronectin receptor subunit *ITGA5* in T47D breast cancer cells. Furthermore, overexpression of *Nm23* can

block the effects of alcohol on *ITGA5* expression. Additionally, results show that suppression of *Nm23* by siRNA increases the expression of *ITGA5* in the cancer cells, thus, indicating that *Nm23* regulates *ITGA5* expression. Furthermore, we show that down-regulation of *ITGA5* is sufficient to block the effects of alcohol on the invasion of T47D cells. Further investigation with other breast cancer cell lines will be necessary before conclusive statements can be made regarding the involvement of the Nm23-ITGA5 pathway in alcohol-induced breast cancer cell invasiveness. Nevertheless, our results indicate that alcohol decreases the expression of *Nm23*, thereby allowing *ITGA5* to be expressed, which in turn allows T47D breast cancer cells to obtain a more invasive phenotype.

Further investigation is also necessary to better understand how alcohol regulates Nm23 expression and how Nm23 regulates ITGA5 expression. It is well accepted that alcohol may promote breast cancer development via the estrogen signaling pathway [131]. As breast cancer cells are able to produce estrogen *in vitro*, the binding of estrogen to the estrogen receptor α (ERα) may activate downstream PI3K/Akt and MAPK/ERK pathways to promote cell migration [137, 138]. In a recent study, it was reported that estrogen negatively regulates Nm23 expression *in vitro* [139]. Thus, the modulation of Nm23 expression shown in this study as a result of alcohol exposure may be mediated by estrogen levels. As a NDP kinase, Nm23 may modify cytoskeleton organization and protein trafficking, possibility through ITGA5, to promote cell migration and adhesion to the extracellular matrix (ECM). Previous studies have shown that Nm23 decreases activity of Rac1, a specific nucleotide exchange factor, through binding of Tiam1 [140, 141]. Reduction of Rac1 activation induces the activity of RhoA, a component in the ITGA5-mediated cellular adhesion and migration signalling pathway [141, 142]. Indeed, estrogen has been found to activate RhoA and this activity is necessary for cytoskeletal

remodelling and for the enhancement of breast cancer cell migration and invasion [143]. Thus, down-regulation of Nm23 by alcohol may promote RhoA activation through estrogen regulation to favor ITGA5-mediated breast cancer progression.

The ECM and adhesion molecules play a critical role in the invasive phenotype of cancer cells [144]. For example, the binding of integrins to ECM proteins stimulates the phosphorylation of focal adhesion kinase (FAK); this activated FAK can activate signaling pathways such as PI3K, MAPK, and ERK [145]. These pathways have been shown to regulate cell adhesion, motility, invasion, and metastasis [146]. Integrins are heterodimer cell surface receptors composed of α and β subunits. The integrin α5 subunit (ITGA5) dimerizes exclusively with the β1 integrin (ITGB1) to form the classic fibronectin receptor (α5/β1 or ITGA5B1) [147]. The interaction of α5/β1 with fibronectin (FN) plays an important role in the adhesion of cancer cells to the extracellular matrix [148]. Moreover, previous studies have shown that interaction of α5/β1 with FN promotes activation of the ERK and PI3K signaling pathways, which in turn stimulates cells to invade and produce MMPs (e.g., MMP-1 MMP-9) to facilitate invasion [149]. In our studies, we show that the integrein α5 subunit expression is necessary for alcohol to increase the invasive ability of T47D breast cancer cells. It is possible that alcohol stimulates signaling pathways such as ERK and PI3K, via α5/β1, which then increases the invasive phenotype of T47D breast cancer cells. Consequently, activated integrins may facilitate the movement and metastasis of breast cancer cells. In future studies, we will determine if alcohol affects signaling pathways such as FAK, ERK, and PI3K via ITGA5 and elucidate the role of estrogen in alcohol-mediated down-regulation of Nm23.

In conclusion, our data suggest that alcohol increases breast cancer cell invasion by regulating the Nm23-ITGA5 pathway. Alcohol exposure in human breast cancer T47D cells down-regulated expression of the *Nm23* metastasis suppressor gene, leading

to increased expression of the *ITGA5* fibronectin receptor subunit, and consequently induced cellular invasion *in vitro*. Results from this work suggest that modulation of the Nm23-ITGA5 pathway may be important for the prevention and treatment of human breast cancers.

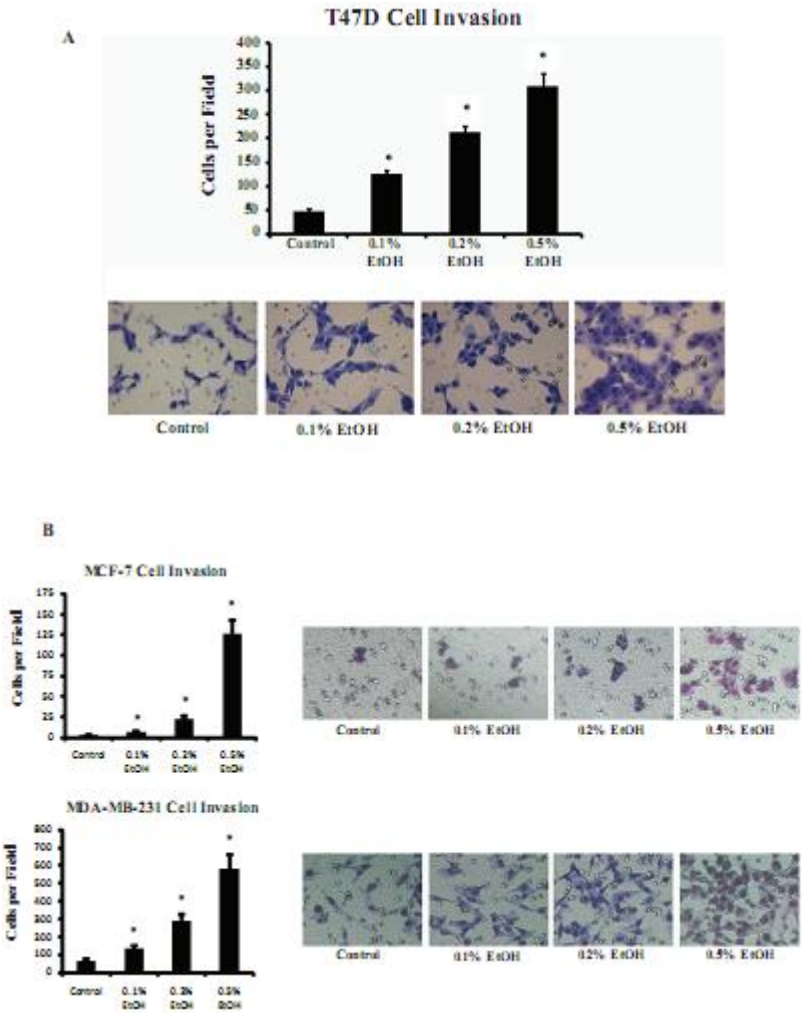

Figure 4.1. Alcohol induces cell invasion in a dose-dependent manner.
Human breast cancer cells were treated with 0.1%, 0.2%, and 0.5% v/v ethanol for the invasion assay. (A) The top panel shows the average number of T47D cells per field that have invaded through the basement membrane-like Matrigel layer and into the lower Boyden chamber following the invasion assay. Diff-Quik staining of the lower chamber following the assay is shown below. The number of cells in the lower chamber is a direct measurement of cell invasion. (B) Invasion assay results are shown using MCF-7 (low invasive potential) and MDA-MB-231 (high invasive potential) breast cancer cells. (*$p<0.05$, as compared to the control cells with no alcohol treatment)

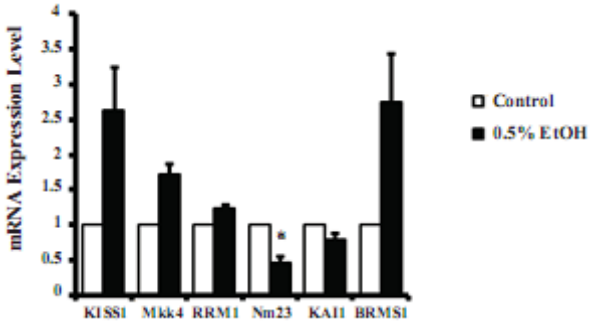

Figure 4.2. Alcohol induces cell invasion by suppressing Nm23 expression.
T47D cells were treated with 0.5% v/v alcohol and the expression of known metastasis suppressor genes was determined by qRT-PCR. *Nm23* mRNA expression levels significantly decreased following treatment. *KAI1*, *RRM1*, and *BRMS1* expression were not affected by alcohol and expression of *KISS1* and *Mkk4* were increased by alcohol. (*$p<0.05$, as compared to the control cells with no alcohol treatment)

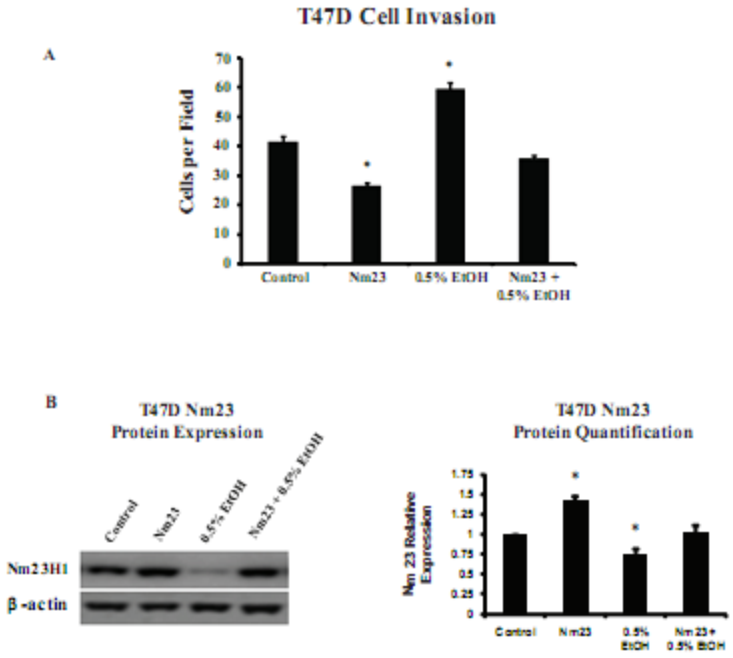

Figure 4.3. Overexpression of Nm23 suppressed cell invasion.
The invasion assay was used to determine the invasive ability of T47D cells treated with 0.5% v/v ethanol and overexpressing *Nm23*, independently and in combination. (A) Alcohol treatment increased the invasiveness of the T47D cells transfected with the empty vector; however, alcohol did not increase invasion in the T47D cells transfected with *Nm23*. (B) Western blot shows Nm23 expression levels following ethanol treatment, Nm23 overexpression, and the combination of ethanol and Nm23 overexpression. Quantification by ImageJ software indicates relative Nm23 expression. (*$p<0.05$, as compared to the control cells transfected with empty vector)

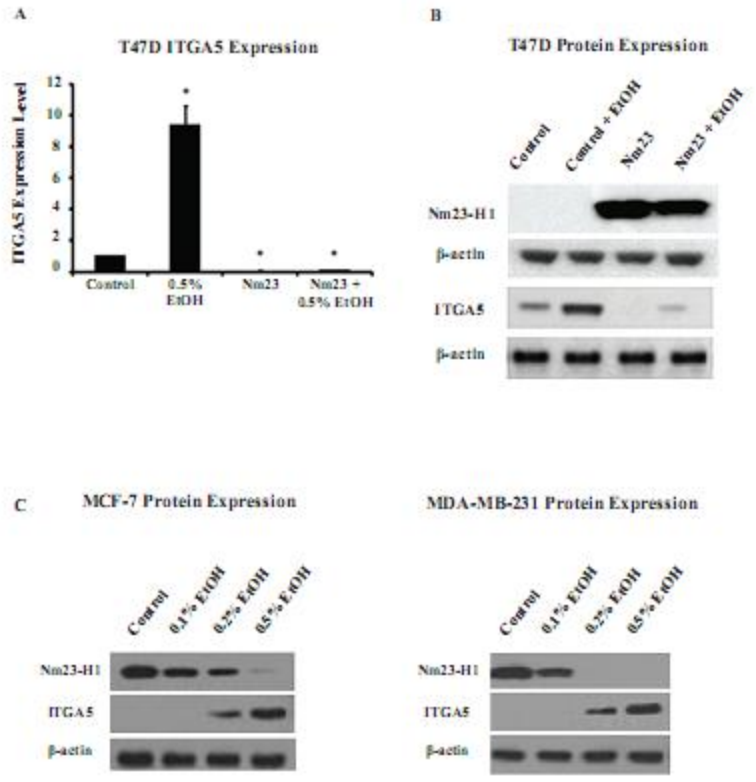

Figure 4.4. Nm23 down-regulates ITGA5 expression.
Nm23 regulates cell invasion through ITGA5 expression. (A) *ITGA5* mRNA levels were determined by qRT-PCR in T47D cells treated with 0.5% v/v ethanol and overexpressing *Nm23*, independently and in combination. Alcohol promotes *ITGA5* mRNA expression approximately nine-fold. This effect was blocked by the overexpression of *Nm23*. (B) Western blot shows Nm23 and ITGA5 protein level in T47D cells with ethanol treatment, Nm23 overexpression, and in combination. (C) Western blots show Nm23 and ITGA5 protein level in MCF-7 and MDA-MB-231 cells following various doses of ethanol treatment. (*$p<0.05$, as compared to the control cells transfected with empty vector)

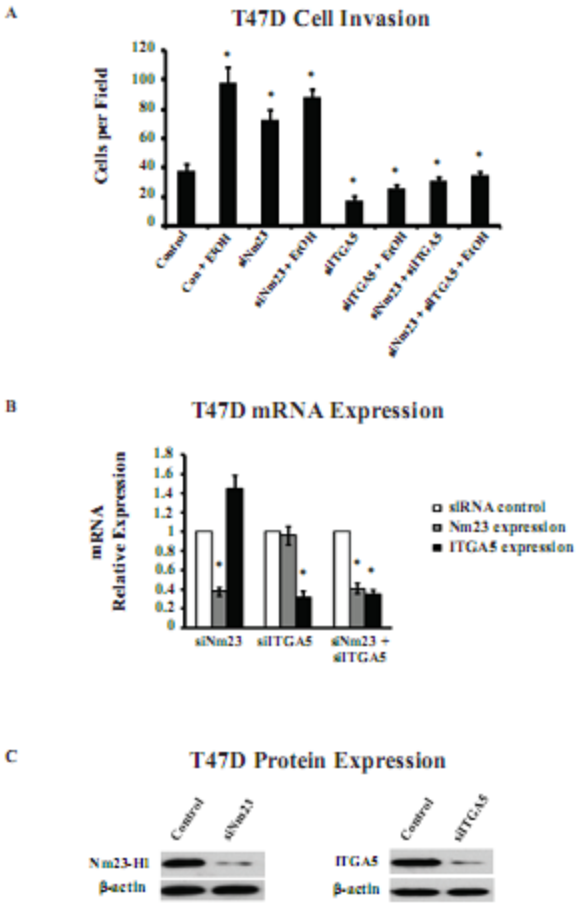

Figure 4.5. Nm23 knock-down promotes cell invasion and increases ITGA5 expression.
Nm23 and *ITGA5* were knocked down via siRNA to determine their effects on T47D cell invasion. (A) The invasion assay showed that alcohol and siNm23 independently increased cell invasion. *ITGA5* knockdown by siRNA suppressed EtOH and siNm23-induced cell invasion in T47D cells. *ITGA5* siRNA decreased cellular invasion. (B) Following siNm23 in T47D cells, mRNA expression of *Nm23* was reduced 62% while *ITGA5* mRNA expression increased relative to the siRNA control. siITGA5 in T47D cells resulted in a 65% knock-down of *ITGA5* expression and *Nm23* levels were not affected. Double siRNA of *Nm23* and *ITGA5* suppressed the expression of both to less than 40%. (C) Western blot shows expression of Nm23 and ITGA5 following siRNA. (*$p<0.05$, as compared to the control cells)

Table 4.1. Primer sequences used for qRT-PCR

Gene name	Sequence
Nm23	F: 5'-ACC TGA AGG ACC GTC CAT TCT TTG C-3'
	R: 5'-GGG TGA AAC CAC AAG CCG ATC TCC T-3'
KISS1	F: 5'-ACC TGC CTC TTC TCA CCA AG-3'
	R: 5'-TAG CAG CTG GCT TCC TCT C-3'
Mkk4	F: 5'-GCA ACT TGA AAG CAC TAA ACC-3'
	R: 5'-CAT GTA TGG CCT ACA GCC AG-3'
RRM1	F: 5'-ACT AAG CAC CCT GAC TAT GCT ATC C-3'
	R: 5'-CTT CCA TCA CAT CAC TGA ACA CTT T-3'
KAI1	F: 5'-CAT GAA TCG CCC TGA GGT CAC CTA-3'
	R: 5'-GCC TGC ACC TTC TCC ATG CAG CCC-3'
BRMS1	F: 5'-ACT GAG TCA GCT GCG GTT GCG G-3'
	R: 5'-AAG ACC TGG AGC TGC CTC TGG CGT GC-3'
MMP1	F: 5'-CTG TTC AGG GAC AGA ATG TGC T-3'
	R: 5'-TCG ATA TGC TTC ACA GTT CTA GGG-3'
MMP2	F: 5'-TCA CTC CTG AGA TCT GCA AAC AG-3'
	R: 5'-TCA CAG TCC GCC AAA TGA AC-3'
MMP9	F: 5'-CCC TGG AGA CCT GAG AAC CA-3'
	R: 5'-CCA CCC GAG TGT AAC CAT AGC-3'
MMP13	F: 5'-TCC TCT TCT TGA GCT GGA CTC ATT-3'
	R: 5'-CGC TCT GCA AAC TGG AGG TC-3'
MMP14	F: 5'-TGC CTG CGT CCA TCA ACA CT-3'
	R: 5'-CAT CAA ACA CCC AAT GCT TGT C-3'
ITGA5	F: 5'-GTC GGG GGC TTC AAC TTA GAC-3'
	R: 5'-CCT GGC TGG CTG GTA TTA GC-3'
18S rRNA	F: 5'-TAC CTG GTT GAT CCT GCC AG-3'
	R: 5'-GAG CTC ACC GGG TTG GTT TTG-3'

Table 4.2. Effects of alcohol and Nm23 overexpression on extracellular matrix and adhesion proteins expression

Gene Name	0.5% EtOH	Nm23-H1	0.5% EtOH + Nm23-H1
VCAN	4.1125	3.1514	4.359
COL8A1	-18.2522	-18.6875	-8.9755
CTGF	-4.3772	-5.712	-4.1296
CTNNA1	-15.455	-20.1681	-14.5808
CTNNB1	5.6569	5.5251	5.9134
CTNND1	-69.551	-18.9483	-26.4647
CTNND2	16.9123	12.9601	17.9262
ITGA1	-1.7777	-2.3168	-1.6771
ITGA2	-6.4531	-8.421	-6.0881
ITGA4	-5.3889	-7.0323	-5.0841
ITGA5	**9.3827**	**-12.0754**	**-9.038**
ITGA6	-1.1408	-1.4886	-1.0762
ITGA7	-8.1681	-7.5371	-5.4869
ITGAL	-6.3643	-8.3051	-6.0043
ITGAV	-2.042	-2.6647	-1.9265
ITGB1	-3.0314	-3.2355	-1.554
ITGB2	-2.3295	-3.0398	-2.1977
ITGB3	-5.2416	-4.8032	-3.8798
ITGB4	-1.021	1.8226	1.6066
ITGB5	-19.4271	-15.3908	-3.62
KAL1	1.454	1.1142	1.5411
LAMA1	1.1096	-1.1761	1.1761
MMP1	4.1487	-1.136	1.2176
MMP10	-12.5533	-11.3451	-5.191
MMP13	24.761	18.9746	26.2455
MMP16	4.1989	4.1583	5.6334
MMP2	3.249	1.7363	2.3685
NCAM1	-3.8106	-4.9726	-3.595
PECAM1	-13.4543	-17.5573	-12.6933
SELE	1.2483	-1.0454	1.3232
SELL	7.0128	5.374	7.4333
SELP	-7.1107	-9.2792	-6.7085
SGCE	1.021	-1.2781	1.0822
SPG7	10.4107	6.0043	8.2477
CLEC3B	-1.4641	-1.9106	-1.3813
TNC	-3.9177	-5.1124	-3.6961
VCAM1	1.0281	1.325	1.0898

Chapter 5: Summary

5.1 SUMMARY

Alcohol consumption is an established risk factor for breast cancer progression. Yet, the mechanism remains unclear. Given that many women drink some form of alcoholic beverage frequently, it is important to understand how alcohol consumption promotes breast cancer development. Determining the mechanism by which alcohol affects breast cancer may provide a therapeutic strategy to prevent alcohol-related breast cancers in women. In terms of the impact on breast cancer prevention, our findings may help define better recommendations on alcohol consumption for women in order to decrease their risk of developing this disease.

In this dissertation, I present several unique findings. In the first study, we provide direct evidence suggesting alcohol increases mammary tumor development via the estrogen pathway. Our data show that alcohol consumption promotes HER2 mammary tumor development in MMTV-neu mice but only in the presence of ovarian hormones. The acceleration of tumor development in the MMTV-neu mice due to alcohol consumption were associated with increased systemic estrogen levels, and increased expression of aromatase and ERα in tumor tissues. Furthermore, we show that the effects of alcohol on tumor development in HER2 mice can be blocked by ovariectomy (removal of the ovaries and ovarian estrogen production). Since alcohol only promoted tumor development in the presence of physiologically normal systemic levels of estrogen, results suggest that the effects of alcohol on HER2 mammary tumor development may be mediated via estrogen. Even though alcohol increased the expression of ERα in the OVX

(low estrogen levels) mice, alcohol did not increase tumor incidence and tumor growth in these OVX mice. However, one caveat in this study is that ovariectomized mice failed to develop tumors in numbers comparable to non-ovariectomized mice. It is possible that this may be due to the OVX mice exhibiting immature mammary glands as a result of having ovariectomy performed at 8-weeks of age. Repeating the study with OVX mice undergoing ovariectomy at 16-weeks of age, when the mice become sexually mature and have fully developed ductal branching in the mammary glands [52], will eliminate this caveat.) Thus, it is possible that alcohol only affects tumor development in the presence of normal circulating estrogen levels, which the OVX mice lacked. This finding is significant because it indicates that drug therapies targeting the estrogen pathway, more specifically the production of estrogen, may be able to block the effects of alcohol on breast cancer development. In humans, pre-menopausal women exhibit normal circulating estrogen levels, whereas post-menopausal women exhibit low estrogen levels [45]. Thus, our results may indicate that drug therapies targeting the estrogen pathway may be effective for pre-menopausal women at risk for alcohol-mediated breast cancer.

Our second study seeks to explain the role of alcohol on ERα expression regulation, a direct follow-up to our first study where we showed that alcohol consumption promoted ERα expression and activation in the primary mammary tumors of MMTV-neu mice, a mouse model which is reported to develop ERα-negative tumors. Here, we examined the methods underlying ERα re-expression and reactivation in the ERα-negative MMTV-neu cell line derived from the tumors of MMTV-neu mice and in the ERα-negative MDA-MB-231 human breast cancer cell line. ERα is an important

prognostic biomarker and therapeutic target in primary breast cancer. ERα-negative tumors typically appear to be more malignant and have poorer prognosis compared witgh ERα-positive tumors [117, 118]. Epigenetic mechanisms have previously been used to explain the repression of ERα expression in ERα-negative breast cancer cells. Findings from this study supports this notion and shows that ERα re-expression and reactivation due to alcohol treatment in ERα-negative breast cancer cells is associated with changes in ERα promoter CpG methylation status, enrichment of active chromatin markers and the increased recruitment the p300 histone acetyltransferase within the pRb2/p130-associated ERα transcription regulation complex. More importantly, we showed that the inhibition of ERα by tamoxifen specifically blocks the effects of alcohol, lending to support of our hypothesis that alcohol mediates breast cancer development and progression through regulation of the estrogen signaling pathway. Along with data from our first study, results from this study indicate that alcohol is affecting cancer cell ERα expression and estrogen signaling, and that these effects may mediate increased breast cancer risk, as seen in the MMTV-neu mice.

In contrast to the first two studies, our third study explores the effects of alcohol on a later stage of cancer cell progression, cellular invasion, and we uncover a possible mechanism to explain the phenomena. The ability of cancer cells to invade through tissue barriers (such as basement membrane and interstitial stroma) is an essential step towards establishing cancer metastasis. Breast cancer cells typically spread from the primary tumor site (the breast) to secondary sites (i.e. lungs, liver, bones, etc.) resulting in an increased likelihood of mortality [132]. Thus, it is critical to understand the mechanism

by which alcohol promotes the invasive ability of breast cancer cells in order to develop prevention and treatment options for cancer metastasis. In this study, we found that alcohol increases breast cancer cell invasion by regulating the Nm23-ITGA5 pathway. Our data show that alcohol increases the invasive ability of breast cancer cells by significantly down-regulating the expression of the *Nm23* metastasis suppressor gene. To verify this, we showed that the invasive ability associated with alcohol can be blocked by regulating *Nm23* levels. We next found that the down-regulation of *Nm23* leads to the subsequent increased expression of *ITGA5* (a fibronectin receptor subunit which facilitates metastasis), which we show consequently induces cellular invasion *in vitro*. Furthermore, we show that down-regulation of *ITGA5* is sufficient to block the effects of alcohol on cell invasive ability. Collectively, our results indicate that alcohol decreases the expression of *Nm23*, thereby allowing *ITGA5* to be expressed, which in turn allows breast cancer cells to obtain a more invasive phenotype. Our findings here suggest that modulation of the Nm23-ITGA5 pathway may be important for the prevention and treatment of human breast cancers.

In conclusion, our studies suggest that alcohol promotes mammary tumor development through regulation of estrogen signaling. While other animal studies have shown alcohol to be a significant breast cancer risk factor, to our knowledge, no study has shown the mechanism(s) by which alcohol affects breast cancer development. Others have suggested alcohol may affect breast cancer via the estrogen pathway; however, no study has shown that estrogen is indeed the key factor by which alcohol affects breast cancer. Most studies on the alcohol-cancer relationship ignore the fact that breast cancer

develops in both pre- and post-menopausal women and that breast cancer cells can be ERα-positive or ERα-negative. Our studies are novel because we demonstrate the effects of alcohol in both the presence and absence of ovarian hormones. Given that many women frequently drink alcoholic beverages, it is important to understand how and why alcohol affects breast cancer so that better recommendations regarding alcohol consumption can be made.

5.2 Future Directions

5.2.1 The role of estrogen receptor alpha in alcohol-mediated breast cancer

In this dissertation, our results strongly suggest that alcohol may affect breast cancer via ERα. To verify that indeed alcohol affects breast cancer via ERα the following set of experiments may be carried out. To verify if the presence of ERα is necessary for alcohol to affect breast cancer development, it would be interesting to examine the effects of alcohol mammary cancers where ERα has been deleted. Therefore, to generate MMTV-neu female mice with various levels of ERα, the following breeding of mice may be done: male MMTV-neu mice may be bred to females with different ERα genotypes. This breeding will produce offspring with varied ERα expression. The offspring produced would be WT/neu; ERα +/-, neu; and αERKO/neu, shown in **Figure 5.1**. In this way, ERα expression can be controlled in female mice and, thus, the effects of alcohol on breast cancer development can be examined in the presence of normal, low and absent ERα levels.

Female mice heterozygous for the ERα gene survive to adulthood with a normal gross external phenotype and respond to estrogen stimulation similar to mice wild-type for the ERα gene [150]. However, the ERα heterozygous mice are reported to be infertile

[150]. The αERKO mouse model exhibits a complete lack of functional ERα proteins and exhibits no significant changes in ERß gene expression [151]. Furthermore, the expected proportion of heterozygous and homozygous offspring are born from matings of αERKO to non-αERKO mice, indicating that no essential aspects of early development depend on ERα and that carrying out the experiment with NOVX and OVX αERKO/neu animals exhibiting the lack of ERα expression from initial development is possible.

Based on data presented in this dissertation, we would expect that, of the female mice groups shown in **Figure 5.1**, alcohol treatment would promote breast cancer development and progression in the WT/neu mice and in the ERα +/-, neu mice compared to their water-treated counterparts. We would expect to see no difference in breast cancer development between alcohol-treated and water-treated αERKO/neu mice. Our data suggest that alcohol mediates breast cancer through the regulation of ERα; thus, the absence of ERα in αERKO/neu mice would result in no significant difference in tumor incidence and progression among alcohol-treated and water-treated animals. On the same note, we may even expect to see alcohol having a less significant effect on breast cancer development in the ERα +/-, neu mice compared to the WT/neu mice, as ERα +/-, neu animals have lower levels of ERα expression. Altogether, this proposed future work would verify our findings that suggest ERα expression and its regulation is critical to alcohol-mediated breast cancer development.

Alternatively, ERα activation can specifically be attenuated in MMTV-neu animals by pharmacological means using tamoxifen. Tamoxifen acts as an ERα antagonist in the breast tissue by binding to ERα and preventing its activation by the estrogen ligand [52]. Currently, tamoxifen is being used as an effective treatment of ERα-positive breast cancer [112, 152]. To verify that the presence (or initial presence) ERα is critical in alcohol-mediated breast cancer, MMTV-neu mice, which are initially ERα-

positive in the mammary epithelium, may be treated with tamoxifen beginning at an early stage. Published reports indicate that tamoxifen can safely be administered in mice at 12-weeks of age [52, 153]. Tumors in MMTV-neu are typically not yet developed by 12-weeks of age and a previous report has suggested that the mammary epithelium of mice at this stage are still ERα-positive and respond to tamoxifen treatment [52]. Thus, beginning tamoxifen treatment in 12-week old ERα-positive MMTV-neu mice will specifically block ERα activation. We would expect alcohol treatment in these tamoxifen-treated animals to have no significant effect on breast cancer development as the activation of ERα is inhibited by the drug. On the other hand, by the time MMTV-neu female mice reach the age of 24-weeks the subclinical tumors found on these mice are ERα-negative; thus, if we feed alcohol to 24-week old MMTV-neu female mice and if alcohol is still able to induce the expression of ERα and tumor promotion, it will suggest that alcohol may also promote the development of ERα-negative tumors. Additionally, if the effects of alcohol in 24-week old mice are inhibited by tamoxifen, then it will strongly suggest that the effects of alcohol on mammary tumor development are indeed mediated via ERα. In women, studies show an association between alcohol consumption and the development of ERα-positive breast cancer. The major weakness of these studies is that the authors had not identified the status of ERα expression in the tumor cells when the women first began to consume alcohol. However, our studies suggest that alcohol causes re-expression of ERα in an otherwise ERα-negative breast cancer subtype; thus, if this is accurate then our studies will suggest that alcohol may have an even greater impact on breast cancer than previously hypothesized. That is, alcohol may influence the development of both ERα-positive and ERα-negative breast cancers. This may also suggest that the number of breast cancers attributable to alcohol consumption may be even greater than previously believed.

5.2.2 THE ROLE OF ESTROGEN IN ALCOHOL-MEDIATED REGULATION OF NM23 AND ITGA5 IN BREAST CANCER CELL INVASION

In this dissertation, we also uncovered two genes, *Nm23* and *ITGA5*, which we show to be important for alcohol-induced breast cancer cell invasion. We found that alcohol increased the invasive ability of human breast cancer T47D cells in a dose-dependent manner through suppressing the *Nm23* metastatic suppressor gene, which, in turn, increased the expression of fibronectin receptor subunit *ITGA5*, subsequently lead to increased cellular invasion. The invasion of cancer cells into surrounding tissues is an initial step in tumor metastasis. As mentioned above, it is often not the primary tumor that leads to cancer patient mortality but, rather, the metastases of the cancerous cells. Further investigation is necessary to better understand how alcohol regulates Nm23 expression and how Nm23 regulates ITGA5 expression.

A recent study reported that estrogen may negatively regulate *Nm23* expression *in vitro.* Thus, the modulation of *Nm23* expression by alcohol exposure *in vitro* may be mediated by estrogen levels. Nm23, an NDP kinase, may modify cytoskeleton organization and protein trafficking, possibility through ITGA5, to promote cell migration and adhesion to the extracellular matrix (ECM). Previous studies have shown that Nm23 decreases activity of Rac1, a specific nucleotide exchange factor, through binding of Tiam1. Reduction of this activation induces the activity of RhoA, a component in the ITGA5-mediated cellular adhesion and migration signaling pathway. Interestingly, estrogen has been found to activate RhoA and this activity is necessary for cytoskeletal remodeling and for the enhancement of breast cancer cell migration and invasion. Thus, down-regulation of Nm23 by alcohol may promote RhoA activation through estrogen regulation to favor ITGA5-mediated breast cancer progression. In future studies, we will

elucidate the role of estrogen in alcohol-mediated down-regulation of *Nm23* and up-regulation of *ITGA5*.

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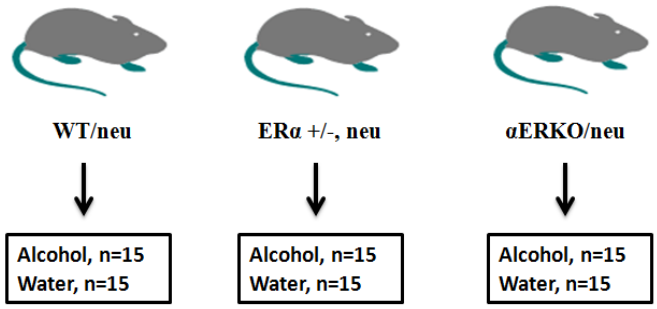

Figure 5.1. Future work to verify the role of ERα in alcohol-mediated breast cancer. Male MMTV-neu mice bred to females with different ERα genotypes will produce offspring with varied ERα expression (shown above). The treatment of these three mice groups with alcohol or water would determine the effects of alcohol on breast cancer in the presence of normal, low and absent ERα levels.

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