Characterization of Alcohol-Mediated Promotion of Breast Cancer Stem Cells

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LOYOLA UNIVERSITY OF CHICAGO

CHARACTERIZATION OF ALCOHOL-MEDIATED PROMOTION OF BREAST CANCER STEM CELLS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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BY
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<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
</tr>
<tr>
<td>ABSTRACT</td>
</tr>
</tbody>
</table>

CHAPTER ONE: INTRODUCTION
- Cancer and Modifiable Risk Factors 1
- Breast Cancer: General Overview 2
- Overview of the Estrogen Receptor Signaling Pathway 4
- Overview of the Notch Signaling Pathway 6
- Notch Signaling and Breast Cancer 9
- Cancer Stem Cell Hypothesis 10
- Maintenance of Breast Cancer Stem Cells by Notch Signaling 11
- Maintenance of Breast Cancer Stem Cells by Estrogen Signaling 12
- Alcohol and the Promotion of Stemness 14

CHAPTER TWO: PRELIMINARY DATA, HYPOTHESES, AND SPECIFIC AIMS 16
- Preliminary Data 16
- Central Hypothesis 18
- Specific Aims 18
  - Aim 1: Elucidate potential mechanisms by which alcohol promotes breast cancer stem cells in naïve ER+ breast cancer cells. 18
  - Aim 1a 18
  - Aim 1b 18
  - Aim 2: Determine the effect of alcohol in long-term estrogen deprived (LTED) ER+ breast cancer stem cells. 19
  - Aim 2 19

CHAPTER THREE: MATERIALS AND METHODS 21
- Materials 21
  - Cell Culture 21
  - Drug Treatments 21
  - Antibodies 22
  - Primers 23
- Experimental Methods 23
  - Western Blotting 23
  - Bicinchoninic Acid (BCA) Assay 23
  - Gel preparation 24
  - Western blot 25
- Reverse Transcription, Real Time Polymerase Chain Reaction (PCR) 26
  - RNA extraction 26
  - Reverse Transcription (RT) 26
  - Quantitative Real Time PCR 27
CHAPTER FOUR: RESULTS
- Acute Alcohol Exposure Induces Expression of CSC-Promoting Genes
- Alcohol Attenuates Transcriptional Regulation of ER Targets by 17β-estradiol
- Alcohol Promotes Expression of Notch4 in a Dose-Dependent Manner
- The Effect of γ-Secretase Inhibition in Bulk and/or BCSCs on BCSC Survival
- Alcohol May Promote the Interaction of the γ-Secretase Complex with Notch4.
- BCSCs are Resistant to GSI Treatment in Bulk Cells Prior to Selection
- Alcohol Promotes Resistance in GSI-Treated BCSCs

Specific Aim 2: Determine the Effect of Alcohol on Long-Term Estrogen Deprived ER+ Breast Cancer Stem Cells

CHAPTER FIVE: DISCUSSION

REFERENCE LIST

VITA
LIST OF FIGURES

Figure 1. Molecular Mechanisms of Estrogen Receptor Signaling to Regulate Gene Expression & Physiological Responses. 4

Figure 2. Structure of the Notch Ligands Receptors. 8

Figure 3. Canonical Notch Signaling Mechanisms to Mediate Transcriptional Activation of Downstream Targets & Pharmacological Inhibitors. 8

Figure 4. The role of ER in Normal Tissue and Breast Cancer Stem Cell Maintenance. 13

Figure 5. Alcohol Metabolism Results in the Production of Acetaldehyde and Increased ROS Formation. 15

Figure 6. Alcohol Enhances BCSC Survival in a Dose-Dependent Manner in Naïve ER+ Cells, Regardless of 17β-estradiol. 17

Figure 7. Alcohol Dose-Dependently Enhances BCSC Size in Naïve ER+ Cells. 17

Figure 8. Expression of HES1 RNA Trends Upwards in Response to Alcohol. 31

Figure 9. Alcohol Treatment Trends Toward Inducing Expression of Pluripotent Genes. 33

Figure 10. Alcohol Alone Induced Expression of Classical ER Target Genes. 34

Figure 11. Alcohol Dose-Dependently Enhances Notch4 Protein Expression. 36

Figure 12. Alcohol Dose-Dependently Enhances DAXX Protein Expression in the Presence of 17β-estradiol. 37

Figure 13. Experimental Design of γ-Secretase Complex Inhibition in Bulk Cells and/or BCSCs. 38

Figure 14. Confirmation of γ-Secretase Complex Inhibition by Target Protein Expression Analysis 39

Figure 15. Accumulation of Notch4 Following γ-Secretase Inhibition is Enhanced by Exposure to Alcohol 40
Figure 16. GSI Treatment in Bulk Cell Populations Results in Resistant BCSC Populations. 43

Figure 17. Alcohol Exposure Trends Towards Enhanced BCSCs Survival in 17β-estradiol treated LTED ER+ Cells. 45

Figure 18. Alcohol Enhances Notch4 and DAXX protein expression in LTED ER+ cells. 46

Figure 19. Regulation of Notch Signaling by 17β-estradiol mediated Activation of the Estrogen Receptor. 48

Figure 20. Alcohol Regulation of 17β-estradiol Mediated ER Activity. 50
LIST OF TABLES

Table 1. Real-time RT-PCR Primers 22
Table 2. Gel Preparation for Western Blot 24
Table 3. Reverse Transcription Reaction (50μL) 27
Table 4. Real-time RT-PCR Reaction (12.5μL) 28
ABSTRACT

Breast cancer is the leading cause of cancer-related death in women globally, and one in eight women will be diagnosed with breast cancer at some point in her life. Alcohol consumption has been linked to increase breast cancer risk and increased risk of tumor recurrence. Understanding the molecular mechanisms of modifiable lifestyle factors such as alcohol consumption will help to lower these risks and design more effective therapeutic approaches.

Estrogen Receptor positive (ER+) breast cancer comprises approximately 70% of all breast cancers, and these tumors are effectively targeted by endocrine therapy in the form of estrogen deprivation with aromatase inhibitors, a selective estrogen receptor modifier (tamoxifen), or a selective estrogen receptor degrader (fulvestrant). However, resistance to endocrine therapy poses a major threat to women with ER+ breast cancer. One proposed mechanism for drug resistance and tumor recurrence is explained by the cancer stem cell hypothesis, which states that tumors are composed of a heterogenous cell population that consists of differentiated and stem-like cells, coined cancer stem cells (CSCs). CSCs are able to evade endocrine therapy and remain dormant until later reactivated by the microenvironment, in which they then re-bulk a tumor, usually at the metastatic site. The effects of alcohol on ER+ breast CSCs have yet to be elucidated. The CSC population is known to be maintained by a variety of signaling pathways. Estrogen signaling is thought to maintain a differentiated state and suppress pluripotent populations, whereas Notch signaling is a known promoter of CSC populations and a necessary component of their survival. Thus, we propose the central hypothesis that alcohol promotes ER+ breast cancer stem cells through the activation of Notch signaling.
Preliminary data show that alcohol enhances ER+ breast CSC survival regardless of the presence of estrogen. Results show that Notch target genes HES1, SOX2, and others are induced upon exposure to 40mM ethanol in naïve ER+ breast cancer cells. On the contrary, exposure to ethanol resulted in attenuated induction of classic ER targets PS2 and PGR in response to treatment with 5nM estradiol. Additionally, inhibition of Notch signaling by a γ-secretase inhibitor had no effect on the ability of ethanol to enhance breast CSC survival when estrogen was present, however under estrogen deprivation conditions Notch inhibition prevented breast CSC survival in the presence of ethanol. Lastly, investigation into the effects of alcohol on an endocrine therapy resistant cell line show a positive trend in breast CSC survival and induced Notch activation.

Taken together, these results indicate that alcohol mediates breast CSC survival partly through Notch activity under estrogen deprived conditions. However, Notch signaling may not be necessary for alcohol’s effect on the CSC population when estrogen is present based on our findings using a GSI in breast CSCs. Further studies are necessary to investigate the exact mechanism of alcohol on estrogen signaling and determine if the Notch activation seen when alcohol is present is due to direct activation of the pathway or through inhibition of its negative regulator, ERα.
CHAPTER ONE
INTRODUCTION

Cancer and Modifiable Risk Factors

Cancer is a significant global burden, with 19.3 million new cases diagnosed and 10 million cancer-related deaths worldwide in the year 2020 (Sung et al., 2021). It is well known that cancer risk is positively impacted by a variety of modifiable lifestyle factors such as physical activity, tobacco use, and diet (Stein & Colditz, 2004, Islami et al., 2018, Gapstur et al., 2018). Among these modifiable factors is alcohol – a classified Group 1 carcinogen as of the year 1988 (Scheilder & Klein, 2018, Testino, 2011). In the year 2019, 69.5% of people aged 18 years or older reported having drank alcohol within the last year, and 54.9% having drank within the last month (SAMHSA Table 2.18B, SAMHSA Table 2.19B). Alcohol is known to increase the risk of at least seven different types of cancer: bowel, oral, pharynx, oesophagus, colon, rectum, and female breast cancer (Connor, 2016, Lopez-Lazaro, 2016, Boffetta and Hashib, 2006, Testino, 2011, Scheilder & Klein, 2018). In young adults, ages 30-34, breast cancers accounted for 18.4% of all alcohol-attributable cancer deaths (Rehm et al., 2020). For every 10 grams of alcohol consumed per day, the risk of breast cancer development increases by 10% in adult women, regardless of menopausal status (Liu et al., 2015) and a 40-50% increase in breast cancer risk seen in women consuming more than three alcoholic beverages a day (Seitz et al., 2021). Drinking 6 grams or more per day also correlates with increased risk of breast tumor recurrence and death, and this risk appears to be elevated in post-menopausal women (Kwan et al., 2010,
Kwan et al., 2013, Simapivapan et al., 2016), however the molecular mechanisms by which alcohol promotes this increased risk have yet to be elucidated.

Early in *vitro* studies suggest that alcohol promotes proliferation of Estrogen Receptor positive (ER+) breast cancer cell lines through the stimulation of estrogen signaling. Cells treated with increasing concentrations of ethanol showed increased proliferation, increased expression of both ERα and aromatase, the enzyme responsible for estrogen synthesis, and intracellular cAMP levels (Fan et al., 2000, Singletary et al., 2001). More recent studies suggest that alcohol regulates transcription of RNA Polymerase III genes in an ERα dependent manner (Zhang et al., 2012), and microarray datasets have shown that alcohol consumption correlated with upregulation of genes associated with recurrence, metastasis, and death in ER+ tumor samples being managed by endocrine therapy (Candelaria et al., 2015).

**Breast Cancer: General Overview**

In the year 2020, female breast cancer became the most commonly occurring cancer globally, overtaking lung cancer (Sung et al, 2021, Siegel et al., 2021). A projected 43,600 women will lose their life to breast cancer in the year 2021 according to recent studies (Siegel et al., 2021). Breast cancer can originate from any of the major tissues found within the breast, resulting in either invasive ductal carcinoma, which comprise approximately 80% of all breast cancer diagnoses, or invasive lobular carcinoma which account for about 10% of breast cancers (Wasif et al., 2010, Barroso-Samous and Metzger-Filho 2016).

There are known genetic mutations that are associated with increased breast cancer risk, such as mutations in the *BRCA1* and *BRCA2* genes (Maric et al., 2011, Rosen et al., 2003), as well as *TP53, PTEN, ATM*, and others (Sheikh et al., 2015, Greenblatt et al., 2001, Broeks et al.,
However, the mechanism by which alcohol contributes to the mutation of these genes or the interplay of these mutations with alcohol consumption to promote breast cancer remain under studied (Freudenheim et al., 2004, Dennis et al., 2010, Scoccianti et al., 2014).

Breast cancer is a heterogeneous disease that is classified by its diverse molecular subtypes as defined by protein expression of the Estrogen Receptorα (ERα), Progesterone Receptor (PR), and/or the Human Epidermal Growth Factor Receptor 2 (HER2) (Dai et al., 2016). These tumors can then be further classified as luminal tumors, which express ERα with or without expressing PR, or basal-like tumors which lack expression of ERα, PR, or HER2 and are generally defined by expression of cytokeratins 5, 14, and 17 (Sims et al. 2007).

Tumors that are classified as ERα+ and/or PR+ can be effectively targeted using endocrine therapy, which includes the use of tamoxifen, a selective estrogen receptor modifier (SERM), letrozole, anastrozole, or exemestane, an aromatase inhibitor (AI), and/or fulvestrant, a selective estrogen receptor degrader (SERD). Aromatase inhibitors have been shown to significantly improve disease-free survival (DFS) after five years of treatment for post-menopausal women (Hussain et al., 2004, Tremont et al., 2017). Tamoxifen is approved for pre-menopausal women and has been shown to significantly increase overall survival after five years of adjuvant therapy (Boccardo et al., 1998, Higgens et al., 2013, Colleoni & Munzone, 2015).

Tumors classified as HER2+ overexpress the receptor tyrosine kinase due to a gene amplification of the proto-oncogene ERBB2. HER2+ targeted therapies include humanized monoclonal antibodies, such as trastuzumab and pertuzumab (Gianni et al., 2016, Howie et al., 2019, Hudis 2007), or second-line therapies such as lapatinib or neratinib, which inhibits tyrosine kinase activity (Jones et al., 2009). Tumors that lack expression or overexpression of ERα, PR, and/or HER2 are classified as triple negative, and these tumors lack approved targeted therapies and
thus are treated with cytotoxic mitotic disruptors (i.e. paclitaxel) or genotoxic (i.e. carboplatin) chemotherapy (Hatzis et al., 2015, Ishikawa et al., 2011, Pal et al., 2011, Sharma et al., 2018).

**Overview of the Estrogen Receptor Signaling Pathway**

The Estrogen Receptor was first discovered in 1958 (Jensen et al., 1967, Jensen et al., 1968), and was later cloned using an ER+ breast cancer cell line, MCF-7 (Green et al., 1986). Shortly after its discovery, the role for ER in breast cancer as a prognostic marker was established (Knight III et al., 1977, Samaan et al., 1981, Pike et al., 1993). The predominant endogenous ligand of ER is 17β-estradiol, which is primarily produced in the ovaries and considered the most biologically active hormone in human breast tissue (Russo & Russo, 2006, Russo et al., 2006). There are two known isoforms of the ER, ERα and ERβ, which are both a part of the nuclear receptor superfamily of transcription factors (Hua et al. 2018).

The ER is capable of signaling through a variety of mechanisms, which have been summarized in Figure 1 (McDevitt et al., 2008). Upon binding of its ligand, the ER undergoes a confirmational change that releases it from the sequestration of heat shock proteins and induces either homo- or heterodimerization with Figure 1: Molecular Mechanisms of Estrogen Receptor Signaling to Regulate Gene Expression & Physiological Responses.
other ERs (Cowley et al., 1997, Jisa & Jungbauer 2003). Its classical signaling mechanism is through direct binding of DNA at the Estrogen Response Element (ERE) upon translocation from the cytosol to the nucleus (Hall et al., 2001). Upon binding to the ERE, ER directs transcription of its downstream targets through the recruitment of co-factors, such as the co-activators that include steroid receptor coactivators (SRC-1, -2, or -3), p300CBP, and histone acetyl transferases (HATs) or co-repressors such as NcoR or SMRT and histone deacetylases (HDACs) (Sommer & Fuqua, 2001). Alternatively, the ER can regulate transcription without needing to directly bind to the ERE. It does so mainly through protein-protein interactions, such as binding directly to AP-1 or Sp-1 via its AF-1 and AF-2 domains (Yasar et al. 2016, Fuentes & Silveyra, 2019). There also exists a non-nuclear ER known as GPER-1, which is a G-protein coupled receptor (GPCR) whose endogenous ligand is also E₂ and is known to increase intracellular calcium levels and cAMP production upon activation (Ranganathan et al., 2019).

Lastly, ER signaling can be activated independent of its ligand by other intracellular signaling pathways. For example, it is well known that MAPKs such as ERK1/2 can phosphorylate ERα at Serine 118 to activate the receptor in response to Epidermal Growth Factor (EGF) stimulation (Coleman & Smith, 2001, Bunone et al., 1996).

As stated previously, expression of the ER defines the molecular subtype of breast cancer tumors, with ER+ tumors comprising approximately 70% of all breast cancer cases (Rosenberg et al., 2015, Ferreira Almeida et al., 2020). Normally, ERα expressing luminal cells in the breast rarely proliferate (Band & Laiho, 2011), yet activation of ERα by E₂ is known to stimulate cell growth and proliferation in ER+ breast cancer cells (Chalbos et al., 1982, Mense et al., 2008). ER status in breast tumors is associated with differences in gene expression patterns (Lu et al., 2008, Gruvberger et al., 2001), specifically ER+ tumors show significantly upregulated genes involved
in cell growth ($CCND1$, $CMYC$) DNA binding, and transcription factor activity (Abba et al., 2005).

**Overview of the Notch Signaling Pathway (Originally published in BeLow & Osipo, 2020)**

Notch signaling is an evolutionary conserved pathway, originally discovered through investigations of *Drosophila* wing development (Artavanis-Tsakonas et al., 1995) and has since grown into an increasingly large field of study for cancer biologists. This intricate pathway mediates normal stem cell differentiation, cell fate, and organ development (Lai, 2004, Chiba, 2006). However, its dysregulation and role in promoting cellular transformation has led to further investigations of the role of Notch in a variety of cancers (Miele et al., 2013).

There exist four known mammalian Notch receptors, Notch1, Notch2, Notch3, and Notch4. Each receptor is translated as a single polypeptide that is subsequently cleaved in the Golgi-apparatus by a furin-like convertase. The resulting cleaved protein is delivered to the plasma membrane as a heterodimeric protein containing an extracellular domain tethered to the transmembrane and intracellular domains by a calcium cation, as shown in Figure 2 (BeLow & Osipo, 2020). Upon interaction of the extracellular domain with one of its ligands that include Jagged-1 (JAG1), Jagged-2 (JAG2), Delta-like 1 (DLL1), Delta-like 3 (DLL3), or Delta-like 4 (DLL4), through cell-to-cell contact (Figures 1 and 2), the extracellular portion of the receptor is pulled away from the transmembrane/intracellular domains by ligand-mediated endocytosis. The remaining transmembrane portion of the receptor (NotchTM) is first cleaved by a disintegrin and metalloprotease (ADAM17 or ADAM10), resulting in a product: Notch extracellular truncation (NEXT). NEXT is subsequently cleaved by the $\gamma$-secretase complex releasing the intracellular portion of Notch (Notch$^{IC}$). Notch$^{IC}$ is translocated from the cytoplasm to the nucleus where it binds to the CSL (CBF-1/RBPJ-κ in *Homo sapiens/Mus musculus*, respectively, Suppressor of
Hairless in *Drosophila melanogaster*, Lag-1 in *Caenorhabditis elegans*) transcription factor. The interaction of NotchIC with CSL replaces corepressors with coactivators including the transcriptional activator Mastermind1 (MAML1) at regulatory sequences of gene targets (Figure 3, BeLow & Osipo, 2020). This allows for transcriptional activation of Notch target genes (Andersson et al., 2011, Kopan et al., 2009).

Some of the earliest known targets of Notch signaling include transcriptional repressors, such as the hairy/enhancer of split (*HES*) genes, as well as the HES subfamily members *HEY1*, *HEY2*, and *HEYL* (Borggrefe et al., 2009, Fischer et al., 2004). These *HES*/HEY genes are critical cell-fate regulators during development and tissue renewal. In addition to this, cell-cycle regulators such as c-Myc (Klinakis et al., 2006) and cyclin D1 (Cohen et al., 2009) are directly activated by Notch signaling. Dysregulation of Notch signaling, such as activating Notch receptor mutations, overexpression of ligands and/or receptors, and/or overexpression of its target genes, contributes to increased proliferation, cell transformation, and increased drug resistance in cancers of the breast, multiple myeloma, prostate, T-cell acute lymphoblastic leukemia, and others (Bolós et al., 2007).
Figure 2: Structure of the Notch Ligands and Receptors.

Figure 3: Canonical Notch Signaling Mechanisms to Mediate Transcriptional Activation of Downstream Targets & Pharmacological Inhibitors.
Notch Signaling and Breast Cancer (Originally published in BeLow & Osipo, 2020)

It has been shown that Notch is an oncogene in the breast, as overexpression of Notch1IC (Dievart et al., 1999, Hu et al., 2006), Notch3IC (Hu et a., 2006), or Notch4IC (Jhappan et al., 1992, Rafaat et al., 2004) is sufficient for transformation of normal breast epithelial cells into cancer cells. Overexpression of Notch1 and/or Jagged1 predicts the poorest overall survival outcome for women with breast cancer (Dickson et al., 2007, Reedijk et al., 2005). Early studies show that normal breast tissue has high expression of the negative Notch regulator, Numb, and that its expression is lost in breast tumors (Pece et al., 2004). Treatment with the proteasome inhibitor MG-132 led to increased Numb expression in primary cultures of human breast tumor cells and decreased Notch transcriptional activity. Based on these findings, Stylianou and colleagues investigated whether Notch was aberrantly activated in breast cancer and how this may impact cellular transformation. Upon stable overexpression of Notch1IC in the non-transformed breast cell line MCF-10A, they were able to demonstrate cellular transformation via changes in cell shape, increased cell growth, colony formation, and resistance to apoptosis. Importantly, overexpression of Numb in the ER+ breast cancer cell line MCF-7 resulted in decreased Notch1IC accumulation, inhibition of colony formation, and accumulation of E-cadherin, suggesting that transformation of these cells had been reversed (Stylianou et al., 2006). Together, these data demonstrate that increased Notch activity and/or deregulation of Notch leads to the transformation of normal breast cells into cancer cells.

Expression and activation of Notch in primary breast tumors has been used to assess if Notch signaling is a prognostic and/or predictive biomarker. For example, overexpression of Notch1 and Jagged1 predict the poorest overall outcome for women with breast cancer, with a predicted mortality of 63% in women with JAG1high-expressing tumors, compared to 32% in
JAG1\textsuperscript{low}-expressing tumors. Furthermore, women with Notch1\textsuperscript{high}-expressing tumors had a 66% mortality rate, compared to 30.5% for Notch1\textsuperscript{low}-expressing tumors (Reedijk et al., 2005). A study conducted by Yao and colleagues (2011) identified that expression of Notch1 and Notch4 proteins was cytoplasmic in ER+ breast tumors, compared to ER− tumors. In conjunction with this, Ki67 expression, a nuclear protein associated with proliferation (Yao et al., 2010), significantly correlated with Notch1 nuclear expression and Notch4 membrane and cytoplasmic expression in ER+ tumors. Further research demonstrated that Notch1 and Notch4 immunoreactivity significantly correlated with tumor grade and Ki67 expression in triple-negative breast tumors (Speiser et al., 2011). These findings and others (Dickson et al., 2007, Reedijk et al., 2007, Xing et al., 2011, Speiser et al., 2013, Pandya et al., 2015, Strati et al., 2017) provided support for Notch as a poor prognostic biomarker in breast cancer.

**Cancer Stem Cell Hypothesis (Originally published in BeLow & Osipo, 2020).**

One proposed theory for drug resistance and tumor recurrence is that a small population of cells referred to as breast cancer stem-like cells (BCSCs) within the bulk primary tumor are inherently resistant to many forms of targeted or cytotoxic therapy. These BCSCs survive therapy and remain dormant until they are reactivated to proliferate, depending on the microenvironment. A small population of CD44\textsuperscript{+/−}/CD24\textsuperscript{−/low} cells were originally isolated from patient tumors in 2003 by Al-Hajj et al., in which these cells were found to have high tumor-initiating potential, 10- to 50-fold greater than CD44\textsuperscript{+/+} cells, when injected into immune-compromised mice. These cells were coined “cancer stem cells” due to their high tumor initiating potential and their ability to form distinct populations of stem-like and differentiated cells within the bulk tumor (Al-Hajj et al., 2003).
Alternatively, stem cells can be identified by measuring the enzymatic activity of Aldehyde Dehydrogenase (ALDH). ALDH is expressed across normal tissue and is responsible for the oxidation of intracellular aldehydes produced from alcohol metabolism by alcohol dehydrogenase (Cederbaum, 2012, Vassali, 2019). However, it’s elevated activity in neural and hematopoietic stem cells (Corti et al., 2006, Armstrong et al., 2004, Storms et al., 1999, Chute et al., 2006), along with multiple myeloma and acute myeloid leukemia cells (Cheung et al., 2007, Matsui, et al., 2008) led researchers to investigate its role in mammary stem cells. Ginestier and colleagues (2007) established that high ALDH activity was found in both normal breast epithelium along with cells collected from human-derived breast cancer xenotransplants. Using the ALDEFLOUR assay, which utilizes a fluorescent aldehyde to detect ALDH activity (Zhou et al., 2019), they were able to establish that ALDEFLOUR positive cells had high tumorigenicity, and ultimately that the isoform ALDH1 was a viable diagnostic biomarker and predictor of poor clinical outcomes (Ginestier et al., 2007, Balicki, 2007).

**Maintenance of Breast Cancer Stem Cells by Notch Signaling**

The mammary glands are derived from mammary stem cells that differentiate into luminal and myoepithelial progenitors, and these further differentiate into luminal and myoepithelial cells. Mammary stem cells were originally investigated using a unique suspension cell culture technique (Dontu et al., 2004). Single cell suspensions were cultured in a mammosphere-forming medium, as the term “mammosphere” is derived from the ability of the cells to proliferate in suspension in the form of a sphere, as previously seen using neuronal stem cells which formed “neurospheres” (Dontu et al., 2004, Reynolds & Weiss, 1996).

Dontu and colleagues were the first to use this assay to show activation of Notch signaling promotes proliferation and self-renewal of mammary stem/progenitor cells. Further, the
investigators demonstrated that Notch signaling was also required for the lineage commitment of mammary progenitors to myoepithelial cells in vivo as either Notch4 blockade or a γ-secretase inhibitor (GSI) inhibited the myoepithelial lineage commitment (Dontu et al., 2004). This technique was then utilized to isolate Ductal Carcinoma in Situ (DCIS) cells from human breast tissues, and researchers were able to demonstrate a direct role for Notch by inhibiting the mammosphere forming ability of these cells, using the GSI DAPT (Farnie et al., 2007).

Interestingly, Notch target genes were shown to be elevated in mammospheres derived from various breast cancer cell lines (Grudzien et al., 2010); and ALDEFLOUR+ breast cancer cells had increased mammosphere-forming efficiency (MFE), compared to ALDEFLOUR− cells, which correlated with an increased expression in Notch2 mRNA, a known promoter of mammary stem cell self-renewal (Charafe-Jauffret et al., 2009).

**Maintenance of Breast Cancer Stem Cells by Estrogen Signaling**

It is known that estrogen plays a critical role in promoting breast epithelial cell differentiation (Bocchinfuso et al., 2000). However, only a small percentage of cells in the mammary gland express ER during development, and these cells were considered to be permanently differentiated due to their lack of proliferative markers (Shoker et al., 1999). Dontu and colleagues proposed an alternative hypothesis that ER+ cells found in the breast represent undifferentiated stem or progenitor cells that are slowly dividing that then give rise to more rapidly dividing cell populations that vary in ER status when stimulated by E₂, as summarized in Figure 4 (Dontu et al., 2004). More recent studies have shown that ER+ cells are exclusively maintained by a lineage-restricted, ER+ stem cell population (Van Keymeulen et al., 2017), thus supporting a role for estrogen signaling in the maintenance of stem cell populations in healthy breast tissue.
Figure 4: The Role of ER in Normal Tissue and Breast Cancer Stem Cell Maintenance.

The mammosphere formation assay that has been previously described allows for a more in-depth study of both mammary stem cells and breast cancer stem cells. Simões and co-workers investigated the effect of estrogen on stem cell populations in both normal mammary epithelial cells, as well as in ER+ breast cancer cells. Their data showed that treatment with E₂ significantly reduced the expression of pluripotent genes, such as NANOG, SOX2, and OCT4 in the differentiated cells compared to the mammosphere populations in both normal mammary cells and breast cancer cells (Simões et al., 2011). Additionally, treatment with the SERM Tamoxifen has been shown to significantly increase the number of mammospheres compared to cells grown in the presence of E₂ (Simões et al., 2011, Raffo et al., 2013). Interestingly, previous studies suggest that this promotion of stemness following estrogen antagonism is mediated by Notch4 activity and correlated with increased expression of ALDH1 (Simões et al., 2015,
Studies have shown that ER+ breast cancer cell lines treated with estrogen-deprivation therapy, Tamoxifen or fulvestrant, have increased expression of the cleaved forms of Notch1 (Notch1IC) and Notch4 (Notch4IC) (Rizzo et al., 2008). Specifically, endocrine-therapy-induced Notch1IC and Notch4IC increased CSL-driven reporter activity in ER+ MCF-7 and T47D cells, suggesting that, while E2-mediated activation of ER maintained low Notch activity, antagonizing ER increased Notch signaling. Together, these data support the further investigation of the interplay of Notch and ER signaling in maintaining the breast cancer stem cell population.

Alcohol & the Promotion of Stemness

The role of alcohol consumption in the differentiation and maintenance of stem cells has been extensively studied specifically in neural stem cells and embryonic stem cells as a model for studying Fetal Alcohol Spectrum Disorders (FADS). Early studies show that exposure to ethanol inhibits proliferation and induces apoptosis in neural stem cells through disruption of growth factor signaling (Crews et al., 2003, Crews et al., 2003). Additional studies showed that ethanol redistributes cell lineage through maintaining elevated expression of the pluripotent genes OCT4, SOX2, and NANOG in both mouse and human embryonic stem cells (Arzumayyan et al., 2009, Sánchez-Alvarez et al., 2013). The effects of alcohol exposure on mammary stem cells have yet to be elucidated.

As mentioned previously, a hallmark for cancer stem cells is elevated expression and/or activity of the enzyme Aldehyde Dehydrogenase, or ALDH. When ethanol enters the cell, it undergoes oxidative metabolism by Alcohol Dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) to form acetylaldehyde and increase the level of reactive oxygen species (ROS). Acetylaldehyde, a known carcinogen, is then further metabolized by ALDH into acetate which is
then finally metabolized into CO$_2$ and water to be eliminated, as reviewed in Figure 5 (Di Rocco et al., 2019). It has been suggested that alcohol contributes to tumorigenesis potentially through the production of ROS to maintain the cancer stem cell pool (Di Rocco et al., 2019, Xu & Luo, 2017). More specifically, a study using HER2 overexpressing breast cancer cells showed that alcohol exposure increased the cancer stem cell population through activation of the p38$_{\gamma}$/MAPK pathway (Xu et al., 2016), thus providing strong rationale to examine the effects of alcohol on ER+ breast cancer stem cells.

Figure 5: Alcohol Metabolism Results in the Production of Acetyaldehyde and Increased ROS Formation.
CHAPTER TWO
PRELIMINARY DATA, HYPOTHESIS, AND SPECIFIC AIMS

Preliminary Data

Previous studies have focused on the role of HER2 signaling and utilized HER2+ breast cancer cell lines to investigate alcohol’s effect on BCSCs (Xu et al., 2016), however little is known about the response of naïve ER+ breast cancer cells to alcohol exposure. We sought to determine whether treatment with increasing concentrations of ethanol would result in changes in the CSC population of the ER+ breast cancer cell line MCF-7. Cells were grown in the presence of 5nM 17β-Estradiol or a DMSO vehicle control in phenol red-free RPMI media while simultaneously being treated with 0, 10, 20, or 40mM ethanol for 72 hours. Cells were then individualized, counted, and seeded at a density of $2.5 \times 10^4$ cells into mammosphere forming medium, and incubated at 37°C for 7 days. After the incubation period was over, cells were imaged prior to extraction, and mammosphere forming efficiency was calculated. In addition to this, we assessed the differences in mammosphere size using ImageJ software analysis. Our findings show that exposure to alcohol enhances mammosphere forming efficiency, with a significant change shown at 20mM ethanol compared to 0mM controls (Figure 6). Interestingly, there was a dose-dependent increase in mammosphere size in response to alcohol in both the presence and absence of 17β-Estradiol (Figure 7).
Figure 6: Alcohol Enhances BCSC Survival in a Dose-Dependent Manner in Naïve ER+ Cells, Regardless of 17β-Estradiol. MCF-7 cells were grown in charcoal-stripped phenol-red free RPMI for 72 hours. Then they were treated with 5nM 17β-Estradiol or DMSO alone or in combination with increasing concentrations of ethanol. 2.5 x 10^4 cells were seeded into mammosphere media and incubated for 7 days, after which %MFE was calculated. Results are presented as the mean + SD of three independent experiments. Two-Way ANOVA with Tukey’s multiple comparisons analysis was used to determine statistical significance. (## = p<0.01).

Figure 7: Alcohol Dose-Dependently Enhances BCSC Size in Naïve ER+ Cells. MCF-7 cells were treated as previously described in Figure 6. Cells were imaged at 20X magnification and size analysis was performed using ImageJ software. Results are presented as the mean + SD of three independent experiments. Two-Way ANOVA with Tukey’s multiple comparisons analysis was used to determine statistical significance. (* = p<0.05, ** = p < 0.01).
Central Hypothesis

Based on previous findings and preliminary data, we propose the following hypothesis: *alcohol promotes cancer stem cell survival in estrogen receptor positive breast cancers through the stimulation of Notch signaling.* The following specific aims have been designed to test potential mechanisms by which alcohol promotes breast cancer stem cells, and how this may change in the context of long-term estrogen deprivation in *vitro.*

Specific Aims

**Aim 1: Elucidate Potential Mechanisms by Which Alcohol Promotes Breast Cancer Stem Cells in Naïve ER+ Breast Cancer Cells.**

**Aim 1A:** To test whether alcohol promotes breast CSC survival through enhancement of the Notch signaling pathway, we utilized RT-PCR to assess changes in expression of the Notch canonical target gene *HES1*, as well as *NOTCH1* following 72 hours of ethanol exposure in conjunction with 5nM E$_2$. Additionally, we assessed changes in pluripotent gene expression, specifically of *SOX2, OCT4*, and *NANOG* as it is known that Notch signaling can regulate the expression of these genes in order to promote CSC survival. Lastly, we tested the hypothesis that Notch signaling is responsible for alcohol-mediated promotion of breast CSCs by attenuating Notch signaling in conjunction with alcohol treatment in the bulk cell population prior to incubation in mammosphere forming media. If alcohol is dependent on Notch signaling for the promotion of breast CSCs, then we would expect that treatment with a GSI would inhibit the enhanced MFE shown in our preliminary experiments following exposure to alcohol in MCF-7 cells.
**Aim 1B:** In order to determine if alcohol promotes breast CSC survival and growth through inhibition of estrogen signaling, we utilized real time PCR to assess changes in Estrogen Receptor gene targets \(PS2\) and \(PGR\) following 72 hours of ethanol treatment, and either 0-, 3-, or 24-hours exposure to 5nM \(E_2\). We also assessed changes in the protein expression of DAXX via western blot, as it is known that inhibition of estrogen signaling results in decreased DAXX expression resulting in increased Notch signaling. In addition to this, low levels of DAXX are correlated with increased mammosphere forming efficiency in MCF-7 cells (Peiffer et al., 2019). Thus, if alcohol mediates breast CSC survival through inhibition of the estrogen signaling pathway, then we would expect to see diminished levels of ER target transcripts and decreased protein expression of DAXX.

**Aim 2:** Determine the Effect of Alcohol in Long-Term Estrogen Deprived (LTED) ER+ Breast Cancer Stem Cells.

Clinical studies have suggested a strong positive correlation between alcohol consumption and increased risk for breast cancer recurrence, specifically in post-menopausal women (Hu et al., 2006, Liu et al., 2015). Thus, it is imperative that a more relevant model be used to assess the effects of alcohol in ER+ breast cancer cell lines. There are currently no published findings of alcohol exposure in an in vitro model of long-term estrogen deprivation in breast cancer cell lines. Long-term estrogen deprived cells have acquired resistance to endocrine therapies and no longer require \(E_2\) to stimulate their growth, thus providing a critically needed model to better investigate the molecular mechanisms behind alcohol-mediated promotion of breast CSCs.

**Aim 2:** We assessed the effects of alcohol exposure on mammosphere formation efficiency in the long-term estrogen deprived cell line MCF-7/5C, derived from the parental
naïve cell line MCF-7. These cells were treated with increasing concentrations of ethanol (0, 10, 20, and 40mM) for 72 hours in the presence or absence of 5nM E2, then seeded at 2.5 x 10^4 cells in mammosphere forming media and left to incubate for 7 days. Mammosphere forming efficiency and size analysis was performed to test whether acquired resistance to estrogen deprivation results in enhanced susceptibility to alcohol-mediated CSC survival. Additionally, we assessed changes in DAXX protein expression as well as Notch4 to determine changes in Notch signaling following ethanol exposure.
CHAPTER THREE

MATERIALS AND METHODS

Materials

Cell Culture

MCF-7 Breast Cancer cells were purchased and obtained from American Type Culture Collection (ATCC). A long-term estrogen deprived (LTED) variant MCF-7/5C were provided by Dr. V. Craig Jordan (University of Texas, M.D. Anderson Center, Houston, TX). MCF-7 cells were cultured in Roswell Park Memorial Institute Medium (RPMI 1640; Corning CellGro) enhanced with 10% Fetal Bovine Serum (FBS, Corning CellGro), 1% (2mM) L-glutamine (Corning CellGro), and 1% (100μM) non-essential amino acids (NEAA, Invitrogen). MCF-7/5C cells were cultured in phenol-red free RPMI 1640 enhanced with dextran charcoal-stripped 10% FBS to remove estradiol from the solution, as well as 1% L-glutamine and 1% NEAA. All cell lines were maintained at 37°C with 5% CO₂ and 95% humidity. Experimental medium was changed daily.

Drug Treatments

17β-estradiol was purchased from Sigma Aldrich (Catalogue #E8875) and suspended in dimethyl sulfoxide (DMSO, Sigma Life Sciences, Catalogue #D2660) to form a 25μM stock which was protected from light and maintained in -20°C. This solution was diluted in growth medium for an experimental working concentration of 5nM. 200 proof molecular grade absolute
ethanol was purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, BP2818-500) and diluted in Phosphate Buffer Saline (PBS, Corning CellGro) to form a stock of 1, 2, or 4 M, which was protected from light and maintained in 4°C. This solution was diluted in growth medium for an experimental working concentration of 10, 20, or 40mM respectively. A γ secretase inhibitor (GSI), MRK-003, was provided from Merck Oncology & Co. (Whitehouse Station, NJ) and re-suspended in DMSO to create a 10mM stock solution. This solution was diluted in growth medium for an experimental working concentration of 5μM.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>ATGAACCAGGTATGACCTTGAT</td>
<td>CCTGTGACTGGTCTATTACAATA</td>
</tr>
<tr>
<td>PGR</td>
<td>AGCATGTGCCCTTAGAAAGTGC</td>
<td>TAGGGCTGGCTTTTCATTG</td>
</tr>
<tr>
<td>PS2</td>
<td>GAGGCCGACACAGACGACGTTG</td>
<td>CCCTGAGAAGTGCTCTAAAATTCA</td>
</tr>
<tr>
<td>HES1</td>
<td>CGGACATTTCTGGAAATGACA</td>
<td>CATTGATCTGGGTCTAGCAG</td>
</tr>
<tr>
<td>SOX2</td>
<td>CACACTGCCCCTCTCAC</td>
<td>TCCATGCTGTCTTACTCTCC</td>
</tr>
<tr>
<td>OCT4</td>
<td>GGCAACCTGGAGAATTTGTTC</td>
<td>GTTACAGAACCACACTCGGAC</td>
</tr>
<tr>
<td>NANOG</td>
<td>AGAGAATGAAATCTAAGAGGTG</td>
<td>GTTGGTAGGAAGAGTAAAGGCTG</td>
</tr>
</tbody>
</table>

Table 1: Real Time RT-PCR Primers

Antibodies

Antibodies and their respective concentrations of use include: DAXX (1:1,000, 25C12, Cell Signaling Technology); NOTCH4 (1:200, H225, Santa Cruz Biotechnologies), NICASTRIN (1:200; Covance); β-AMYLOID (1:500, H-43, Santa Cruz Biotechnologies); and β-ACTIN (1:10,000, A5411, Sigma Aldrich. β-ACTIN served as a loading/endogenous control for total protein western blots. Secondary antibodies used for band visualization include anti-rabbit and
anti-mouse Horseradish Peroxidase (HRP) conjugated secondary antibodies purchased from Cell Signaling (Cell Signaling Technologies).

**Primers**

All primers used in Reverse Transcription, quantitative real-time Polymerase Chain Reaction (qRT-PCR) were designed using the NCBI Primer Blast and purchased from Invitrogen. All primers were re-suspended in Nuclease-Free water to create a stock solution of 50μM. Sequences for each primer used are listed in Table 1.

**Experimental Methods**

**Western Blotting**

**Bicinchoninic Acid (BCA) Assay:** 300μL of lysis buffer (1% Triton X-100, 50mM HEPES, 150mM sodium chloride, 5mM ethylene-diaminetetraacetic acid (EDTA), 1mM phenylmethylsulphonyl fluoride (PMSF), 1mM sodium orthovanadate, 10mM sodium fluoride, and 200x Protease Inhibitor Cocktail) was used to lyse cells. Cells were trypsinized and pelleted prior to the addition of lysis buffer, then incubated on ice for 10 minutes after being suspended in the lysis buffer. After incubation, lysates were sonicated twice for ten seconds at 20% amplification using the Sonic Dismembrator (Model 100, Thermo-Fisher Scientific). Protein standards of bovine serum albumin (BSA) ranging from 0 to 10μg/mL were plated in duplicate at 10μL per well in a 96-well plate, in addition to 10μL of each lysate in duplicate. 200μL of BCA working solution (50:1 Reagent A:Reagent B, Thermo-Fisher Scientific) was added to each well. The 96-well plate was incubated at 37°C for twenty minutes, then read by a fluorescent plate reader. Total protein concentrations were calculated via comparison of absorbance values of the lysates to that of the standard curve generated, with an R² value of 0.98 or greater. 20μg of total-
protein containing samples were prepared using 2X Laemmeli Buffer (Bio-Rad, Catalogue #1610737) and β-mercaptoethanol (Thermo-Fisher Scientific, Catalogue #BP-176-100), and additional lysis buffer as needed. Samples were then boiled at 95°C on a shaking hot plate for 10 minutes and stored at -20°C until use.

**Gel preparation:** Sodium Dodecyl Sulfate (SDS) gels were prepared to separate proteins based on their molecular weight. A clean, short glass plate was secured against a 1.5mm tall glass spacer plate using a casting frame with the bottom of the plates completely flat and level. The casting frame was then secured in place in a casting stand for gel preparation. Resolving and Stacking solutions were prepared as described in Table 2 for 10% acrylamide gels. 8mL of resolving solution was poured between the two glass plates and topped with 1mL of isopropanol to flatten the top of the gel. Once the resolving solution solidified, the isopropanol was rinsed off with nuclease-free water and 2mL of the stacking solution was poured onto the resolving layer. A 1.5mm gel comb containing 10 wells was then inserted into the stacking solution, and the gels were left to solidify. Once solidified, gels were wrapped in paper towels soaked in nuclease-free water and stored in 4°C overnight before use.

<table>
<thead>
<tr>
<th>Table 2: Gel Preparation for Western Blot</th>
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<tbody>
<tr>
<td><strong>10% Resolving Solution</strong></td>
</tr>
<tr>
<td><strong>Reagent Name</strong></td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
</tr>
<tr>
<td>30% Acrylamide</td>
</tr>
<tr>
<td>1.5M Tris Buffer (pH 8.8)</td>
</tr>
<tr>
<td>10% SDS</td>
</tr>
<tr>
<td>10% Ammonium Persulfate (APS)</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
</tbody>
</table>
**Western blot:** Samples were boiled for 10 minutes at 95°C prior to being ran for western blot analysis. Proteins were separated by molecular weight using SDS-PAGE buffered with 8% tris-glycine along with HiMark Prestained protein standard (Thermo-Fisher Scientific, Catalogue #LC5699). Proteins were separated at 150V for 60 minutes in 1X Tris-glycine SDS Running Buffer. Proteins were then transferred to a polyvinylidene fluoride membrane that had been pre-wetted with methanol, then water, then transfer buffer prior to use, at 100V for 60 minutes. Following transfer, the membranes were rocked in 5% non-fat dry milk for 60 minutes at room temperature to prevent non-specific binding of primary antibodies. After blocking, membranes were incubated in the designated primary antibody at 4°C overnight with constant agitation. The following day, membranes were washed three times with 1X Tris-buffered Saline plus Tween-20 (TBST) for 10 minutes while rocking. After the final wash, the appropriate HRP-conjugated secondary antibody was added to the membrane for 60 minutes at room temperature while rocking. After the secondary antibody incubation is complete, the membranes are washed three times with TBST for ten minutes, and then detected using enhanced chemiluminescence (ECL) Western Blotting Substrate (Pierce), or SuperSignal West Extended Duration Substrate (Thermo-Fisher Scientific) if needed, in a 1:1 volume. Protein bands were visualized by exposing the membranes to X-ray film for 30 seconds to 10 minutes, depending on however long was necessary to visualize the bands. Reprobing was performed by washing the membrane with stripping buffer for five minutes two times, then washed with PBS two times for five minutes, then washed with TBST three times for ten minutes. Membranes were then blocked for thirty minutes and incubated with primary antibody overnight. Densitometry analysis was conducted using ImageJ software, samples were normalized to β-ACTIN.
Reverse Transcription, Real Time Polymerase Chain Reaction (PCR)

**RNA extraction:** 300µL Ambio TRIZol Reagent was either added directly to tissue culture plates or to cells pelleted after being trypsinized, and samples were stored in -80°C until extraction was performed. In order to extract RNA, 50µL of 1-Bromo-3-chloropropane (BCP) was added directly to the cells in the 300µL TRIZol Reagent and were vortexed for 15 seconds to mix the solution thoroughly. Samples were incubated at room temperature for 5 minutes, then centrifuged at 14,000rpm for 15 minutes at 4°C. After this, the Zymo Research Direct-zol RNA Miniprep kit was utilized with a few modifications to the established protocol. The clear aqueous phase was removed from each sample and placed in a clean 1.5mL Eppendorf tube, and equal volume of 200 proof molecular grade absolute ethanol was added. The samples were vortexed for 10 seconds to mix the solution thoroughly, then the entire sample was added to the Zymo-Spin IIICG Column placed within a collection tube. Samples were centrifuged at 15,000rpm for 30 seconds. 200µL of Direct-Zol Prewash Buffer was added directly to the column and samples were spun as previously described. Then, 500µL of RNA Wash Buffer was added to the column, and samples were spun as previously described. After this, the columns were spun once again at the same speed and time to ensure the column was completely dry of any wash buffers. Then 40µL of DNase/RNAs-free water was added to the column and left to incubate for 3 minutes at room temperature. Samples were then spun at 15,000rpm for 30 seconds to elute the RNA, then placed directly onto ice. The concentration of the RNA and 260/280 ratios were measured using the NanoDrop 2000.

**Reverse Transcription (RT):** 0.5µg of RNA from each sample was utilized to generate 25ng/µL of cDNA. If necessary, RNA samples were diluted in nuclease-free water to achieve the
desired concentration for the Reverse Transcriptase reaction. The components of each reaction are listed in Table 3. Once the reactions are prepped, the reverse transcriptase reaction was conducted in a thermal cycler under the following parameters: 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C, 60 minutes at 25°C, then held at 4°C until use.

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Amount (µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer</td>
<td>5</td>
<td>1X</td>
</tr>
<tr>
<td>25mM MgCl2</td>
<td>11</td>
<td>5.5nM</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10</td>
<td>500µM/dNTP</td>
</tr>
<tr>
<td>Random Hexamers</td>
<td>2.5</td>
<td>2.5µM</td>
</tr>
<tr>
<td>RNAse Inhibitor</td>
<td>1</td>
<td>0.4U/L</td>
</tr>
<tr>
<td>RT Enzyme</td>
<td>1.25</td>
<td>1.25U/L</td>
</tr>
<tr>
<td>RNA + Nuclease Free Water</td>
<td>19.25</td>
<td>0.5µg RNA</td>
</tr>
</tbody>
</table>

Quantitative Real Time PCR (qRT-PCR): cDNA generated from the reverse transcriptase reaction was used in qRT-PCR analysis, which utilized the iTaq™ SYBR® Green Enzyme Supermix with ROX (BioRad) in a 96-well optical PCR plate. Components of the reaction and their volumes are listed in Table 4. Once prepared, the qRT-PCR was performed using the Applied Biosystems Step One Plus Real-Time Thermocycler as follows: initial denature at 95°C for 10 minutes, PCR cycling for 10 seconds at 95°C for 40 cycles, and annealing for 45 seconds at 60°C. The average cycle threshold ($C_t$) was used to determine relative gene expression for each experimental condition. $C_t$ values are the number of cycles necessary for the fluorescent signal to overcome the background threshold of fluorescence. $C_t$ were normalized to the housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase (HPRT), resulting in the $\Delta C_t$, which was then used to determine the $\Delta\Delta C_t$ by normalized all
experimental samples to the control sample’s ΔCt. Lastly, relative fold changes were calculated by using the $2^{-\Delta\Delta Ct}$ method.

<table>
<thead>
<tr>
<th>Table 4: Real Time RT-PCR Reaction (12.5µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent Name</strong></td>
</tr>
<tr>
<td>Nuclease Free Water</td>
</tr>
<tr>
<td>50µM Forward Primer</td>
</tr>
<tr>
<td>50µM Reverse Primer</td>
</tr>
<tr>
<td>25ng/µL cDNA</td>
</tr>
<tr>
<td>2X SYBR Green Master Mix</td>
</tr>
</tbody>
</table>

**Mammosphere Formation Assay**

Cells were seeded at a density of 5 x 10⁵ per dish in a 6 cm² tissue culture dish or 1 x 10⁶ in a 10 cm² tissue culture dish. MCF-7 cells were grown in charcoal-stripped RPMI 1640 for 72 hours prior to being plated into experimental conditions. 24 hours after seeding, cells were treated with DMSO or 5nM 17β-estradiol in conjunction with a specified concentration of ethanol or PBS for 72 hours. For GSI experiments, cells were also treated with 5µM GSI or equal volume DMSO during the 72-hour treatment period. After the treatment period concluded, cells were trypsinized and harvested in charcoal stripped RPMI 1640. Cells were counted using Trypan blue staining on the Countess Automated Cell Counter (Life Technologies). 2.5 x 10⁴ cells were seeded in a 6-well non-adherent tissue culture plate coated in mammosphere forming media [196mL DMEM F12 (Life Technologies), 3g methyl cellulose (Sigma Aldrich), 3mL B27 (Life Technologies), 3µL recombinant EGF (Sigma Aldrich)]. These plates were left to incubate at 37°C and 5% CO₂ for 7 days undisturbed. Following the incubation period, mammospheres
were imaged using a Nikon Diashot TMD Fluorescence Phase Contrast Inverted microscope (Nikon) at 4X and 10X magnification for size analysis. In order to calculate mammosphere forming efficiency, mammospheres were extracted by adding 6mL of PBS to each well to dissolve the mammosphere forming media. This was done twice, then the 12mL of PBS-mammosphere media mixture was spun down at 1200rpm for 3 minutes to pellet the extracted mammospheres. The supernatant was then aspirated down to 1mL, and this was transferred to a clean 2mL Eppendorf tube. The samples were then spun down at 1200rpm for 5 minutes. The PBS was then aspirated off, and the mammospheres were resuspended in a fresh 2mL of PBS. 50μL of each sample was then placed in a flat bottom 96-well plate in addition to 200μL of PBS. Pictures were then taken of each well at 4X magnification using the same microscope to collect the images used for size analysis. Mammosphere forming efficiency (% MFE) was determined by counting the number of mammospheres greater than 50μm with % MFE = [(number of spheres > 50microns counted X dilution factor/number of cells plated) X 100].
CHAPTER FOUR

RESULTS


According to our preliminary data, when naïve ER+ breast cancer cells are treated with increasing concentrations of ethanol, there is a significant increase in cancer stem cell survival, as measured by %MFE regardless of the presence of 17β-estradiol. These data are supported by previous findings in HER2 overexpressing breast cancer cells (Xu et al., 2016), however previous work has not investigated the role of 17β-estradiol under these conditions. As described previously, 17β-estradiol has been shown to drive differentiation of progenitor and stem cells within the breast, and this may be through inhibition of the Notch signaling pathway. Thus, the goal of this aim is to test the mechanism by which alcohol promotes BCSCs, with the hypothesis being that alcohol prevents the differentiating effects of 17β-estradiol within the bulk cell population thus promoting survival of BCSCs.

Acute Alcohol Exposure Induces Expression of CSC-Promoting Genes

We first sought to determine how exposure to ethanol directly effects the Notch signaling pathway. Notch1 expression is known to be significantly lower in BCSCs, while Notch4 expression is significantly higher compared to the bulk cell population (Harrison et al., 2010). Additionally, Notch1-mediated transcription is known to be negatively regulated by ER signaling, and this inhibition is alleviated when ER is antagonized (Rizzo et al. 2008). Therefore,
we deprived MCF-7 cells of estrogen by culturing them in charcoal-stripped RPMI for 72 hours. Cells were then treated in the presence or absence of 17β-estradiol in conjunction with 40mM ethanol for an additional 72 hours, then collected and processed for qRT-PCR analysis. Changes in the canonical Notch target, \textit{HES1}, as well as the gene encoding for the Notch1 receptor were assessed. If alcohol promotes BCSC survival through the activation of Notch signaling, then we would expect to see an induction of \textit{HES1} transcripts when ethanol is present, additionally we would expect to see decreased levels of \textit{NOTCH1} transcripts.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Expression of \textit{HES1} RNA Trends Upwards in Response to Alcohol. MCF-7 cells were grown in charcoal-stripped phenol-red free RPMI containing 10\% FBS for 72 hours. Cells were then seeded in a 6cm$^2$ dish and treated with either 5nM 17β-estradiol or DMSO, as well as 40mM ethanol or Phosphate Buffer Saline (PBS) for 72 hours. RNA was extracted from the cells following treatment and cDNA was synthesized. The relative expression of canonical Notch target \textit{HES1} and \textit{NOTCH1} was measured by qRT-PCR. Results are presented as the mean ± SD of five independent experiments. All groups were compared to DMSO 0mM EtOH by Two Way ANOVA to determine statistical significance (** = p < 0.01).}
\end{figure}

As shown in Figure 8, treatment with 5nM 17β-estradiol for 72 hours significantly inhibits \textit{HES1} transcript levels. Though not statistically significant, there is a trend towards induction of \textit{HES1} following 72 hours of ethanol exposure under estrogen deprived conditions,
and in the presence of 17β-estradiol, though not as robust, there appears to be a slight increase in HES1 transcript levels when cells are treated with ethanol. On the contrary, NOTCH1 transcripts are relatively unchanged in the absence of ethanol treatment, regardless of 17β-estradiol treatment. Interestingly, there is an induction of NOTCH1 transcripts when both ethanol and 17β-estradiol are present in the bulk cell population, though not statistically significant, similar to that of HES1. Taken together, these data suggest that under estrogen deprived conditions, alcohol may slightly activate the Notch signaling pathway, at least at the level HES1. However, upon addition of 17β-estradiol, it may be competing with ER to regulate transcription of these genes.

To further test this hypothesis, transcript levels of pluripotent genes SOX2, OCT4, and NANOG were measured following 72 hours of ethanol exposure in the presence or absence of 17β-estradiol. As previously mentioned, 17β-estradiol is known to inhibit the expression of these genes, and alcohol may promote the BCSC population by enhancing transcription of these genes within the bulk cell population. We see in Figure 9, that all three genes investigated are inhibited when cells are treated with 17β-estradiol in the absence of ethanol. Similar to what was observed with HES1, there is a trend towards induction of SOX2, OCT4, and NANOG transcripts when cells are exposed to ethanol under estrogen deprived conditions. Additionally, when 17β-estradiol is present, ethanol again appears to alleviate some of 17β-estradiol’s inhibitory effects on expression of these genes. Collectively, these data together suggest that alcohol tends to protect CSCs and possibly reverse the differentiating effects of 17β-estradiol.
Figure 9: Alcohol Treatment Trends Toward Inducing Expression of Pluripotent Genes.
MCF-7 cells were treated and processed as described previously for qRT-PCR analysis of the pluripotent genes SOX2, OCT4, and NANOG following 72 hours of treatment with 40mM ethanol in the presence or absence of 5nM 17β-estradiol. Results are presented as the mean + SD of five independent experiments. All groups were compared to DMSO 0mM EtOH by Two Way ANOVA to determine statistical significance (** = p < 0.01).

Alcohol Attenuates Transcriptional Regulation of ER Targets by 17β-estradiol

Our previous findings suggest that alcohol may interfere with the ability of 17β-estradiol to induce transcriptional changes of its target genes. We saw that when MCF-7 cells were treated with alcohol and 17β-estradiol in combination for 72 hours, the repressive effects of ER on pluripotent genes and HES1 were partly alleviated, though not significantly. It is possible that alcohol may be directly inhibiting ER, so to test this, we deprived MCF-7 cells of 17β-estradiol for 72 hours in charcoal-stripped RPMI. We then treated these cells for 72 hours with 40mM ethanol and added 5nM 17β-estradiol for either 0, 3, or 24 hours, as ER is known to have immediate effects on transcription, as quickly as 30 minutes following 17β-estradiol exposure (Stossi et al., 2004, Jagannathan & Robinson-Rechavi, 2011). Analysis of canonical ER early
target genes PS2, or Trefoil Factor 1, and PGR, the Progesterone Receptor, was conducted by qRT-PCR as previously described. If ethanol promotes CSCs through inhibition of immediate ER transcriptional regulation, then we would expect that PS2 and PGR transcript levels would be lower in ethanol treated cells compared to untreated controls.

As shown in Figure 10, treatment with 5nM 17β-estradiol induces expression of both PS2 and PGR transcripts in as little as 3 hours, and further increased by 24 hours. Interestingly, 40mM ethanol also induces expression of these transcripts in the absence of 17β-estradiol. Further, addition of 17β-estradiol to alcohol-treated cells did not increase transcripts compared to the absence of 17β-estradiol. Taken together these data suggest that alcohol alone is sufficient to activate classical ER signaling and may compete for regulation of ER target genes when 17β-estradiol is present in ER+ breast cancer cells.

Figure 10: Alcohol Alone Induced Expression of Classical ER Target Genes. MCF-7 cells were grown in charcoal-stripped phenol-red free RPMI containing 10% FBS for 72 hours. Cells were then seeded in a 6cm² dish and treated with either 40mM ethanol or PBS for 72 hours. Additionally, cells were treated with 5nM 17β-estradiol for either 0, 3, or 24 hours, then collected and processed for qRT-PCR analysis of the classical early ER target genes PS2 and PGR. Results are presented as the mean ± SD of three independent experiments. All groups were compared to 0 hours 5nM estradiol + 0mM EtOH by Two-Way ANOVA to determine statistical significance. (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).
Alcohol Promotes Expression of Notch4 in a Dose-Dependent Manner

Notch signaling is activated when a ligand-expressing cell comes into contact with one of the four Notch receptors on the surface of a neighboring cell. Upon ligand activation, the receptor then undergoes cleavage by ADAM10 or ADAM17, which then exposes the two cleavage sites for the γ-secretase complex S3 and S4 (Schweisguth, 2004). As mentioned previously, Notch4 is known to be a critical promoter of ER+ BCSC survival, thus we decided to assess changes in Notch4 expression within the bulk cell population following exposure to ethanol. MCF-7 cells were deprived of 17β-estradiol for 72 hours, then treated with increasing concentrations of ethanol in the presence or absence of 5nM 17β-estradiol for an additional 72 hours. They were then collected and processed for protein expression analysis via western blot. If alcohol promotes BCSC survival through Notch4 activation, then we would expect to see increased protein expression of the Notch4 receptor.

Figure 11 demonstrates that when cells are grown in the presence of 17β-estradiol, Notch4 expression is downregulated compared to cells grown under estrogen deprived conditions, as indicated by the 70kDa band. Interestingly, alcohol appears to upregulate Notch4 expression regardless of whether 17β-estradiol is present or not. If the multiple bands located between 55 and 40kDa are specific to Notch4, then this would also indicate an increase in the cleaved receptor, and indicate activation of the Notch4 signaling pathway, which would further suggest that alcohol promotes the expression and activation of Notch4 in naïve ER+ cells.
Figure 11: Alcohol Dose-Dependently Enhances Notch4 Protein Expression. MCF-7 cells were grown in charcoal-stripped phenol-red free RPMI for 72 hours, then treated with increasing concentrations of ethanol in the presence or absence of 5nM 17β-estradiol for an additional 72 hours. Total protein was isolated from the cells and lysates quantified for western blot analysis of Notch4 using β-Actin as the loading control.

We next sought to determine potential mechanisms by which alcohol enhances Notch4 in these cells. Peiffer and colleagues established that Notch4 is negatively regulated by the Death Domain Associated Protein 6 (DAXX), which is stabilized by 17β-estradiol and also known to inhibit BCSCs (Peiffer et al., 2019). Thus, if alcohol promotes BCSC survival through the activation of Notch4, then this may be through downregulation of its repressor, DAXX. As our previous findings show, alcohol appears to disrupt regulation of ER targets at the level of transcription, however it is unknown how alcohol affects ER activity at the protein level. Using the same membrane for Notch4 analysis, the blot was stripped and re-probed using a primary antibody against DAXX.

As shown in Figure 12, DAXX protein expression is depleted under estrogen deprived conditions and stabilized in the presence of 17β-estradiol. Interestingly, alcohol appears to
enhance DAXX expression when 17β-estradiol is present in a dose-dependent manner yet has little effect on its expression under estrogen deprived conditions. Collectively, these data suggest that alcohol enhances Notch4 expression in a dose-dependent manner, and this effect may be independent of ER-mediated regulation of the Notch4 receptor by DAXX.

![Figure 12: Alcohol Dose-Dependently Enhances DAXX Protein Expression in the Presence of 17β-estradiol.](image)

**The Effect of γ-Secretase Inhibition in Bulk and/or BCSCs on BCSC Survival**

It is known that inhibition of Notch signaling within the BCSC population alone is sufficient to inhibit their survival, as shown by a decrease in MFE (Grudzein et al., 2010). However, the effect of pre-treating bulk cell populations alone with a γ-secretase inhibitor (GSI) on BCSC survival, and whether this is affected by exposure to alcohol, has yet to be elucidated. To test this, MCF-7 cells were deprived of 17β-estradiol for 72 hours, then treated with 20mM ethanol in the presence or absence of 17β-estradiol for 72 hours. During the ethanol/estradiol treatments, cells were additionally treated with 5μM GSI (MRK-003) or a DMSO vehicle. After completing their treatments, cells were seeded in mammosphere forming medium in the presence or absence of the GSI at a density of 2.5 x 10⁴ and incubated for 7 days at 37°C (Figure 13).
Figure 13: Experimental Design of γ-Secretase Complex Inhibition in Bulk Cells and/or BCSCs. MCF-7 cells were treated as previously described with DMSO or 5nM 17β-estradiol (E2) in combination of GSI alone, 20mM ethanol alone, or a combination of the two. 2.5 x 10^4 cells were seeding in mammosphere media and treated with 5μM GSI or DMSO for 24 hours, then incubated for an additional 6 days, following which %MFE was determined.

Alcohol May Promote the Interaction of the γ-Secretase Complex with Notch4

We confirmed inhibition of the γ-secretase complex by assessing the protein expression of the classical γ-secretase substrate, β-amyloid precursor protein (APP), as well as Nicastrin, a component of the γ-secretase complex that serves as a gatekeeper for substrate interaction with Presenilin’s active site (Bolduc et al., 2015, Wolfe, 2020). Our findings demonstrate that treatment with the GSI results in decreased APP expression and cleavage, as well as decreased expression of Nicastrin. This suggests that these cells are sensitive to GSI, and the effect of the GSI on classical γ-secretase complex substrates is not altered by co-treatment with ethanol.
Figure 14: Confirmation of γ-Secretase Complex Inhibition by Target Protein Expression Analysis. MCF-7 cells were grown in charcoal-stripped phenol-red free RPMI for 72 hours. Then they were treated with 5nM 17β-estradiol or DMSO alone or in combination with 5µM GSI (MRK-003) and/or 20mM ethanol. Total protein was isolated from the cells and lysates quantified for western blot analysis of β-Amyloid Precursor Protein and Nicastrin using β-Actin as the loading control.

Knowing that GSIs act as pan-Notch inhibitors and with a lack of data to suggest whether the γ-secretase complex shows specificity towards one Notch receptor over another (Olsauskas-Kuprys et al., 2013), we next investigated changes in Notch4 protein expression under these conditions. In Figure 15, we see an accumulation of Notch4 protein, specifically at 70kDa, when cells are treated with GSI, in the presence and absence of 17β-estradiol (lanes 1-4). We also see that when cells are treated with 20mM ethanol, Notch4 expression is enhanced compared to untreated controls (lanes 1, 3, 5, & 7), as shown previously. Interestingly, when cells are treated with the GSI in the presence of 20mM ethanol, there is a significant accumulation of Notch4 protein at the 70kDa molecular weight compared to cells grown in the absence of ethanol (lanes 2, 4, 6, & 8). Because alcohol had no effect on the classical γ-Secretase complex substrate APP, yet we see a marked changed in Notch4 expression following GSI treatments when alcohol is present, these data may suggest that alcohol promotes γ-secretase-mediated cleavage of Notch4.
through increased expression of Notch4 and thus increased availability of this substrate compared to others.

**Figure 15: Accumulation of Notch4 Following γ-Secretase Inhibition is Enhanced by Exposure to Alcohol.** Total protein was isolated from MCF-7 cells as previously described, and western blot analysis of Notch4 was performed using β-Actin as the loading control.

**BCSCs are Resistant to GSI Treatment in Bulk Cells Prior to Selection**

As our findings suggest, MCF-7 cells are sensitive to GSI treatments in the bulk cell population, as shown by the decreased protein expression of the classical γ-secretase substrate APP. However, how inhibition of γ-secretase complex in the bulk cells affects BCSC survival has yet to be elucidated. As described previously (Figure 13), after 72-hour co-treatments of GSI and 20mM ethanol in the presence or absence of 17β-estradiol, MCF-7 cells were plated in mammosphere forming medium and treated with 5μM GSI or equal volumes of DMSO vehicle for 24 hours. If Notch activation in the bulk cells is required for BCSC survival, then we would expect that pre-treatment with the GSI in the bulk cells alone would inhibit mammosphere formation. Additionally, if alcohol-mediated promotion of BCSC survival is dependent on Notch
activation in the bulk cell population, then we would expect to see a decrease in mammosphere forming efficiency (MFE) following pre-treatment with the GSI in bulk cell populations. In contrast, if Notch activation is not required for alcohol-mediated promotion of BCSCs, then we would expect to see no change in MFE in BCSCs that are treated with the GSI for 24 hours, compared to untreated controls.

As shown in Figure 16, cells grown in the presence of 17β-estradiol have a lower MFE compared to cells grown under estrogen deprived conditions (red bars 1 and 2). Additionally, treatment with 20mM ethanol enhanced the MFE of cells grown in the presence of 17β-estradiol, however we failed to see this in estrogen deprived cells unlike our previous experiments (red bars 3 and 4). When bulk cells are pre-treated with the GSI alone, we see no significant change in MFE in the presence or absence of 17β-estradiol (pink bars 1 and 2). Interestingly, pre-treatment with the GSI in combination with ethanol does not affect MFE when 17β-estradiol is present (pink bar 3), however co-treatment with GSI and ethanol under estrogen deprived conditions show a downward trend in MFE, though not statistically significant (pink bar 4).

Overall, BCSCs appear to be resistant to γ-secretase inhibition in bulk cell populations prior to selection.

**Alcohol Promotes Resistance in GSI-Treated BCSCs**

We further sought to determine if GSI pre-treatment in combination with direct treatment of the BCSCs would affect alcohol’s ability to promote BCSC survival. We hypothesized that if alcohol promotes BCSC survival through the activation of Notch signaling, then we would see a decrease in MFE in BCSCs pre-treated with ethanol and plated in GSI containing media. If Notch signaling is not required for alcohol-mediated BCSC survival, then we would anticipate seeing little change in MFE in GSI-treated BCSCs compared to untreated controls.
As expected, GSI treatment in BCSCs alone results in complete inhibition of mammosphere formation in the presence or absence of 17β-estradiol (Figure 16, dark green bars 1 & 2). The addition of 20mM ethanol prior to GSI treatment in the BCSCs does not appear to protect against this effect (dark green bars 3 & 4). Of note, pre-treatment of the bulk cell population with 5μM GSI prior to GSI treatment in the BCSC population shows a positive trend in MFE in cells grown in the absence of ethanol (light green bars 1 & 2). When co-treated with 20mM ethanol, this effect is unchanged for cells grown in the presence of 17β-estradiol (light green bar 3), however co-treatment with the GSI and 20mM ethanol in bulk cells deprived of estrogen prior to GSI treatment in the BCSC population show no resistance to the GSI (light green bar 4).

Collectively, these data suggest that alcohol promotes the expression of Notch4 which may increase its availability to interact with the γ-secretase complex. This interaction appears to be necessary for alcohol-mediated promotion of BCSC survival when 17β-estradiol is absent. Interestingly, pre-treatment of bulk cell populations with GSI prior to 24-hour treatment of BCSCs with GSI results in a γ-secretase independent population. This is most pronounced when cells are grown in the absence of estrogen and ethanol or in the presence of both. However, when cells are grown in the absence of estrogen, but the presence of ethanol, this resistance is not seen. These data suggest that γ-secretase complex activity is a necessary component of alcohol-mediated BCSC survival in the absence of 17β-estradiol, but not required for alcohol-mediated BCSC survival in the presence of 17β-estradiol.
Specific Aim 2: Determine the Effect of Alcohol on Long-Term Estrogen Deprived ER+ Breast Cancer Stem Cells

As mentioned previously, there is a critical need for investigation into the effects of alcohol on a clinically relevant model of endocrine therapy. The MCF-7/5C cell line was originally derived from the naïve ER+ MCF-7 cell line, but this clone has required resistance to estrogen deprivation and no longer depends on 17β-estradiol for its proliferation. The clinical data currently available suggests that post-menopausal women or women who are currently or successfully completed endocrine therapy are at most risk in terms to the negative impacts of
alcohol consumption on breast cancer recurrence. Thus, the goal of this aim was to verify whether exposure to ethanol in a long-term estrogen deprived (LTED) cell line would alter the BCSC population as we see in the naïve parental cells. Specifically, we examined how the changes seen in the parental cell line differed in the LTED cells in response to ethanol treatment in the presence or absence of 17β-estradiol. We hypothesized that if alcohol poses a greater risk under estrogen deprived conditions, then we would expect these cells to be more sensitive to alcohol mediated BCSC survival, potentially through the activation of Notch signaling.

We treated MCF-7/5C cells with increasing concentrations of ethanol in the presence or absence of 17β-estradiol for 72 hours, then seeded 2.5 x 10^4 cells in mammosphere forming medium, as previously described. Mammosphere forming efficiency was then calculated after the 7-day incubation period. As shown in Figure 17, 17β-estradiol inhibits BCSC survival in these cells compared to estrogen-deprived conditions. Alcohol does not have any apparent effect on BCSC survival under estrogen-deprived conditions, however there is a positive trend in %MFE when cells are treated with increasing concentrations of ethanol in the presence of 17β-estradiol, though not statistically significant.

We next investigated whether this trend seen in the 17β-estradiol treated cells could be explained by changes in Notch signaling. As we saw in the naïve ER+ cells, increasing concentrations of ethanol increased Notch4 expression and cleavage, and Notch activity is known to be elevated in LTED cells (Faronato et al., 2015). We collected cells for protein analysis as previously described and assessed changes in both Notch4 and DAXX protein expression following 72 hours of treatment with increasing concentrations of ethanol. If alcohol promotes BCSC in LTED cells in the presence of 17β-estradiol, then we would expect to see increases in Notch4 expression and cleavage in ethanol treated cells compared to controls.
Additionally, we would expect to see decreased DAXX expression under conditions when Notch4 expression is high.

Figure 18 shows that treatment with ethanol upregulates expression of Notch4 in LTED ER+ cells, though there appear to be no significant changes in the cleavage of this receptor. In contrast to this, alcohol dose-dependently upregulates DAXX expression in both the presence and absence of 17β-estradiol. Collectively, these data suggest that alcohol may promote BCSC survival in the presence of 17β-estradiol independently of Notch4, but possibly through changes in 17β-estradiol regulation at the protein level.

Figure 17: Alcohol Exposure Trends Towards Enhanced BCSCs Survival in E2 treated LTED ER+ Cells. MCF-7/5C cells were grown in charcoal-stripped phenol-red free RPMI and treated with either 5nM 17β-estradiol or DMSO, as well as increasing concentrations of ethanol or PBS for 72 hours. 2.5 x 10^4 cells were seeded in mammosphere media and incubated for 7 days, following which %MFE was calculated. Results are presented as the mean ± SD of three independent experiments. Two-Way ANOVA with Tukey’s multiple comparisons analysis was used to determine statistical significance.
Figure 18: Alcohol Enhances Notch4 and DAXX protein expression in LTED ER+ cells.
MCF-7/5C cells were grown in charcoal-stripped phenol-red free RPMI and treated with 5nM 17β-estradiol or DMSO alone or in combination with increasing amounts of ethanol. Total protein was isolated from the cells and lysates quantified for western blot analysis of Notch4 and DAXX using β-Actin as the loading control.
CHAPTER FIVE
DISCUSSION

Notch signaling is a known contributor to BCSC survival and has been implicated in resistance to endocrine therapy as well as tumor recurrence (Rizzo et al., 2008, Peiffer et al., 2019), thus providing a rationale to investigate changes in this pathway in response to alcohol exposure. ER is a known inhibitor of Notch signaling shown in Figure 19 (BeLow & Osipo, 2020), and demonstrated in our findings by the significant decrease in HES1 transcripts in 17β-estradiol treated cells compared to DMSO controls. However, the effect of alcohol on Notch activity and on 17β-estradiol’s ability to regulate this pathway had yet to be elucidated. Our findings demonstrate there is a trend towards Notch activation following alcohol exposure in the presence or absence of 17β-estradiol. This pattern is also reflected in the pluripotent genes SOX2, OCT4, and NANOG, suggesting that alcohol helps to maintain a stem-like state within the bulk cell population through activation of Notch signaling, which correlates with increased expression of pluripotent genes. Additionally, these data suggest that alcohol interferes with the ability of 17β-estradiol to negatively regulate its downstream targets at least in part. Due to the level of variance seen in the induction of these transcripts, it would be advantageous to directly assess changes in expression of these transcription factors using a fluorescent reporter to determine changes in their promoter activity. Alternatively, we can assess if the induction that is seen is due to Notch activation by targeting the Notch receptors via siRNA and determine if this positive trend towards induction is still seen.
To determine if there is a direct effect of alcohol on ER activity, we investigated changes in classic ER target genes $PS2$ and $PGR$ in response to 72 hours of treatment with 40mM ethanol prior to 0-, 3-, or 24-hour treatments of 5nM 17β-estradiol. This allows us to determine whether alcohol prevents the activation of ER target genes in response to 17β-estradiol. As expected, both $PS2$ and $PGR$ transcripts were significantly induced with 3 hours of 17β-estradiol, and this was further enhanced at 24 hours of 17β-estradiol exposure. Interestingly, treatment with 40mM ethanol alone for 72 hours resulted in a significant increase in $PS2$ and $PGR$ transcripts in the absence of 17β-estradiol. However, when 17β-estradiol is introduced to ethanol-treated cells, its ability to further enhance these transcript levels appears to be attenuated, though not statistically significant. Studies that have investigated the effect of alcohol on ER signaling use significantly higher doses of ethanol (80-100mM) which may not be physiologically relevant in the breast (Fan et al., 2000), and several studies have reported increased total protein expression of ERα in response to increasing concentrations of ethanol (Fan et al., 2000, Etique et al., 2004).

In order to determine if changes in these target genes are in fact due to ERα, a knockdown approach using siRNA targeted against $ESR1$ would be necessary. If alcohol-mediated induction of $PS2$ and $PGR$ is due to activation of ERα independent of 17β-estradiol, then we would anticipate that knockdown of the $ESR1$ gene would prevent the induction of these transcripts by alcohol alone, similar to that of estrogen deprived non-alcohol treated controls. It would also be beneficial to assess changes in the phosphorylation of ERα following treatment with ethanol, as it is known that ERα can be activated independent of estradiol through phosphorylation by MAP kinases (Yue et al., 2002). Interestingly, a study conducted by Weitsman and colleagues found that increases in reactive oxygen species (ROS) in HER2-
overexpressing MCF-7 cells resulted in activating phosphorylations of ERα on serine 118 and 167 (Weitsman et al., 2009), which may suggest an alternative mechanism by which alcohol promotes an estrogenic effect within these cells in the absence of 17β-estradiol.

Knowing that Notch signaling is regulated by 17β-estradiol, more specifically ER-mediated stabilization of DAXX at the protein level directly correlates with decreased expression of Notch4 protein, as shown in Figure 19 (Peiffer et al., 2019), we sought to determine whether alcohol disrupted 17β-estradiol-mediated regulation of Notch. To do so, we examined changes in Notch4 expression, as well as the protein expression of DAXX, in response to increasing concentrations of ethanol in the presence or absence of 17β-estradiol. When 17β-estradiol is present, Notch4 expression is inhibited compared to estrogen deprived conditions, whereas DAXX expression is enhanced. Interestingly, we see that Notch4 expression is upregulated by increasing concentrations of ethanol, regardless of whether or not 17β-estradiol is present. In addition to this, alcohol also increases the protein expression of DAXX, but only in the presence of 17β-estradiol. These finding support the hypothesis that alcohol helps to promote BCSC survival through upregulation of Notch4 in the bulk cell population. However, these data suggest that this is independent of alcohol’s effects on estrogen signaling. It is possible that alcohol disrupts 17β-estradiol – mediated transcriptional regulation by the prevention of nuclear translocation of the ER, or its protein targets such as DAXX (Figure 20).

If alcohol prevents the translocation of DAXX to the nucleus, then it will no longer be able to bind to the NOTCH4 promoter to inhibit its transcription, thus resulting in enhanced Notch4 expression when alcohol is present. Alternatively, alcohol may compete with estradiol for the ER itself, and thus we see diminished ER transcriptional activity due to competition of
the ligands for the receptor (Figure 20). To directly test whether alcohol disrupts translocation of either ERα or DAXX, we could use immunofluorescence to determine changes in the cellular localization of either protein following exposure to ethanol. Similarly, we could perform nuclear fractionation to determine if the changes seen in the protein expression of DAXX, or ER, are reflective of increased DAXX in the cytosol versus the nucleus. Thus, if alcohol prevents the translocation of ERα and/or DAXX to the nucleus and thus preventing their ability to regulate transcriptional activity, then we would expect to see an increase in the protein levels of cytosolic DAXX or ERα, or increased fluorescence within the cytosol, compared to that of the nucleus following exposure to ethanol.

**Figure 19: Regulation of Notch Signaling by 17β-estradiol -mediated Activation of the Estrogen Receptor.** Upon activation by 17β-estradiol, ER is known to stabilized DAXX protein expression. DAXX translocates to the nucleus upon 17β-estradiol mediated activation of ER, where it acts as a transcriptional repressor of pluripotent genes and Notch4, thus resulting in inhibition of BCSC survival. Upon estrogen deprivation, DAXX repression of Notch4 transcription is alleviated, and BCSC survival is enhanced.
Figure 20: Alcohol Regulation of 17β-estradiol Mediated ER Activity. In the presence of 17β-estradiol, alcohol partially disrupts the ability of ER to regulate transcription of its downstream targets, including classical targets such as PS2 and PGR. This may be explained by competition between alcohol and estradiol for occupation of the ER. Additionally, although DAXX protein expression is enhanced in the presence of 17β-estradiol and alcohol, its ability to repress transcription of its targets such as SOX2 and HES1, and presumably NOTCH4, is diminished thus resulting in increased expression and activity of the Notch4 receptor in the presence of alcohol.

We can assess whether Notch activation is necessary for alcohol-mediated BCSC survival through the use of a pan Notch inhibitor (GSI), MRK-003, which inhibits the catalytic activity of the γ-secretase complex. We first sought to determine if alcohol was increasing overall Notch activity in the bulk cell population to enhance the BCSC population, and thus MCF-7 cells were pre-treated with the GSI for 72 hours prior to plating into mammosphere media. Our findings show that BCSCs are resistant to Notch inhibition in the bulk cell population
alone in the absence of ethanol. Additionally, when alcohol is added to GSI, in the presence or absence of 5nM 17β-estradiol in bulk cell populations, this resistance is maintained.

We further explored this by adding GSI treatment directly to the mammosphere media for 24 hours to determine if Notch activation within the BCSC population was necessary for alcohol mediated BCSC survival. GSI treatment of BCSC with no prior pre-treatment of the bulk cell population with GSI or alcohol completely inhibits mammosphere formation, which is supported by previous findings (Harrison et al., 2010). Interestingly, when the bulk cell population is pre-treated with the GSI followed by GSI treatment of the BCSC population, a GSI-resistant population persists within the BCSCs regardless of 17β-estradiol. The addition of 20mM ethanol further enhances this resistant population when 17β-estradiol is present, however when ethanol is given under estrogen deprived conditions and the γ-secretase complex is inhibited in both the bulk and BCSC populations, there is virtually no mammosphere formation observed.

Collectively, these data suggest that alcohol promotes ER+ BCSC survival by upregulation of the Notch4 receptor in bulk cell populations, making it more available for activation by its ligands as well as increased likelihood of interaction with the γ-secretase complex. This effect appears to be necessary for alcohol-mediated promotion of BCSC survival when 17β-estradiol is absent. However, in the presence of 17β-estradiol, alcohol-mediated BCSC survival appears to be independent of γ-secretase complex activity. We can further assess whether alcohol promotes the BCSC population within the bulk cells by performing flow cytometry to sort out cell populations based on stem cell markers. More specifically, we could sort based on CD44+/CD24- flow expression, ALDH+ vs. ALDH- populations, or we can assess changes in the surface expression of the various Notch receptors and/or their ligands. This would further clarify if alcohol promotes an increase in BCSCs within the bulk cell population as well
as directly assess changes in the Notch signaling pathway based on surface expression of each receptors and their ligands.

It is plausible that alcohol may be acting independently of Notch signaling to promote BCSCs. Studies have shown that alcohol can induce activation of Wnt signaling to promote alcohol-associated hepatocellular carcinoma (Hennig et al., 2006), and Wnt/β-catenin signaling has been shown to promote BCSCs (Tang et al., 2019, Cai et al., 2013, and Jang et al., 2015). Alternatively, BCSCs can be promoted through activation of NF-κB (Yamamoto et al., 2013, Zhou et al., 2008), Hippo YAP/TAZ pathway (Cordenonsi et al., 2011), and others (Zhou et al., 2007, Bai et al., 2020). Thus, utilizing unbiased approaches such as RNA Sequencing may prove to be essential in narrowing down the pathways necessary for alcohol-mediated BCSC survival independent of the γ-secretase complex.

In addition to our work in naïve ER+ cells, we investigated the effects of alcohol on BCSCs using a long-term estrogen deprived ER+ cell line MCF-7/5C. There is a deficit in the knowledge of how estrogen deprivation changes the effects of alcohol on breast cancer, yet the clinical data suggests that post-menopausal women or women undergoing endocrine therapy are most at risk for the cancer-promoting effects of alcohol consumption (Kwan et al., 2013, Simapivapan et al., 2016). Although our data did not show a statistically significant change in overall BCSC survival following treatment with ethanol, the trends in both Notch4 and DAXX protein expression were similar. Due to time constraints, we were unable to pursue the hypothesis that alcohol disrupts transcriptional regulation mediated by 17β-estradiol in these cells, however our current data supports that this may also be true for LTED ER+ cells. This is of particular importance in terms of pre-menopausal women who have already undergone endocrine therapy and then choose to consume alcohol later in life.
In summary, this project has demonstrated that alcohol exposure in ER+ breast cancer poses a significant risk to breast cancer patients through the promotion of BCSCs. Potentially, alcohol may promote BCSC survival through disruption of estrogen-mediated transcriptional regulation, resulting in increased Notch activity which has been correlated with cellular transformation, endocrine therapy resistance, and tumor recurrence. Future studies should investigate whether exposure to alcohol disrupts the translocation of ER and/or DAXX to the nucleus, and how this correlates with the changed expression in their target genes as shown in this study. Additionally, more work is necessary to determine whether alcohol exposure promotes enhanced tumorigenicity of BCSCs or increases tumor recurrence using an in vivo model. Furthermore, future experiments should be aimed towards reducing the level of variance seen within these experiments by identifying specific cell populations through sorting via flow cytometry, or through genetic knockout of prospective alcohol-regulated targets such as Notch4 or ERα. Together, these data will provide further insight into the molecular mechanisms by which alcohol promotes ER+ breast cancer development and recurrence.
REFERENCE LIST


VITA

McKenna Noelle BeLow was born on December 5th, 1994, to Sandra and Steven BeLow in Arlington Heights, Illinois. McKenna earned her Bachelor of Science degree in Psychology at Grand Valley State University in Grand Rapids, Michigan in 2017. During her undergraduate career, McKenna worked under Dr. Elizabeth Flandreau who mentored her in behavioral neuroscience. During her time in the Flandreau lab, McKenna studied the effect of a Western diet on stress and anxiety-like behaviors in a rodent model of post-traumatic stress disorder. McKenna was given the opportunity to present her data at Grand Valley’s university wide research symposium and publish her findings in the Journal of Neurobiology.

Following her undergraduate career, McKenna spent a year at the University of Iowa working for Dr. John Freeman at the Iowa Neuroscience Institute. During her time in the Freeman lab, McKenna learned how to perform various rodent surgeries for experiments using optogenetics as well as cerebellar learning behavioral assays.

McKenna entered into the Interdisciplinary Program of Biomedical Sciences PhD program at Loyola University of Chicago in 2018. After completing her qualifying exam, McKenna made the decision to transition into the Masters Program of Integrated Cell Biology, and she has completed her graduate studies under the mentorship of Dr. Clodia Osipo. In the Osipo lab, McKenna initiated the first project within the lab to investigate the effects of alcohol on breast cancer. Upon completion of her graduate studies at Loyola, McKenna intends to pursue a Masters in Genetic Counseling, where she can apply her knowledge of cancer biology to a patient-focused career as a genetic counselor.