Signaling Networks as Possible Therapeutic Implications in Breast Cancer

DISSERTATION

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Abstract

In 2014 there will be an estimated 235,030 new cases of invasive breast cancer in the United States and breast cancer is the most frequently diagnosed cancer in women with 1 breast cancer diagnosis occurring in every 3 new cancer cases. Even with the advances in breast cancer therapies, such as tamoxifen and trastuzumab, resistance remains a huge problem and the major limitation to overall survival. The research presented in this dissertation attempts to investigate breast cancer signaling pathways, alternative strategies for therapeutic implications and potential mechanisms of resistance to drugs.

The second chapter investigates the influence of MAPK signaling on Pol II positioning and activation in association with the immediate early gene, JUNB’s proximal promoter and JUNB expression in human breast cancer cell lines. The results of this chapter indicate that constitutive RAS/MAPK signaling enhances the association of Pol II phosphorylated on serine 5 (Pol IIser5p) with the JUNB proximal promoter and treatment with U0126, a MAPK pathway inhibitor, reduces Pol IIser5p association with the JUNB proximal promoter and reduces JUNB expression. This demonstrates that MAPK signaling plays a primary role in the control of JUNB gene expression by promoting the association of Pol IIser5P with the JUNB promoter.
As JUNB induction was found to be reliant on the MAPK control of Pol II, the third chapter aims to investigate the influence of kinase inhibitors on immediate early gene expression in breast cancer cells. The results demonstrate that the immediate early gene JUNB is induced by lethal doses of flavopiridol (CDK9 inhibitor), U0126 (MEK1 inhibitor) and sorafenib (a multi-kinase inhibitor) in multiple breast cancer cell lines. Functional studies revealed that JUNB plays a pro-survival role in kinase inhibitor treated breast cancer cells. These results demonstrate a unique induction of JUNB in response to kinase inhibitor therapies that may be among the earliest events in the progression to treatment resistance.

To understand how treatment combinations can affect breast cancer patients, the fourth chapter investigates the role of neoadjuvant chemotherapy in combination with both lapatinib and trastuzumab compared to neoadjuvant chemotherapy and trastuzumab alone. Randomized clinical trials (RCT) that evaluated safety and efficacy of adding lapatinib to trastuzumab plus neo-adjuvant chemotherapy (NAC) in patients with HER-2/neu over-expressing, operable breast cancer reveal mixed results. Some studies showed statistically significant improvement in pathologic complete response (pCR) rate with dual anti-HER-2 therapy while others showed only a trend towards improvement in pCR which was not statistically significant. We therefore performed a meta-analysis of published or presented prospective RCTs that studied the effect of adding lapatinib to trastuzumab and NAC on pCR rate in patients with operable HER-2/neu positive breast cancer. Addition of lapatinib to trastuzumab in combination with NAC improves pCR
rates in patients with HER2-positive breast cancer regardless of its definition. The addition of lapatinib to trastuzumab containing neoadjuvant chemotherapy on pCR rates defined as no residual invasive carcinoma in breast only benefits both hormone receptor positive and hormone receptor negative patients.

To understand how breast cancer cells develop resistance to small molecule inhibitors, resistant triple negative breast cancer cells were developed and characterized in chapter 5. Triple negative breast cancer (TNBC) is a subtype of breast cancer that lacks the estrogen receptor, progesterone receptor or HER2 producing tumors that are unresponsive to available targeted breast cancer therapies. TNBC is characterized by an up-regulation of the EGFR-RAS-MEK-ERK signaling pathway and a clinical trial is currently ongoing investigating the use of a MEK inhibitor trametinib. This study is the first to investigate trametinib resistance in TNBC cell lines. PTEN status can accurately predict trametinib sensitivity. Interestingly, PTEN wild type cells are sensitive to trametinib, dramatically reducing the expression of pERK, and causing an induction of pAKT, while PTEN levels are unaffected. This induction of pAKT persists through the resistant cells but PTEN levels appear to decrease. Trametinib resistant cells proliferate similar to the parental cell and can withstand a dramatic increase of trametinib. Using kinase antibody arrays, PRAS40, HSP60 and AKT seem to be highly activated in the resistant lines relative to the parental lines. To date this is the first characterization of trametinib resistance in TNBC.
In summary, the results uncovered in this dissertation provide clues into possible resistance mechanisms to targeted therapy in breast cancer cells for both flavopiridol (JUNB) and trametinib (PRAS40, HSP60, AKT, c-JUN), the pivotal role of MAPK signaling on Pol II activation on the JUNB proximal promoter and the clinical benefit of neoadjuvant chemotherapy in combination with lapatinib and trastuzumab for HER2-amplified breast cancers on pathological complete response. Information gained from these studies can help design rationale drug combinations to prevent disease recurrence and cancer related deaths.
Dedication

This dissertation is dedicated to my parents Mell and Laura, and Benjamin Nixon for always supporting me through these trying times. All I have and could accomplish are due to their love and support. I could not have done it without every Sunday dinner and every phone call.
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# Table of Contents

Abstract........................................................................................................................................... ii

Acknowledgments......................................................................................................................... vii

Vita................................................................................................................................................ viii

Table of Contents ............................................................................................................................. ix

List of Tables ................................................................................................................................. xiii

List of Figures ............................................................................................................................... xiv

Chapter 1: Introduction ...................................................................................................................... 1

1.1 General Introduction .................................................................................................................... 1

1.2 Cancer Signaling ........................................................................................................................ 3

1.2.1 MAPK Signaling ..................................................................................................................... 6

1.2.2 PI3K pathway ......................................................................................................................... 7

1.3 Anatomy and Development of the Breast ................................................................................. 9

1.3.1 Anatomy of the Breast ......................................................................................................... 9

1.3.2 Development of the Breast .................................................................................................. 12

1.4 Breast Cancer .......................................................................................................................... 16

1.4.1 Stage Classification of Breast Cancer ................................................................................. 17
1.4.2 Breast Cancer Treatment options ................................................................. 20
1.4.3 Chemotherapy .............................................................................................. 21
1.4.4 Chemotherapy Resistance ........................................................................... 25
1.5 JUNB and Cancer ............................................................................................ 26
1.6 Overall Goal of Dissertation ............................................................................ 28

Chapter 2: Mitogen Activated Protein Kinase signaling controls constitutive Oncostatin M mediated induction of JUNB gene expression .......................................................... 31
2.1 Introduction ...................................................................................................... 31
2.2 Methods ........................................................................................................... 35
2.3 Results .............................................................................................................. 38
2.4 Discussion ........................................................................................................ 44

Chapter 3: JUNB promotes the survival of Flavopiridol treated human breast cancer cells ................................................................................................................. 52
3.1 Introduction ...................................................................................................... 52
3.2 Methods ........................................................................................................... 54
3.3 Results .............................................................................................................. 57
3.4 Discussion ........................................................................................................ 61

Chapter 4: Neoadjuvant Dual HER-2/neu-targeted therapy with lapatinib and trastuzumab improves pathologic complete response in patients with early stage HER-2/neu-positive breast cancer. A meta-analysis of randomized prospective clinical trials ............... 69
List of Tables

Table 1. Characteristics of the Randomized Control Trials Included in the Meta-Analysis ....................................................................................................................................................................................... 87

Table 2. Incidence and Relative Risk (RR) of pCR Stratified by definition of pCR and hormonal status. ........................................................................................................................................................................... 88
List of Figures

Figure 1. MAPK and PI3K signaling................................................................. 6
Figure 2. Anatomy of a human adult breast......................................................... 11
Figure 3. JUNB expression is constitutively elevated in human breast cancer cell lines. 48
Figure 4. OSM induces JUNB expression and activates MAPK and STAT3 signaling. 49
Figure 5. MAPK inhibitor U0126 reduces JUNB gene expression and Pol II association with the JUNB proximal promoter. .......................................................... 50
Figure 6. U0126 blocks OSM mediated MAPK induction, Pol II ser5p association with the JUNB proximal promoter and JUNB expression........................................... 51
Figure 7. JUNB is induced by flavopiridol treatment........................................... 64
Figure 8. JUNB is induced in multiple human breast cancer cell lines in response to flavopiridol treatment............................................................................. 65
Figure 9. JUNB plays a pro-survival role in FP treated MCF10A^met cells. .............. 66
Figure 10. JUNB gene expression is induced by treatment with sorafenib and U0126, but not doxorubicin.............................................................. 67
Figure 11. JUNB plays a role in survival of breast cancer cells treated with kinase inhibitors. .................................................................................. 68
Figure 12. Selection process for the randomized controlled trials included in the meta-analysis........................................................................ 86
Figure 13. Odds ratio of incidence of pathological complete response definition of no invasive disease in the breast and lymph nodes. ........................................... 89

Figure 14. Odds ratio of incidence of pathological complete response definition of no invasive disease in the breast. .......................................................... 90

Figure 15. Odds ratio of incidence of pathological complete response definition of no invasive disease in the breast based on hormone receptor status. ............................................. 90

Figure 16. PTEN status can predict sensitivity to trametinib. .................................. 109

Figure 17. PTEN wild type MDA-MB-231 TNBC cells develop an acquired resistance to trametinib, in contrast to the intrinsically resistant PTEN null MDA-MB-468 TNBC cells. ........................................................................................................ 110

Figure 18. Trametinib resistant TNBC cells have an altered kinase profile relative to parental cells and intrinsically resistant cells have a different profile relative to TNBC cells that acquired resistance................................................................. 111

Figure 19. TNBC cells are sensitive to the AKT inhibit LY294002. ......................... 132

Figure 20. Cell morphology changes with trametinib resistance............................. 133

Figure 21. E-cadherin is reduced in trametinib resistant MDA-MB-468 cells........... 134
Chapter 1: Introduction

1.1 General Introduction

The National Institutes of Health estimate that in 2009 the overall cost of cancer in the United States was $216.6 billion, with $86.6 billion in direct medical costs and $130 billion in indirect mortality costs [1]. 1,665,540 new cancer cases are expected to be diagnosed in the United States in 2014. Cancer is the second leading cause of death behind heart disease with 1,600 people dying every day from cancer [1]. Ohio is ranked 6th for number of new cancer cases with 67,000 cases expected for 2014 and 6th for number of cancer deaths with 25,260 deaths. The five year survival rate for all cancers from 2003 to 2009 is 68%. This has increased from 49% from 1975-1977 which is due to improvements in both early detection and treatment [1].

Cancer is defined as uncontrolled growth of cells with the ability to metastasize within the body. Cancer can be caused by environmental factors such as tobacco smoke or genetic factors such as viruses or genetic mutations [2]. According to Hanahan and Weinberg, there are eight hallmarks of cancer including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy
metabolism and evading immune destruction [2]. These hallmarks, according to Hanahan and Weinberg, are underlined by genome instability and inflammation caused by the microenvironment to foster these hallmarks [2]. These hallmarks induce carcinogenesis, promote growth of the primary tumor and aid in the ability of these malignant cells to metastasize to distant sites. Complicating this further, most tumors contain cellular heterogeneity [3]. Tumors contain many cell types, such as epithelial, stromal and immune cells, all playing different but important roles in carcinogenesis. Within each of these cell types there can also be genetic heterogeneity where subgroups of cells exist within a cell type that contain genetic diversity. This genetic diversity could explain the difficulty in treating many cancers, as cancer treatments often target a specific genetic alteration that may not be shared by all cells in a tumor. Tumor heterogeneity is not new as Dr. Virchow noted infiltrating leukocytes in neoplastic tissue in 1863 [4].

Genetic heterogeneity occurs when the DNA of cancer cells is altered to either be silenced or expressed in abundance. This can be done by splice variants, mutations, promoter methylations and copy number variants, to name a few [5]. These genetic alterations can be difficult to target therapeutically as they occur in the nucleus of cells and are therefore difficult to target with existing technology [6]. The alterations can however be targeted at the protein and post-translational level, predominately by altering signaling pathways.
1.2 Cancer Signaling

Specific alterations in signaling pathways can vary based on the context and specific type of cancer. The most common alteration in signaling pathways are triggered by growth factors. Growth factors are small molecules that bind to the extracellular component of a receptor. These receptors usually span the plasma membrane, with an extracellular component to bind to the extracellular growth factor, connected to an intracellular cytoplasmic domain by a short hydrophobic helix transmembrane component [5]. The cytoplasmic domain has regulatory sequences and conserved receptor tyrosine kinase (RTK) domains which transfer a phosphate group from adenosine triphosphate (ATP) onto a protein substrate (usually the amino acid tyrosine). This domain also can phosphorylate itself post ligand binding, causing a conformational change to facilitate dimerization of two receptors, either two of the same receptor (homo-dimer) or two different related receptors (hetero-dimer) to allow full catalytic ability [5]. This dimerization causes an intramolecular phosphorylation within the catalytic domain and other domains to open the kinase activation loop which allows access to ATP. Other phosphorylation regions create docking sites for further downstream signaling. In the case of cancer, this signaling pathway can be hijacked by loss of auto-inhibitory mechanisms thus creating ligand independent activation of the catalytic activity of the kinase [5].
The epidermal growth factor (EGF) and the epidermal growth factor receptor 1 (EGFR or HER1) are one example of a receptor tyrosine kinase and ligand that can be hijacked by cancer. Under normal conditions, EGF must bind to HER1 on the surface of the cells. HER1 can then dimerize with another HER1 receptor or one of its other family members such as HER2. This dimerization causes signaling to cause the cell to grow and proliferate. 25% of non-small cell lung cancers have an activating mutation in the EGFR/HER1 receptor [7]. In contrast to HER1, HER2 receptors do not have the extracellular domain and therefore if a HER2: HER2 dimer is formed, it can signal in a ligand independent manner. There is a sub-type of breast cancer called HER2 amplified which occurs in 20% of cases, where the HER2 receptor is amplified, increasing the concentration of activated dimers (HER2: HER2) resulting in continuous catalytic activity and inappropriate proliferation. A normal breast cell contains 20,000 to 50,000 HER2 molecules, compared to a HER2 amplified cell which can have up to 2,000,000 HER2 molecules [8].

In addition to the receptor tyrosine kinases, there is also down-stream signaling that can be used by cancer cells [5]. Cytoplasmic proteins are rapidly associating proteins that form complexes to transmit information within the cell. These adaptor proteins contain Src Homology 2 (SH2) or Phospho-tyrosine binding domains (PTB) to recognize tyrosine phosphorylation sequence motifs. These proteins dock to the RTK on phosphorylated tyrosine using their SH2 domains. They then transmit the signal using another motif, such
as Src Homology 3 (SH3), to bind to another close by molecule. Growth Factor Receptor Bound-2 (grb-2) is an example of these adaptor proteins[5]. The SH2 domain of grb-2 associates with the receptor while its SH3 domain binds to a proline based motif on the Son of Sevenless (SOS) protein. This SOS protein is a guanine nucleotide exchange protein which can activate proteins like Ras [5]. While “activator proteins” are not necessarily targets of cancer dysregulation, they can be circumvented by targeting GTPases like Ras.

Ras is a membrane associated molecular that can actively signal when bound to guanine triphosphate (GTP). The Ras proteins are GTPases that cycle between GTP bound active molecules and GDP inactive molecules in response to external stimuli. Ras is activated by proteins such as SOS that release Ras from the Ras:GDP complex, allowing Ras to bind to GTP causing a conformational change. This allows other signaling molecules such as B-Raf to bind and further transmit the signal. Binding of other signaling proteins recruits the GTPase activating protein (GAP) to increase the weak GTPase activity of Ras thus causing a conformational change in Ras to dissociate the effector molecules like B-Raf. In many cancers, including breast cancer, Ras has an activating mutation which causes constitutive activation and cellular transformation [9]. Ras mutations are common in codons 12, 13, and 61 which prevent GAP from inducing Ras GTPase activity and allowing ligand independent constitutive signaling [9]. This constitutive Ras signaling
can then activate the mitogen activated protein kinase (MAPK) pathway or the phosphatidylinositol-3-kinase (PI3K) pathway.

![Figure 1. MAPK and PI3K signaling. Figure Adapted from Gordon et al, 2013.[10]](image)

1.2.1 MAPK Signaling

A common pathway that is altered in cancer is the MAPK pathway. This pathway can be activated by growth factor signaling in addition to chemotherapeutic drugs and ionizing radiation [11]. The MAPK pathway is activated by phosphorylation of threonine and tyrosine residues in a T-X-Y motif in the activation loop. There are three distinct MAPK
pathways that each regulates different pathways. In cancer, the most commonly mutated pathway is the RAS-RAF-MEK-ERK pathway which translocates to the nucleus to interact with transcription factors, such as the AP-1 complex or Ets-2, to drive proliferation, differentiation, and survival (Figure 1) [5, 12]. MAPK can also signal stress responses through the c-Jun N-terminal kinase or stress activated protein kinase (JNK/SAPK) and the p38 pathways. It is important to note that both Ras and Raf are comprised of three family members (H-Ras, K-Ras, N-Ras and B-Raf, c-Raf and A-Raf respectively) [13, 14]. In melanoma, it has been found that up to 50% of tumors have a B-Raf mutation affecting valine 600, changing it to glutamic acid (V600E) [15]. Furthermore 30% of all cancers are thought to have hyperactivation in extracellular-signal regulated kinase (ERK) [16] [17].

1.2.2 PI3K pathway

In addition to the MAPK pathway, activator proteins can bind to phosphatidylinositol-3-kinase (PI3K) in order to phosphorylate the 3’ hydroxyl group of an inositol ring which activates secondary messengers in the pathway [18]. PI3K is a heterodimeric protein made up of a catalytic subunit p110 (transcribed from the PIK3CA gene), and an adaptor regulator protein p85, which contains 2 SH2 domains. The p85 SH2 domain binds to the docking site on the RTK causing allosteric activation of the p110 subunit causing phosphorylation of 4,5-phosphoinositide (PIP2) to 3,4,5-phosphoinositide (PIP3) [5, 10]. The 3-phosphoinositide-dependent protein kinase (PDK1) is activated by PIP3 resulting
in the phosphorylation of AKT on Thr308. mTOR complex 2 is also activated and phosphorylates the ser473 on AKT making this protein fully activated for downstream signaling [19]. The PIK3CA gene is commonly mutated or amplified in human cancers [20]. Specifically the PI3KCA mutation is found in 30% of breast cancers [18]. The Phosphatase and tensin homolog (PTEN) protein is a negative regulator of the PI3K pathway. PTEN has diminished expression due to loss of heterozygosity, oxidation, negative phosphorylation, or epigenetic silencing that can be observed in up to 48% of all breast cancers [21]. This pathway, typically signaling through the PI3K- PIP-AKT- mTOR, promotes survival and anti-apoptotic effects within the cell [11]. Interestingly Cowden’s syndrome patients have a germline PTEN mutation which causes a predisposition to breast cancer [22].

Extensive cross-talk has been observed in cancer signaling between the MAPK and PI3K pathways [16]. The Ras family member KRAS can stimulate signaling through the PI3K pathway in addition to the canonical MAPK pathway [16]. Both pathways have also been known to modulate the activity of mammalian target of rapamycin (mTOR) kinase [23]. Both pathways can also regulate other important signaling pathways such as Jak/STAT, Nf-κB and TGF-β [8]. mTORC1 and mTORC2 proteins are involved in protein translation and are activated by both the MAPK and PI3K pathways [11]. Increased expression of the Ras/Raf/Mek/Erk pathway can result in decreased PTEN expression, allowing activation of the PI3K pathway [24]. Both ERK and AKT have been shown to
phosphorylate the anti-apoptotic protein Bim, targeting the protein for proteosomal degradation [11]. When mTOR is inhibited, it has been found that PI3K can activate the MAPK pathway through Ras [25]. Also the blockade of the PI3K/AKT signaling by PTEN causes breast cancer cells to become more dependent on the MAPK pathway [19].

### 1.3 Anatomy and Development of the Breast

A proposed mechanism in cancer development is incorrect replication due to mutations. The average mutation rate in the human genome $2.5 \times 10^{-8}$ mutations per nucleotide per generation [26]. Normally cells grow and divide during development, but stop during adulthood. Mammary glands are one of the few organs that are constantly proliferating throughout a person’s lifetime. With each menstrual cycle, cells divide and proliferate implying an increase in the probability for a mistake compared to other organs. Therefore it makes sense that mammary glands (breasts) are at high risk for carcinogenesis compared to other organs and tissues. To understand cancer of the breast it is first important to understand the anatomy and development of the breast.

#### 1.3.1 Anatomy of the Breast

A major characteristic that sets mammals apart from other species is the presence of a mammary gland. Mammary glands are highly modified sweat glands designed to produce milk, fat and antibodies for offspring [27]. Humans have two mammary glands called
breasts that contain 15-20 lobes and are split into 4 quadrants; the upper outer, upper inner, lower outer and lower inner. In adult women, each of these quadrants contains a network of ducts and lobules and are seemingly identical but for unrecognized reasons, most breast cancer occurs in the upper outer quadrant (Figure 2) [28] [29]. Human breasts weigh on average 200g and are located between the second and sixth rib vertically and the sternal edge to the mid-axillary line, laterally[30, 31]. While breasts are seemingly identical, they often differ in size between the left and right breast and in a study including 55 women, the right breast was found to be smaller than the left breast which was not correlated to handedness. In a study done using the Swedish Cancer Registry, 80,784 cases of invasive and 3,835 cases of pre-invasive breast cancer in women, breast cancer was found to be more common in the left breast compared to the right after the age of 45[32]. There is no correlation between breast mass and cancer risk as larger breasts do not always contain more glandular parenchyma [33]. There is however an increase in breast cancer risk as the percent of dense area in the breast increases [34].

The mammary gland is a complex organ and is made up of a variety of cell types including epithelial cells which make up the ductal network; adipocytes, to make the fat pad which provides the matrix for the ductal network to embed in; vascular endothelial cells to provide oxygen and nutrients via the blood stream; immune cells, which travel in through the vasculature to provide protection against pathogens; and stromal cells such as fibroblasts which provide support. There are two major types of epithelial cells; the luminal epithelium which forms ducts and secretory alveoli and basal epithelium which
are a type of myoepithelium responsible for forcing the secretions through the ductal networks [35]. Most breast cancers form from the luminal epithelium, but those developing from the basal epithelium tend to have a worse prognosis [36].

![Diagram of the human adult breast](image)

**Figure 2. Anatomy of a human adult breast. Adapted from American Cancer Society book.[37]**

Starting from the nipple, there are around 20 orifices of collecting ducts in the nipple (Figure 2) [37]. These lactiferous ducts are surrounded by bundles of smooth muscle that can interlace among the collecting and lactiferous ducts. The portion of the duct system immediately below the collecting duct is the lactiferous sinus where milk accumulates
during lactation. This sinus connects to the segmental ducts which ultimately subdivide into the terminal duct to drain into the lobule. Each lobule is composed of groups of small glandular structures, where during lactation, milk is produced [31]. Basal epithelium (myoepithelium) surrounds the ducts and is responsible for contractility to move secretions out toward the nipple. The basement membrane is found immediately outside of the myoepithelium and divides the glands from the stroma. Beyond the basement membrane is the stroma made up of predominately fibroblasts and adipocytes to make up the scaffolding in which the ductal network lies. There are vasculature and lymphatic systems found within the breast to supply blood, nutrients and immune cells, and take away waste. More than 75% of the lymph drained from the breast enters the axilla lymph nodes. The axillary lymph nodes are important in breast cancer because surgeons and pathologists can test them to determine the extent of invasive disease in breast cancer, as most metastatic cells pass through these lymph nodes in order to spread to the rest of the body. Presence of invasive disease in this area dictates the type of treatment, progression and outcome of the patient, with presence of disease in the lymph nodes correlating to a worse prognosis [38].

1.3.2 Development of the Breast

There are three main stages of mammary gland development: embryonic, pubertal and adult. The mammary gland begins to develop at week 6 of gestation with multiple bilateral thickenings of the ectoderm on the ventral aspect of the fetus along the milk line.
Each mammary gland parenchyma arises from a single epithelial ectodermal bud [29]. There are 10 distinct stages of prenatal development of the mammary gland which are reviewed in greater detail by Russo et al. [29]. During weeks 23-28 the basal epithelium (myoepithelium) begins to develop. During this time the mesenchyme also begins to develop into fat. In the last 8 weeks the epidermis depresses, forming the lactiferous ducts. The newborn breasts contain ducts lined with one or two layers of lobular epithelium and one layer of basal epithelium (myoepithelium). After birth, breast formation remains dormant until puberty.

Mammary glands are identical in males and females until puberty. Puberty occurs in females around age 13 where estrogen is released from the ovaries. During puberty, the rudimentary mammae begin to show growth in the glandular tissue via division of primary and secondary ducts and the surrounding stroma begin to proliferate causing increased density of connective tissue thus increasing breast size [29, 35, 39]. The ducts form club-shaped terminal end buds providing the foundation for new branches [29]. The terminal end buds will then give rise to alveolar buds and cluster around a terminal duct to form the type 1 or virgin lobule [29, 39]. There are two more types of lobules, type 2 and type 3, which form from continued sprouting of new alveolar buds. With each ovulatory cycle, the mammary gland will continue to grow in lobule structures until around the age of 35 [29]. The Breakthrough Generations Study from the United Kingdom determined breast cancer risk by different pubertal variables and found that
there was an increased risk of breast cancer with earlier thelarche (age of breast development), earlier menarche (initiation of menses) and increased time between thelarche and menarche [40].

The breast of nulliparous women contain more undifferentiated structures like terminal end buds and type 1 lobules compared to parous women where the predominant structure are the more differentiated type 3 lobule [29]. This is significant as it has been shown that nulliparous women have a higher risk to develop malignancies compared to parous women and type lobules 1 have the highest proliferative index, the higher concentration of estrogen receptors and number of blood vessels, which makes them the perfect target for malignancy [29, 35, 41]. It is important to note, that while men do not undergo changes in the mammary gland during puberty, they do contain a network of large ducts but not lobules, and these ducts can be subject to malignancy similar to women [31]. Male breast cancer only accounts for 1% of all breast cancers and 430 breast cancer related deaths on average in the United States [42].

As the breast matures during puberty, it is preparing to produce milk for offspring. There are two major phases of breast development during pregnancy; early and late. Early stage development consists of proliferation of the distal elements of the ductal tree resulting in formation of type 3 lobules into type 4, also known as acini. The epithelial cells in the acini grow not only in number, but also size due to cytoplasmic enlargement [43]. As the
lobular epithelium begins to grow, the ductal cells that are surrounded by the lobules being to thicken making then equal in size and therefore indistinguishable from the terminal duct. During the late stage of development the secretory mechanisms are set in place. Further branching continues with little to no bud formation and the secreting units differentiate [44]. The lumen of already formed acini becomes vacuolated with the accumulation of lipids, marking the end of differentiation [29].

Lactation begins after postpartum withdrawal of the placental lactogen and other sex steroids and continues to secrete milk indefinitely until weaning [44]. There are no major morphological changes during lactation [29]. Weaning causes an accumulation of milk which prevents future milk production and thus begins the process of involution.

Involution is the process consisting of a multifocal asynchronous process to reduce the number of secretory cells. The lobules undergo atrophy and adipose tissue accumulates in the stroma [44]. The acinar structures begin to collapse and phagocytes, such as macrophages, infiltrate the area. The ducts and branches remain but the lobules and acini shrink and collapse [44]. This is important to breast carcinogenesis because the microenvironment during involution shares many similarities with inflammation including expression of matrix metalloproteinase, which is known to promote carcinogenesis [45]. During involution, up to 80% of the epithelium can undergo apoptosis [35].
The final change in development to mammary glands in humans is menopause.

Menopause happens in women around the age of 51 when ovarian follicle atresia occurs and gonadotropin releasing hormone is secreted, thus causing amenorrhea or the cessation of estrogen and progesterone production by the ovaries [29]. This causes a regression period in the mammary gland where there is a marked decline in type 2 and 3 lobules and an increase in the number of type 1 lobules. Even with this increase in type 1 lobules in both nulliparous and parous women, only nulliparous women have a marked increase in the incidence of breast cancer, indicating there is an unknown biological difference in these seemingly identical type 1 lobules [46, 47]. Women who have a delayed onset of menopause have an increased risk of developing breast cancer [48].

1.4 Breast Cancer

In 2014 there will be an estimated 235,030 new cases of invasive breast cancer in the United States, with 2,360 male cases [49]. Breast cancer is the most frequently diagnosed cancer in women with 1 diagnosis in every 3 cancer cases. One case of breast cancer is diagnosed every 2 minutes and one woman will die of breast cancer every 13 minutes in the United States. Breast cancer is second only to lung cancer in cancer related deaths among women in the United States [42].
There are many different risk factors that are associated with breast cancer. Age, family history, genetic alterations (BRCA1/2), obesity and alcohol consumption rank among the highest risk factors [50]. Women between the ages of 45 and 74 have the highest breast cancer diagnosis with 68.1% of cases diagnosed in this age range [42]. Women who have not had children, or had children after the age of 30 have a higher risk of developing breast cancer [51].

1.4.1 Stage Classification of Breast Cancer

There are 5 major stages of breast cancer set by the American Joint Committee on Cancer (AJCC) TNM system, each with their own treatment recommendations [52]. Staging begins at stage zero, which is a pre-cancer known as carcinoma in situ. This stage is characterized by abnormal cells in the lining of the lobule (lobular carcinoma in situ, LCIS) or more commonly the lining of the duct (ductal carcinoma in situ, DCIS). These cells have not invaded the surrounding tissue but could accumulate mutations and transition to an invasive cancer. Stage I is the earliest stage of breast cancer characterized by a primary tumor no larger than 2 centimeters in diameter and has not spread beyond the breast. The 5 year relative survival rate for stage 0 and stage 1 is 100% [42]. Stage II has three definitions based on the size and lymph node involvement and overall has a 93% 5-year survival rate. Stage II is defined as a primary tumor no more than 2 cm in diameter with lymph node involvement, a tumor 2-5 cm with or without lymph node involvement or a tumor greater than 5 cm with no lymph node involvement [42]. Stage
III is considered a locally advanced cancer, has three major types and a 72% overall 5-year relative survival rate [42]. Stage IIIA is a tumor smaller than 5 cm and positive lymph nodes that are or are not attached to each other or to other structures. Stage IIIB is characterized by involvement of the chest wall or skin of the breast, or the cancer has spread to lymph nodes beyond the breastbone, or diagnosed as inflammatory breast cancer or a tumor of any size that has spread to other lymph nodes. Inflammatory breast cancer is a very rare cancer characterized by red breasts that are swollen and feel warm due to lymph blockage. Stage IV is characterized by distant metastasis and is usually associated with the worst prognosis (22% 5 year overall survival rate [42]).

Due to advances in breast cancer research, we are also able to classify breast cancer based on molecular features, dividing them into four subtypes. These subtypes are not used clinically to dictate treatment but can be used to determine prognosis and overall survival trends [53, 54]. Current research is ongoing in this area to provide more personalized medicine approach for each of these subgroups of patients [55]. The first subgroup accounts for 40% of all breast cancers, called luminal A, is estrogen receptor (ER) positive tumor with a low grade, low recurrence and the best prognosis. Luminal B subtype accounts for 20% of all breast cancers, is also ER positive, but has high expression of Ki67 (a proliferation marker) or HER2+ and has a poorer prognosis. The HER2 overexpressed subtype represents 10-15% of all breast cancer, can have hormone receptor expression and has an amplification of the HER2 receptor indicated by an IHC
score of 3+. These tumors have a high grade and have a worse prognosis. Finally, there is basal or triple negative breast cancer (TNBC) which is often used to describe the basal like breast cancer. This cancer lacks the hormone receptors and amplification of the HER2 receptor. This subtype is associated with a younger age, African American ancestry, and \textit{BRCA1} and \textit{p53} gene mutations. TNBC accounts for 15-20% of all breast cancer, has a high grade and poorest outlook, as TNBC are not responsive to current targeted therapies such as trastuzumab and tamoxifen. Recently, a subtype of triple negative breast cancer has been identified called claudin-low, which is characterized by low expression of claudin genes 3,4 and 6, low cell-cell junction markers, high lymphocyte infiltration and increased expression for stem cell and EMT markers [56]. This subtype of TNBC tends to be less responsive to chemotherapy and may be responsible for the increased cancer deaths within TNBC.

Thanks to recent therapy advances targeting the ER and HER2, and earlier detection methods, there has been a decline in the death rates among those diagnosed with breast cancer as a whole [49]. Of all diagnosed cases, 61% are localized and confined to the primary site. This diagnosis has a 98.6% relative survival rate. Unfortunately if the cancer cannot be staged or is cancer with metastasis at a distant site, the relative 5 year survival rate is 50% and 24.3% respectively [42]. Due to more intensive screening measures, such as mammographies and breast self-detection, cancers are diagnosed earlier therefore increasing survival and prognosis for those patients [50].
1.4.2 Breast Cancer Treatment options

Once breast cancer has been diagnosed, the treatment options are decided based on the grade of the tumor (how differentiated the tumor cells are), the stage of the tumor (size, involvement of lymph nodes and metastases), general health status, and the expression of hormone (estrogen and progesterone) and HER2 receptor status. Expression of the receptors is measured using either immunohistochemistry (IHC) or fluorescent in situ hybridization (FISH). Clinicians may also recommend treatment based on gene pattern expression tests such as Oncotype DX or MammaPrint. These assays are a set of genes that have been found to predict recurrence or risk of breast cancers [57-60]. Thanks to improvements in these therapies 89.2% of patients diagnosed with breast cancer are surviving more than 5 years [42].

The three major treatment options for breast cancer are surgery, radiation and chemotherapy. There are two major types of surgery, breast sparing mastectomy (lumpectomy) or total mastectomy. There are also two forms of radiation therapy to destroy cancer cells. Radiation can be administered by either an external beam, where radiation is focused from a machine outside the body onto the affected area, or brachytherapy where the patient receives internal radiation by pellet implantation. DCIS patients have breast sparing surgery followed by radiation therapy. These patients may also take tamoxifen to reduce the risk of relapse. Patients with stage I, II, IIIA and operable IIIC will usually have breast sparing surgery followed by radiation therapy.
Stage IIIB, inflammatory and inoperable IIIC breast cancer will have surgery and chemotherapy followed by radiation therapy. Patients with Stage IV breast cancer will receive chemotherapy and radiation. In stage IV the treatments are unlikely to cure the disease, but can help prevent metastases and prolong life. Breast cancer metastases usually occur in the bones, liver, lungs and brain.

1.4.3 Chemotherapy

For the purpose of this thesis, chemotherapy will remain the focus of breast cancer therapy. There are three major types of chemotherapy; cytotoxic, hormonal and targeted. Patients can either receive neoadjuvant therapy (treatment before surgery) or adjuvant therapy (after surgery). Neoadjuvant therapy may help to shrink the tumor to increase surgical success rates and recently the Federal Drug Administration has included a new approval process for drugs in the neoadjuvant setting using pathological complete response as an endpoint to fast-track drug approval [61].

Cytotoxic chemotherapy is used to chemically kill cancer cells. This type of treatment is usually not specific to tumor cells, and therefore is toxic to all rapidly dividing cells, including blood cells, cells in the hair root and cells that line the digestive tract. Most patients receive cytotoxic chemotherapy. Recent advances in treatment modalities allow cytotoxic chemotherapy to be given in a lower dose in combination with other types of therapy to reduce the toxic side effects. Doxorubicin (Adriamycin) is a cytotoxic
chemotherapy that has been used for over 30 years and has been called the most potent of the FDA-approved cytotoxic chemotherapy drugs [62]. Doxorubicin is a non-selective class I anthracycline injected intravenously and metabolized by the liver. The mechanism of action is to bind to DNA associated enzymes such as topoisomerase enzymes I and II and intercalate with base pairs on DNA to cause DNA damage and cell death [63]. Unfortunately significant damage can occur to major organs with the use of the drug including cardiac toxicities [63]. To prevent this, drugs like doxorubicin must be combined with hormonal or targeted therapies. Doxorubicin will be used in Chapter 3 to show that the results are selective for kinase inhibitors and not general to all chemotherapies. Doxorubicin will also be examined in Chapter 4 in combination with trastuzumab and lapatinib for HER2 amplified breast cancer in the preoperative setting to determine if the combination can cause a pathological complete response.

Hormonal chemotherapy in breast cancer is meant to lower estrogen levels as hormone receptor positive tumors are dependent on estrogen. Aromatase inhibitors, such as letrozole, are a class of drugs that prevents the formation of estradiol, the female hormone produced by the ovaries. Along with this inhibitor, patients also can undergo a hysterectomy if they are pre-menopause to prevent the production of estrogens from the ovaries. Selective estrogen receptor modulators (SERMs) such as Tamoxifen, bind to estrogen receptors to prevent the binding of estrogen. Tamoxifen can also be used as prevention in women with high risk of developing breast cancer [64]. Hormonal therapy
revolutionized the way in which we treat breast cancer. Unfortunately expression of the hormone receptor is a requirement to receive this therapy and breast cancer subtypes such as Triple Negative Breast Cancer and some HER2 overexpressing tumors do not express hormone receptors. To solve this clinical disparity drugs against other common alterations in breast cancer were developed.

Targeted therapies have been designed to target abnormalities that basic scientists have discovered in breast cancer. This is the most active field of chemotherapy research due to the successes of drugs like trastuzumab. One of the most obvious targets is signaling pathway kinases. The first kinase inhibitor to hit the cancer market was imatinib, which was approved by the Federal Drug Administration (FDA) in 2001 for use in chronic myelogenous leukemia (CML) [15, 18]. With the market success of this tyrosine kinase inhibitor, hundreds of new small molecular inhibitors were developed and began to enter clinical trials for cancers. One of the most successful targeted therapies is trastuzumab (Herceptin). Trastuzumab is a monoclonal antibody developed at UCLA in collaboration with Genentech [65]. This drug blocks the effects of HER2 by binding to its domain IV of the extracellular component, disrupting receptor dimerization and downstream signaling, thus killing the cancer cells [66]. Trastuzumab can also harness the immune system to target the cancer via antibody dependent cell-mediated cytotoxicity [67]. Lapatinib (Tykerb) is also a HER2 targeted therapy but instead of binding to the extracellular domain, it binds to the ATP binding pocket of the tyrosine kinase domain on
the cytoplasmic portion [68]. This drug is currently FDA approved for use in metastatic breast cancer in combination with other drugs. Chapter 4 of the thesis will examine if patients receive a benefit from dual anti-HER2 therapy (lapatinib and trastuzumab) in the neo-adjuvant setting by performing a meta-analysis with pathological complete response as the outcome.

There are other drugs that were used in experimental section of this thesis that are not currently approved for use in breast cancer. First, in Chapter 2 and 3, U0126 was used as a small molecular inhibitor of MEK1, part of the MAPK pathway. This drug is used as a model for generic MAPK inhibition to establish proof of principle. Unfortunately U0126 has a low bioavailability and cannot be used clinically [69]. Another kinase inhibitor in Chapter 3 is flavopiridol (Alvocidib). Flavopiridol is a semisynthetic flavonoid originally isolated from the stem bark of the Indian plant *Dysoxylum binectariferum* and is a pan-cyclin dependent kinase (CDK) inhibitor with greatest affinity for CDK9 [70, 71]. Flavopiridol was the first CDK inhibitor to enter clinical trials and many phase I and II trials have been conducted with flavopiridol including trials for multiple myeloma, melanoma, and endometrial adenocarcinoma with little success [70]. Many patients on these clinical trials developed a life threatening condition called tumor lysis syndrome and were forced to withdraw [72]. Recent work with flavopiridol has decreased the dose when used in combination with trastuzumab and another kinase inhibitor Sorafenib with promising results [73, 74]. In Chapter 5 a drug called trametinib (Mekinist) is used.
Trametinib is a MEK1 inhibitor recently FDA approved for metastatic melanoma patients and is currently under investigation in clinical trials for triple negative breast cancer [75].

1.4.4 Chemotherapy Resistance

Even with the advances in breast cancer therapies, resistance remains a huge problem and the major limitation to overall survival. It has been estimated that 30% of breast cancer will progress to a metastatic breast cancer with a 30-70% response rate to cytotoxic chemotherapy [76]. Even with an initial response to cytotoxic chemotherapy, there is a 6 to 10 month progression rate, yielding patients with limited treatment options [76, 77]. Because signaling pathways are so intertwined and compensatory pathways are often activated upon inhibition to another, resistance seems unavoidable. Finding ways to prevent resistance are in a sense a game of “Wac-A-Mole” where you start by treating with an inhibitor against a specific target, causing another to “pop up” [78]. If you move to that target, another one will pop up and so on and so forth. By studying cancers that have become resistant to a drug, it is possible to understand how they became resistant in the first place and either how to prevent the development of resistance or gain insight into the next “mole” to target.

There are two major types of resistance, innate and acquired [77]. Innate or de novo resistance requires that cancer cells already have the genetic make up to be resistant to the drug, whether that is a mutation, deletion, or overexpression of a certain gene.
Acquired resistance on the other hand, requires a change in the presence of a drug to overcome the drug’s mechanism of action. This can also come in the form of mutations, deletions and over-expression of genes, but it can also be altered phosphorylation of proteins and signaling pathways to circumvent the original inhibition. This circumvention can either reactive the once inhibited pathway, or it can activate a compensatory pathway to compensate for the inhibited pathway [79]. It is also possible to activate drug pumps such as multidrug resistance protein-1 or breast cancer resistance protein which can prevent the drug from entering the cell [80]. Proteins such as cytochrome P450, glutathione S-transferase, or aldehyde dehydrogenase can inactivate the drug once inside the cell preventing the drug from reaching its target [81]. A morphology change from epithelial to mesenchymal, can also alter the drugs ability by activating TGFβ and IL-6 [82].

1.5 JUNB and Cancer

JUNB is a transcription factor that plays a role in converting a pathway signal to gene expression. JUNB is also a member of the activator protein -1 (AP-1) family. As a member of this family, JUNB must form hetero-dimers with other family members such as FOS, ATF, Fra and other JUN proteins or homo-dimers with itself. Once a dimer is formed it binds to a DNA recognition sequence 5’-TGA[CG]TCA-3’ to regulate transcription in response to stress, virus and growth factors [83]. All of the AP-1 family members contain a conserved bZIP domain which contains a basic DNA-binding domain
and a leucine zipper region responsible for dimerization[83]. As a component of the AP-1 complex JUNB has been implicated in the control of fundamental cell programs including, cell survival, cell proliferation, programmed cell death and senescence [84-86]. JUNB is also a member of the immediate early genes (IEG), a class of transcription factors regulated by “paused” Pol II control [87]. The purpose of IEGs are to quickly respond to external environmental factors and most IEGs, especially cJUN, MYC and FOS are also aberrantly expressed in human cancers [88, 89]

The exact role for JUNB in cancer remains controversial, as expression and function of JUNB appear to be context and cancer specific. For example, JUNB is highly expressed in primary breast tumors compared to normal breast tissue [90]. JUNB is also among a subset of 27 genes up-regulated in primary inflammatory breast tumors [91]. JUNB is over expressed and associated with transformation, proliferation and invasion in human gastric cancer cell lines [92]. JUNB plays a well-established role as an oncogene in Hodgkin’s lymphoma and anaplastic large cell lymphoma [93]. Tight control of JUNB protein levels is critical as persistent JUNB expression during G2/M induces mitotic defects and genetic instability [84]. In addition, JUNB protects pancreatic beta cells from cytotoxic fatty acid and cytokine treatments by inducing the expression of pro-survival target genes including Bcl-xl, activating transcription factor 3 (ATF3), CEBPD and X-box protein1 (XBP1) [85, 94].

27
In addition to inducing the expression of anti-apoptosis related genes, JUNB has also been associated with autophagy. Limited reports suggest that JUNB inhibits autophagy in response to non-physiological serum and growth factor starvation \textit{in vitro} [95]. An extensive literature has accumulated in support of autophagy as a protective, pro-survival mechanism that promotes chemotherapy resistance [96]. Recent studies in chronic lymphocytic leukemia (CLL) cell lines and CLL patients demonstrate that FP induces a complex response that includes ER stress and a protective induction of autophagy that is associated with chemotherapy resistance [97]. In addition, direct evidence linking JUNB with chemotherapy resistance was reported in a global screening study in which JUNB was among a limited subset of 170 out of 15,906 human cDNAs tested for their ability to confer resistance to kinase inhibitor therapy [98].

1.6 Overall Goal of Dissertation

The overarching goal of the work described in this dissertation is to expand on the possible mechanisms of resistance to drugs, to uncover new possible therapeutic targets for breast cancer and to understand new therapy combinations to improve pathological complete response of patients. With so many mechanisms of resistance, it is easy to see why so many drugs fail in the clinical setting. It is important to understand how the drug works and sometimes why they don’t work in order to truly prevent breast cancer related deaths.
In chapter 2 our goal is to understand the signaling pathways activated by Oncostatin M that contribute to activation of RNA Polymerase II for epigenetic modulation in breast cancer cells. We also plan to investigate the Oncostatin M induced transcriptional control mechanisms that regulate JUNB expression in breast cancer.

In chapter 3 we investigate the ability of the kinase inhibitor flavopiridol to inhibit immediate early genes in human breast cancer cells. We will then investigate the functional role of JUNB as a pro survival protein against a lethal dose of flavopiridol. JUNB’s induction will then be investigated using other chemotherapeutic drugs.

In Chapter 4, we investigate the benefit of neoadjuvant chemotherapy plus the combination of lapatinib and trastuzumab on pathological complete response in HER2-amplified breast cancer patients, compared to neoadjuvant chemotherapy plus trastuzumab alone by conducting a meta-analysis of five randomized clinical trials. We will examine both definitions of pathological complete response as well as the effect of hormone receptor status on the dual anti-HER2 therapy.

In Chapter 5, we will attempt to develop the first trametinib resistant cancer cell lines. We will investigate the role of PTEN status on trametinib sensitivity and investigate the role of the PI3K /AKT pathway on the development of resistance. We will then examine
altered kinase signaling in trametinib resistant cells using a phospho-kinase antibody array.

It is my hope that the work provided here will provide insights into possible therapeutic targets, mechanisms of resistance, and possible ways to overcome this resistance to dictate future treatments for breast cancer patients.
Chapter 2: Mitogen Activated Protein Kinase signaling controls constitutive Oncostatin M mediated induction of JUNB gene expression

2.1 Introduction

In recent years there has been an increased focus on the pathological role of the microenvironment in promoting tumor development and metastasis [99, 100]. Cytokines, chemokines and growth factors secreted by the resident stroma and infiltrating cells in the tumor microenvironment activate cancer promoting intracellular signaling pathways and induce the expression of oncogenic target genes [101-103]. In breast cancer, the presence of infiltrating monocyte/macrophage populations has been well-documented and is associated with a poor prognosis [102]. Oncostatin M (OSM) is an IL-6 family cytokine that is secreted by monocytes and macrophages [104]. In vitro, OSM treatment is associated with breast cancer cell growth arrest, enhanced migration/invasion and the promotion of epithelial-to-mesenchymal (EMT)-like phenotypic changes [105-107]. In vivo, increased levels of circulating OSM and increased expression of the OSM receptor are associated with reduced recurrence-free and overall survival in breast cancer patients [108]. OSM activates multiple intracellular signaling pathways, including the MAPK and the signal transducer and activator of transcription3 (STAT3) pathways [107]. Co-activation of the MAPK and STAT3 pathways is common in human cancers but the
The mitogen activated protein kinase (MAPK) intracellular signaling pathway plays a major role in the cellular response to exogenous growth factors and cytokines [111, 112]. Aberrant activation of the MAPK pathway has been demonstrated in approximately 30% of human cancers, including breast cancer [88, 113]. Activation of the MAPK pathway is associated with the downstream activation of effectors that impact target gene transcription, including mitogen and stress-activated kinase1/2 (MSK1/2) and ELK1, a member of the ETS family of transcription factors [114]. Activation of the MSK1/2 is associated with the phosphorylation of key transcriptional regulatory factors including cyclic AMP response element binding protein (CREB) and histone H3 [88, 109, 114]. Alterations in histone H3 phosphorylation and post-translational modification status promotes the recruitment of pre-initiation complex components and the phosphorylation of proximal promoter-bound RNA Polymerase II (Pol II) on the carboxy terminal domain (CTD) at serine 5 (Pol IIser5p) by the TFIIH-associated kinase cyclin dependent kinase 7 [88]. The presence of Pol IIser5p in the immediate downstream region of a gene transcription start site (TSS) is an indicator that a gene is in the early stages of transcription [115]. Pol IIser5p is converted to the active, transcription elongating form of Pol II by phosphorylation on serine 2 (Pol IIser2p) of the Pol II CTD by cyclin dependent...
kinase 9 (CDK9), a component of the positive transcription elongating factor b (pTEFb) complex [115].

Activation of the MAPK signaling pathway results in the transcription of a wide range of cellular genes including “immediate early genes” (IEGs) [116, 117]. IEGs are rapidly and transiently expressed in response to external ligands [87, 88]. Many IEGs function as transcription factors and play a major reprogramming role in the initial cellular response to extracellular ligands [87].

JUNB is among the IEGs that are expressed in response to MAPK activation [118-120]. JUNB is a member of the activator protein1 (AP1) complex and JUNB has been implicated in the control of fundamental cell programs including cell survival, cell proliferation, programmed cell death and senescence [84-86]. Tight control of JUNB protein levels during the cell cycle is critical as persistent JUNB expression during G2/M induces mitotic defects and genetic instability [84]. The role of JUNB in cancer is complex with reports describing both oncogene and tumor suppressor functions [95]. In breast cancer, JUNB is highly expressed in primary breast tumors compared to normal breast tissue [90]. JUNB is also among a subset of 27 genes up-regulated in primary inflammatory breast tumors[91]. JUNB is over expressed and associated with transformation, proliferation and invasion in human gastric cancer cell lines [92]. In malignant pleural mesothelioma, an extremely aggressive human cancer, JUNB is among
13 genes included in a “mesothelioma gene” signature that has been implicated in mesothelioma pathogenesis and resistance to chemotherapy [121]. The oncogenic role of JUNB has been extensively reported in Hodgkin’s lymphoma and anaplastic large cell lymphoma [93]. Taken together, the available evidence supports an oncogenic role for JUNB in a number of human cancers.

The goal of this study was to investigate the intracellular signaling pathways and transcriptional control mechanisms that regulate JUNB expression in transformed human mammary epithelial cells. The results demonstrate that MCF10A<sup>met</sup> and SKBR3 human breast cancer cell lines express elevated levels of JUNB compared to nontransformed MCF10A cells. OSM treatment further augments JUNB expression in human breast cancer cell lines. OSM treatment induces a cyclic activation of MAPK signaling that is associated with a parallel cyclic induction of JUNB gene expression. In constitutive JUNB over expressing breast cancer cells, treatment with the MEK inhibitor U0126 reduces JUNB expression and also reduces the association between Pol II<sup>ser5p</sup> (“transcription initiating” form of Pol II) and the JUNB proximal promoter. In OSM treated breast cancer cells, U0126 treatment reduces OSM induction of Pol II<sup>ser5p</sup> binding to the JUNB proximal promoter and also reduces JUNB expression. Inhibition of MAPK signaling with U0126, however, did not reduce OSM-mediated STAT3 activation or the association of STAT3 with the JUNB proximal promoter. These results demonstrate the primary role of MAPK signaling in the control of the sequence of
molecular interactions that result in the positioning/activation of Pol II in association with the JUNB proximal promoter and JUNB gene expression.

2.2 Methods

Cell culture

The MCF10A nontransformed human mammary epithelial cells were cultured in complete growth media (G) containing DMEM/F12 supplemented with 5% horse serum (HS), 10μg/ml bovine insulin, 10ng/ml epidermal growth factor, 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml Fungizone. MCF10A<sup>met</sup> cells, derived from the MCF10A cell line were cultured in 5% HS, 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml Fungizone. SKBR3 and MDA-MB-231 cells were cultured in DMEM, 10% fetal bovine serum (FBS), 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml Fungizone. To induce growth arrest, near confluent cells were cultured in media deficient in serum and growth factors (growth arrest medium, A, 0.1% serum). Cells were cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Oncostatin M was suspended in sterile water and used at a concentration of 25 ng/ml.

Real Time PCR

Total RNA was isolated using the Roche High Pure RNA Isolation Kit (Indianapolis, IN) according to the manufacturer’s instruction. Total RNA (2μg) was used for reverse transcription using the Roche transcriptor first strand cDNA synthesis kit using both
oligo-dT and random hexamer primers. The reverse transcription products were amplified by Real-time PCR using the Roche LightCycler® 480 II Real-Time PCR System. Amplification was performed in a total volume of 20 μL containing 10 μL of a Roche Light Cycler 480 SYBR Green 1 master mix, 1 each of forward and reverse primers and 5 μL cDNA and 3 μL RNase free water in each reaction. PCR specificity was verified by assessing the melting curves of each amplification product. Total JUNB primers were 5’-CACCAAGTGCCGGAAGCGGA-3’ and 5’-AGGGGCAGGGGACGTTCAGA-3’ and JUNB 5’-TSS primers are 5’-TCCCCCTCCCCACGCTCGAG-3’ and 5’-GCGTCGTTTCCCCAGCTCC-3’. The fold change in specific mRNA levels was calculated using the comparative CT (ΔΔCT) method [122]. Results presented as mean ± SEM of the fold changes derived from three experiments with triplicate analyses performed for each treatment. Statistical analysis of data was carried out by one way ANOVA with Dunnett’s test or the Student’s t test where the fold change in mRNA levels was considered significant at p<0.05. Primer sequences are in Table 1.

Western blot analysis

Cells were rinsed with PBS, scraped from the plastic plate with a rubber policeman, and lysed in RIPA buffer containing protease, kinase and phosphatase inhibitors for 30 minutes at 4°C. Total cell lysates were isolated by centrifugation and the soluble supernatant was collected and protein levels quantified by BCA microprotein assay kit (Pierce). Protein lysates (30μg) were resolved by sodium dodecyl sulfate-polyacrylamide
gel electrophoresis (SDS-PAGE), transferred to Immobilon-P PVDF membrane (Millipore), and membranes were blocked for 1hr with PBS containing 10% non-fat dry milk and 0.5% Tween 20. Membranes were incubated with PBST containing 5% non-fat dry milk and the following primary antibodies JUNB (1:1,000, Cell Signaling), pSTAT3 (Tyr705) (1:2500, rabbit, Cell Signaling), STAT3 (1:3000, rabbit, Cell Signaling), pERK (1:5,000, rabbit, Cell Signaling), ERK (1:1,000, rabbit, Cell Signaling), or β-actin (1:2000, rabbit, Cell Signaling). After washing with PBS, membranes were probed with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:10,000, Cell Signaling) for 1 hr. Membranes were developed using ECL plus chemiluminescence detection reagent (Pierce). Results are representative of 3 independent experiments.

**Chromatin Immunoprecipitation (ChIP) Assay**

ChIP assays were carried out essentially as described [123]. Briefly, ChIP experiments were performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit and protocols (Upstate, Biotechnology, Lake Placid, NY). Briefly, cells were cross-linked with 1% formaldehyde (10 minutes, 37 °C), washed 2X with PBS (4°C), pelleted by centrifugation and re-suspended in 600 µl SDS lysis buffer supplemented with protease, kinase and phosphatases inhibitors. Genomic DNA was sonicated (Biorupter) to ~300-500bp fragments (verified by agarose gel analysis). Sonicated lysates were centrifuged to remove debris, diluted 1:10 in dilution buffer and immunoprecipitated using a Salmon Sperm DNA/Protein A Agarose Slurry. Immunoprecipitation was performed with RNA
Polymerase II phospho ser 5 (Abcam), RNA Polymerase II phospho ser 2 (Abcam) antibodies and pSTAT3 (Cell Signaling). After immunoprecipitation, pellets were washed with 1 ml Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer and LiCl Immune Complex Wash Buffer and TE buffer. Bead precipitates were eluted twice with fresh elution buffer (1% SDS, 0.1 M NaHCO3), pooled, and heated at 65 °C for 4 hours to reverse protein-DNA crosslinks. DNA was purified by phenol extraction and ethanol precipitation. RT-PCR analysis (≈35 cycles) was performed with 5 µl of the 50 µl DNA preparation plus the appropriate primers. PCR amplification products were analyzed by agarose gel electrophoresis. Real Time PCR analysis was performed on ChIP isolated DNA using The Applied Biosystems Step One PLUS Real Time PCR System. Results presented are representative of one to five independent experiments performed with 2-3 replicates per experiment or are presented as % input ± standard error of the mean.

2.3 Results

*JUNB expression is elevated in human breast cancer cell lines*

Previous studies investigating JUNB expression levels and functional activity indicate that JUNB can function as an oncogene or a tumor suppressor gene depending on the cell context [124]. We used real time PCR analysis to investigate JUNB expression in MCF10AΔmet and SKBR3 breast cancer cell lines and compared to nontransformed MCF10A cells. The results indicated that JUNB mRNA levels were 2-5 fold higher in
metastatic breast cancer cell lines compared to nontransformed MCF10A cells (Figure 3A). Preclinical and clinical studies demonstrate that OSM promotes breast cancer cell migration and invasion and elevated serum OSM and breast cancer cell OSM receptor expression is associated with a poor prognosis [106-108]. To assess the influence of OSM treatment on JUNB gene expression we treated MCF10A\textsuperscript{met}, SKBR3 and MDA-MB-231 human breast cancer cell lines with OSM (25ng/mL, Peprotech, 30 minutes). OSM induced JUNB mRNA levels 3–14 fold in the three breast cancer cell lines (Figure 3B). These results indicate that JUNB is constitutively expressed in human breast cancer cell lines, relative to a normal mammary epithelial cell line, and that JUNB gene expression can be further induced by OSM treatment, an exogenous cytokine associated with breast cancer progression.

**OSM activates a cyclic pattern of MAPK signaling**

OSM activates multiple intracellular signaling cascades including MAPK and STAT3 pathways [105, 107]. Previous reports indicate that the JUNB proximal promoter contains a STAT3 consensus binding site, STAT3 activates JUNB gene expression and JUNB is among the genes included in an oncogenic STAT3 gene signature in human tumors [125, 126]. In addition, MAPK signaling has been shown to activate a signaling network that promotes the activation of Pol II in association with the JUNB promoter [88, 118-120]. To determine the effects of OSM on intracellular signaling we assessed MAPK and STAT3 activation in MCF10A\textsuperscript{met} cells at selected time points from 0 to 24 hours
following OSM treatment. The results demonstrated that phosphorylated STAT3 (pSTAT3) levels increased 30 minutes after OSM treatment and then gradually declined over the next 24 hours (Figure 4A). MAPK signaling was assessed by determining phosphorylated extracellular receptor kinase (pERK) levels. pERK levels increased at 30 minutes, decreased at 1 hour, then increased and remained detectable for 24 hours (Figure 4A). The influence of OSM on JUNB mRNA levels was assessed with the initial induction of JUNB mRNA detectable 30 minutes after initiating OSM treatment (Figure 4B). JUNB mRNA levels then declined at 2 hours post OSM treatment, and increased at 4, 8 and 16 hours before declining slightly at the 24 hour time point (Figure 4B). These results demonstrate that following the initial synchronous induction of pSTAT3 and pERK, the pSTAT3 and pERK activation profiles diverge, with pSTAT3 activation levels gradually declining over the 24 hour OSM treatment period and pERK levels exhibiting an oscillating activation pattern. These results demonstrate a difference in the regulation and suggest a difference in the functional roles of the two OSM-induced signaling pathways. JUNB mRNA levels are also rapidly induced following exposure to OSM. After the initial induction, however, JUNB mRNA levels exhibit a cyclic response over the 24 hour OSM treatment that more closely follows pERK activation rather than pSTAT3. A similar, cyclic induction pattern was also observed for CEBPD mRNA levels in response to OSM (data not shown). CEBPD is a well-established STAT3 target gene [105, 127, 128]. These results demonstrate a variable induction of JUNB mRNA levels that suggests a greater dependence on MAPK rather than pSTAT3 signaling.
Inhibition of MAPK signaling reduces JUNB gene expression and reduces the recruitment/activation of Pol II on the JUNB proximal promoter.

Aberrant activation of the MAPK pathway is common in a number of human cancers, including breast cancer [109, 111-113]. To investigate the role of MAPK pathway activation in controlling JUNB expression MCF10A<sup>met</sup> cells were treated with U0126, a MAPK pathway inhibitor that specifically inhibits MEK1. Treatment of MCF10A<sup>met</sup> cells with U0126 inhibited MAPK signaling as evidenced by the reduction in pERK levels (Figure 5A). In contrast, pAKT levels were unaffected; indicating that short term U0126 treatment had no effect on AKT pathway activity (Figure 5A). U0126 mediated inhibition of MAPK signaling resulted in the reduction of JUNB protein levels (Figure 5A). To determine if MAPK inhibition influenced JUNB at the mRNA level MCF10A<sup>met</sup> cells were treated with U0126 and JUNB mRNA levels were assessed by RT-PCR and by real time PCR. The results demonstrated that U0126 treatment reduced JUNB mRNA levels (Figure 5BC). The time course in which the JUNB mRNA content declined in response to U0126 treatment approximated the JUNB mRNA t½, (data not shown). This suggests that inhibition of MAPK signaling reduces JUNB gene expression at the level of transcription. Next, we determined the role of MAPK signaling on the association of Pol II<sup>Ser5p</sup> (transcription “initiating” form of Pol II) and Pol II<sup>Ser2p</sup> (transcription “elongating” form of Pol II) with the JUNB gene proximal promoter. The results demonstrated that U0126 inhibition of MAPK signaling significantly reduced the
association between Pol IIser5p and Pol IIser2p with the JUNB proximal promoter (Figure 5D). These results are consistent with a model in which the MAPK signaling pathway controls the recruitment and/or activation of Pol II in association with the JUNB proximal promoter.

Inhibition of MAPK signaling reduces the recruitment/activation of Pol II on the JUNB proximal promoter.

Exogenous cytokines in the tumor microenvironment promote breast cancer pathogenesis [103]. As a product secreted by infiltrating monocytic cells, OSM activates the MAPK pathway, induces breast cancer cell EMT, and increases migration [106, 108]. To investigate the role of MAPK signaling in response to OSM, MCF10A<sup>met</sup> cells were pretreated with the MEK inhibitor U0126 for 2 hours prior to OSM treatment. U0126 pretreatment blocks OSM mediated MAPK activation, but has no effect on pSTAT3 levels (Figure 6A). Importantly, U0126 pretreatment reduces OSM mediated induction of JUNB protein levels (Figure 6A). At the mRNA level, U0126 treatment reduces JUNB mRNA levels by ~70% compared to untreated MCF10A<sup>met</sup> controls (Figure 6B). OSM treatment significantly induces JUNB mRNA levels (~5fold), but U0126 pretreatment effectively blocks OSM-mediated induction of JUNB mRNA levels (Figure 6B). The expression of CEBPD, a well-established STAT3 target gene [105, 128-130] is also blocked by U0126 pretreatment (data not shown). These results demonstrate that inhibition of MAPK signaling blocks OSM-mediated induction of JUNB gene
expression. To further address the mechanism in which U0126 inhibits OSM-mediated
*JUNB* gene expression we performed Pol IIser5p and pSTAT3 ChIP assays. The results
demonstrated that U0126 reduces the association of Pol IIser5p with the JUNB proximal
promoter region by ~50% (Figure 6C). OSM increases Pol IIser5p association with the
JUNB proximal promoter region by ~5fold, however, pretreatment with U0126,
dramatically reduces OSM-induced Pol IIser5p association with the JUNB proximal
promoter region (Figure 6C).

OSM induces a significant increase in pSTAT3 levels and *JUNB* gene expression (Figure
6AB). ChIP assays demonstrated that OSM increased the association of pSTAT3 with the
JUNB proximal promoter region by approximately 5 fold, consistent with the
aforementioned role of pSTAT3 as a transcriptional activator of *JUNB* gene expression.
Remarkably, U0126 pretreatment further increased the levels of pSTAT3 associated with
the JUNB proximal promoter region to ~20fold compared to controls (Figure 6D). Despite
significant accumulation of pSTAT3 in association with the JUNB proximal promoter region, inhibition of MAPK signaling blocked *JUNB* gene expression. These
results indicate that the MAPK pathway controls *JUNB* expression by regulating the
complex sequence of events that result in the association of Pol IIser5p with the JUNB
proximal promoter region. Inhibition of the MAPK pathway blocks OSM induction of
*JUNB* gene expression but does not reduce pSTAT3 levels or the association of pSTAT3
with the JUNB proximal promoter region. The results are consistent with a model in
which OSM-mediated JUNB gene expression is primarily controlled by MAPK signaling at the level of Pol II recruitment or activation in association with the JUNB proximal promoter.

2.4 Discussion

In this report we demonstrate that JUNB expression is elevated in metastatic MCF10A\textsuperscript{met} and SKBR3 cells. These results are consistent with previous reports and suggest that constitutively elevated JUNB expression could contribute to aberrant growth in breast cancer cells. In addition to promoting growth, persistent JUNB expression could contribute to breast cancer pathogenesis by inducing mitotic defects and genetic instability[84]. Aberrant JUNB expression in MCF10A\textsuperscript{met} cells may be associated with an increased frequency of genetic alterations that was reported in the early studies of the MCF10A\textsuperscript{met} (MCF10CA1) cell line [131]. In addition to promoting cell growth and genetic instability, aberrant JUNB expression is associated with alterations in mammary epithelial cell morphology and the formation of multiacinar-like structures [132]. Taken together, these results suggest a number of pathologic mechanisms in which persistent JUNB expression could promote the development and malignant progression of transformed mammary epithelial cells.

The results reported in this work demonstrate that JUNB expression is “super-induced” in breast cancer cells by exposure to OSM, an IL-6 family cytokine that is secreted by
monocytic cells that infiltrate breast tumors [102, 133]. OSM activates STAT3 and JUNB is among a 12 gene cohort of STAT3 target genes that mediate STAT3-induced oncogenesis in human cancers, including breast cancer [105, 126, 129]. In addition, expression of the OSM receptor in primary breast tumors is associated with a poor prognosis [107]. Interestingly, OSM treatment induced an oscillating pattern of JUNB expression that more closely paralleled MAPK activation than STAT3 activation. CEBPD mRNA levels also fluctuated in response to OSM in a pattern that was similar to JUNB (data not shown). We previously reported that CEBPD gene expression is induced in mammary epithelial cells and breast cancer cells by pSTAT3 in response to IL-6 and OSM [105, 129]. We also reported that Pol II is recruited to the CEBPD gene proximal promoter region under nonexpressing conditions, consistent with “paused” Pol II transcriptional regulation [127]. The results from the current study are consistent with a model in which MAPK pathway activation promotes the enhanced recruitment or activation of Pol II in association with the JUNB proximal promoter. Increased STAT3 activation has been reported in a wide range of human cancers, including breast cancer, and a number of STAT3 inhibitors are in preclinical development and clinical trials [134]. Available evidence, however, suggests that reduced cell permeability and drug stability have limited the clinical utility of STAT3 inhibitors tested to date [135]. The results reported in this study suggest that MAPK inhibitors may be an alternate therapeutic avenue as a monotherapy, or in combination with STAT3 inhibitors, to inhibit STAT3–related oncogenesis.
U0126 inhibition of constitutive MAPK signaling in MCF10A \textsuperscript{met} cells significantly reduced JUNB expression and this reduction in JUNB expression correlated with reduced association of Pol II\textsuperscript{ser5p} with the JUNB proximal promoter. Inhibition of MAPK signaling could influence Pol II\textsuperscript{ser5p} association with the JUNB proximal promoter by reducing the recruitment/activation of pre-initiation complex components, such as TFIH/CDK7 or the pTEFb complex [119, 120]. In addition to influencing the recruitment and activation of pre-initiation complex components, MAPK signaling also promotes histone H1 and H3 phosphorylation and induces an “open” or “relaxed” chromatin structure [88, 114]. These results suggest that MAPK pathway inhibitors can negatively impact a wide critical range of events that affect Pol II recruitment/activation in association with the gene promoters.

The molecular processes that promote cancer cell migration and invasion are among the most pathogenic in cancer biology as ~90% of all cancer deaths are due to complications from the metastases, not the primary tumor [136]. Previous reports have demonstrated that OSM activates multiple intracellular signaling pathways, including MAPK and STAT3, and that OSM treatment increases the migration of human breast cancer cells [106, 107]. Our data demonstrate that inhibition of MAPK signaling is associated with a the reduced association of Pol II\textsuperscript{ser5p} with the JUNB proximal promoter and reduced JUNB expression, while having little or no effect on the activation of STAT3 or the
association of pSTAT3 with the \textit{JUNB} proximal promoter region. This novel observation demonstrates that MAPK signaling drives the primary events in \textit{JUNB} gene expression, ie, the recruitment or positioning of Pol II on the \textit{JUNB} proximal promoter and suggests a secondary role for STAT3 activation in the induction of \textit{JUNB} gene expression in breast cancer cells.
Figure 3. JUNB expression is constitutively elevated in human breast cancer cell lines

(A) JUNB mRNA levels were compared between MCF10A, MCF10A<sup>met</sup>, and SKBR3 cells respectively. * Indicates statistically different from control (MCF10A) (p<0.05). (B) MCF10A<sup>met</sup>, SKBR3 and MDA-MB-231 breast cancer cells were cultured under exponentially growing (G) conditions or treated with OSM for 30 minutes (O). JUNB expression was assessed by Real Time PCR. Data presented as mean +/- SD of three independent experiments performed in triplicate. * indicates statistical significance (p<0.05).
Figure 4. OSM induces JUNB expression and activates MAPK and STAT3 signaling.

(A) MCF10A<sup>met</sup> cell signaling was assessed by Western blot following OSM treatment for 0.5, 1, 2, 4, 8, 16, and 24 hours. (B) MCF10A<sup>met</sup> cells were treated with OSM for the indicated time (0, 0.5, 1, 2, 4, 8, 16, and 24 hours) and JUNB mRNA was assessed by Real Time PCR.
Figure 5. MAPK inhibitor U0126 reduces *JUNB* gene expression and Pol II association with the *JUNB* proximal promoter.

(A) MCF10A<sup>met</sup> cells were treated with vehicle (-) or U0126 (+) (50µM, 4 hours) and protein levels were assessed by Western Blot. (B) MCF10A<sup>met</sup> cells were treated with U0126 and JUNB mRNA levels were assessed by RT-PCR. (C) MCF10A<sup>met</sup> cells were treated with vehicle or U0126 and JUNB mRNA levels were assessed by Real Time PCR. The level of JUNB mRNA in U0126 treated MCF10A<sup>met</sup> cells are expressed as fold change compared to vehicle treated controls. (D) MCF10A<sup>met</sup> cells were treated with vehicle (-) or U0126 (+). ChIP assays were performed using primers that amplify a region within the JUNB proximal promoter. Results are expressed as “% Input”. “Input” was obtained from PCR amplification of MCF10A<sup>met</sup> genomic DNA isolated from vehicle or U0126 treated MCF10A<sup>met</sup> cells (positive control). “IgG” results were obtained from PCR amplification of MCF10A genomic DNA isolated from ChIP assays performed with non-specific rabbit IgG (negative control). Real Time PCR data presented as mean +/- SD of three independent experiments performed in triplicate; * p<0.05 vs control. Representative ChIP data presented as mean +/- SD of 3 independent experiments performed in triplicate; * p<0.05 vs control.
Figure 6. U0126 blocks OSM mediated MAPK induction, Pol II ser5p association with the JUNB proximal promoter and JUNB expression.

(A) MCF10A<sup>met</sup> cells were treated with vehicle (-), U0126 (+) (50µM, 4 hours), OSM (25 ng/ml, 30 minutes) or pretreated with U0126 (+) and then treated with OSM. Protein levels were assessed by Western Blot. (B) JUNB mRNA levels were assessed by Real Time PCR and expressed as fold change compared to vehicle treated controls. Real Time PCR data presented as mean +/- SD of three independent experiments performed in triplicate; * p<0.05 vs control. (C and D) MCF10A<sup>met</sup> cells were treated as described in panel A. ChIP assays performed using anti-Pol II ser5p antibodies (C) or anti-pSTAT3 antibody (D). Real Time PCR analysis was performed using primers that amplify a region within the JUNB proximal promoter. Results are expressed as “% Input”. “Input” was obtained from PCR amplification of MCF10A<sup>met</sup> genomic DNA isolated from untreated. “IgG” results were obtained from PCR amplification of genomic DNA isolated from ChIP assays performed with non-specific rabbit IgG (negative control). * p<0.05 vs vehicle treated control.
Chapter 3: JUNB promotes the survival of flavopiridol treated human breast cancer cells

3.1 Introduction

Breast cancer is the most common cancer diagnosed in women in the United States [137]. Treatment of breast cancer patients has shifted in recent years from cytotoxic chemotherapy to therapies that target hormone and growth factor receptors and oncogenic signaling pathway components [138]. Targeted therapies have contributed to the improved survival rates of breast cancer patients in recent years, but resistance to targeted therapies is common and remains a significant problem in the treatment of breast cancer patients [79, 138].

Cyclin dependent kinases (CDKs) are emerging as attractive targets for anti-cancer therapeutics due to their key roles in the control of cell cycle progression, cell survival and gene transcription [139, 140]. Results from a recent phase II clinical trial demonstrated a dramatic improvement in progression free survival of ER+ breast cancer patients treated with palbocyclib, a CDK4/6 inhibitor [140]. As a component of the positive transcription elongation factor b complex, CDK9 plays a key role in gene expression by phosphorylating the RNA polymerase II (Pol II) C-terminal domain (CTD) on serine 2 (Pol IIser2p) and activating transcriptional elongation [141, 142]. Flavopiridol
(FP) is a semisynthetic alkaloid that inhibits CDK9 phosphorylation of Pol II, dramatically reducing Pol II mediated transcription [71, 143]. FP was the first CDK inhibitor in clinical trials and FP has been extensively investigated in human lymphoma patients and more recently in pre-clinical and clinical studies in combination with trastuzumab and sorafenib in breast cancer [71, 73, 144].

Immediate early genes (IEGs) are rapidly and transiently expressed in response to extracellular ligands and cell stressors [87]. Aberrant expression of IEGs, such as MYC, JUNB and FOS has been linked to a variety of human cancers, including breast cancer [88, 145, 146]. In this report we investigated the influence of FP treatment on CDK9 phosphorylation of Pol II and IEG expression in human breast cancer cell lines. The results indicated that FP treatment initially reduces the Pol IIser2p levels as well as the expression of MYC, JUNB and CEBPD. In addition, FP treatment reduces the expression of MCL1, consistent with FP mediated programmed cell death [147]. We also demonstrate a unique delayed induction of JUNB gene expression in response to FP treatment that occurs in multiple human breast cancer cell lines. JUNB plays a well-established role as an oncogene in Hodgkin’s lymphoma and anaplastic large cell lymphoma [93]. In breast cancer patients JUNB was identified among a subset of 27 genes up-regulated in primary inflammatory breast tumors [91]. In this report we demonstrate that JUNB plays a pro-survival role in breast cancer cells. The delayed induction of JUNB in response to FP treatment extends to other kinase inhibitors
including sorafenib and U0126, but does not include cytotoxic chemotherapy, i.e., doxorubicin. These results demonstrate a unique induction of JUNB in response to kinase inhibitors that may be among the earliest events in the progression to treatment resistance.

3.2 Methods

Cell lines and reagents

The human breast cancer cell lines, SKBR3, MDA-MB-231, and MDA-MB-468 were purchased from ATCC and cultured in DMEM medium plus 10% FBS, 50 U/ml streptomycin, 50 U/ml penicillin, and 500 ng/ml Fungizone. MCF10CA1a (MCF10A<sup>met</sup>) human breast cancer cells were a kind gift from Dr. Tsonwin Hai (Ohio State University) and were cultured in DMEM-F12 supplemented with 5% horse serum, 100 U/ml penicillin, 100 U/ml streptomycin and 500 ng/ml Fungizone. Cells were plated at ~60% confluence prior to treatment with the designated doses of Flavopiridol (FP), Actinomycin D, U0126 (Sigma); Doxorubicin and Sorafenib (Selleckchem).

RNA Extraction and RT-PCR/Real Time PCR

Total RNA was isolated using the Roche High Pure RNA Isolation Kit. cDNA was synthesized using the Roche transcriptor first strand cDNA synthesis kit using both the oligo-dT and random hexamer primers. For Reverse Transcriptase PCR (RT-PCR) mRNA products were amplified (25-40 cycles) and the PCR products were
electrophoresed using a 1% agarose gel containing 0.01% ethidium bromide and imaged using an Alphalmager (Alpha Innotech). For Real Time PCR, the reverse transcription products were amplified using the Roche LightCycler® 480 II Real-Time PCR System with SYBR green mastermix. PCR specificity was verified by assessing the melting curves of each amplification product. The fold change in specific mRNA levels was calculated using the comparative CT (ΔΔ\text{CT}) method [148]. The PCR primers (Sigma) are as follows: JUNB (forward: 5′-CACCAAGTGGCGGAAGCAGGA -3′; reverse: 5′-AGGGGCAAGGGACGTTTCTAGA-3′), CEBPD (forward: 5′-AGAAGTTGGAGCTGTCG-3′; reverse: 5′-GGATGGGTCGTTGCTGAGT-3′), MYC (forward: 5′-AGAAGTGGTTGGAGCTGTCG-3′; reverse: 5′-GGATGGGTCGTTGCTGAGT-3′), MCL1 (forward: 5′-AGAAGTTGGAGCTGTCG-3′; reverse: 5′-GGATGGGTCGTTGCTGAGT-3′), GAPDH (forward: 5′-AGAAGTGGTTGGAGCTGTCG-3′; reverse: 5′-GGATGGGTCGTTGCTGAGT-3′), β-ACTIN (forward: 5′-GCTCGTCGACCAACGGGCTC-3′; reverse: 5′-CAAACATGATCTGGGTCATCTCTTCTC-3′).

Western blotting

Cell lysates were harvested using RIPA buffer containing protease, kinase, and phosphatase inhibitors. Protein lysates (30μg) were resolved by SDS-PAGE and transferred to Immobilon-P PVDF membrane (Millipore). Membranes were blocked with
5% milk in Tris-buffered saline with 1% Tween-20 (TBST). Anti-JUNB (1:1,000, Cell Signaling), anti-RNA Polymerase II Ser2p (1:1,000, Abcam) anti-cyclin D1 (1:5,000, Cell Signaling), anti-cleaved caspase 3 (1:1,000, Cell Signaling), anti-Tubulin (1:1,000, Cell Signaling), anti-β-actin (1:2000, rabbit, Cell Signaling) antibodies were diluted in 5% milk-TBST overnight. After washing with TBST, membranes were probed with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:10,000, Cell Signaling) (1 hour). Membranes were developed using Pierce ECL 2 western blotting chemiluminescence substrate (Thermo Scientific).

**siRNA based knockdown assay**

JUNB siRNA interference was induced with short interfering RNA (siRNA) (Dharmacon) directed against the human JUNB gene. Controls received non-targeting siRNAs (NC siRNA) (Dharmacon). siRNAs were transfected into cells using the Amaxa Nucleofector II and Amaxa transfection kits (Lonza). Following siRNA nucleofection, cells were re-plated and cultured in complete media for 12 hours followed by FP treatment.

**Crystal Violet staining**

MCF10A^met^ breast cancer cells were grown to ~60% confluence and treated with JUNB or control siRNA, allowed to rest for 12 hours, and treated with FP for 24 hours. Cells were harvested, washed and stained with 0.5% crystal violet in 20% methanol.
Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of the fold changes for three independent experiments. Differences between samples were analyzed by t-tests using JMP. P < 0.05 was regarded to be statistically significant. Multiple treatment analyses were performed by one way ANOVA with Dunnett’s test or the Student’s t test where the fold change in mRNA levels was considered significant at p<0.05.

3.3 Results

*JUNB* mRNA levels, exhibit a delayed induction in response to Flavopiridol (FP) treatment.

To determine the influence of FP on IEG expression MCF10A*met* cells were treated with FP and the expression of selected IEGs (JUNB, CEBPD and MYC) was assessed. FP treatment reduced *JUNB*, *CEBPD* and *MYC* mRNA levels (Figure 7A). In addition, FP treatment reduced *MCL1* mRNA levels, which is a marker for FP response (Figure 7A). The initial decline in mRNA levels was maintained after 4 hours of FP treatment for all genes except *JUNB*. After an initial decline, JUNB mRNA levels dramatically increased at 4 hours post FP treatment (Figure 7A). Co-treatment of MCF10A*met* cells with FP + Actinomycin D blocked the delayed induction of JUNB mRNA levels, suggesting that the re-emergence of JUNB mRNA was mediated at the level of transcription (Figure 7B).
The delayed induction of JUNB mRNA levels is also reflected in JUNB protein levels in FP treated MCF10A<sup>met</sup> cells (Figure 7C). These results demonstrate that JUNB mRNA levels are uniquely induced in MCF10A<sup>met</sup> cells despite the effective FP mediated reduction of Pol IIser2p levels.

*JUNB mRNA levels increase despite FP mediated reduction in Pol IIser2p levels*

FP is a highly selective CDK9 inhibitor [149]. To determine if FP treatment resulted in reduced levels of Pol IIser2p the levels Pol IIser2p were assessed in FP (300 nM) treated MCF10A<sup>met</sup> cells. The results indicate that FP treatment reduces Pol IIser2p levels at every time point (2-24 hours) (Figure 8A). To determine if the effects of FP treatment on JUNB mRNA levels extended to additional human breast cancer cell lines JUNB mRNA levels were assessed in FP treated MCF10A<sup>met</sup>, SKBR3, MDA-MB-231 and MDA-MB-468 human breast cancer cell lines. FP treatment induced an initial decline in JUNB mRNA levels which were followed by the delayed induction of JUNB mRNA levels in all five human breast cancer cell lines (Figure 8B-E). The delayed induction of JUNB in response to FP treatment varied from ~5-12 fold (Figure 8B-E). MYC mRNA levels were not induced by FP treatment in any of the breast cancer cell lines tested demonstrating the relative specificity of the delayed induction response for JUNB (Figure 8B-E).
**JUNB plays a pro-survival role in FP-treated MCF10A^met cells**

JUNB expression has been linked to the regulation of fundamental cell programs including cell cycle progression, genetic stability, programmed cell death and autophagy [84, 95, 124, 150]. To assess the potential role of JUNB on MCF10A^met cell proliferation JUNB levels were reduced by siRNA knockdown and cyclin D1, an indicator of cell proliferation was assessed. JUNB siRNA treatment had no effect on cyclin D1 levels, indicating that reducing JUNB levels did not alter cell proliferation (Figure 9A). We next verified that FP treatment induced apoptosis by treating MCF10A^met cells with FP and assessing cleaved caspase 3 levels. Cleaved caspase 3 was detected as early as 6 hours after FP treatment and cleaved caspase 3 was clearly visible after 12 hours of FP treatment (Figure 9B). To assess the role of JUNB in FP-induced apoptosis MCF10A^met cells were treated with JUNB siRNA or noncoding siRNA and the induction of cleaved caspase 3 was assessed in response to FP (300nM). The results demonstrated that treatment with JUNB siRNA effectively reduced JUNB protein levels but reducing JUNB expression alone did not induce caspase 3 cleavage (Figure 9C). In contrast, reducing JUNB protein levels was associated with a marked induction of cleaved caspase 3 in response to FP treatment compared to noncoding siRNA treated MCF10A^met controls (Figure 9C). We next assessed the survival of JUNB siRNA treated MCF10A^met cells exposed to FP using crystal violet staining. The results documented the increase in FP mediated cell death in JUNB siRNA treated MCF10A^met cells compared to controls
Collectively, these results demonstrate that JUNB plays a pro-survival role in FP treated MCF10A<sup>met</sup> cells.

*JUNB is induced by kinase inhibitors (sorafenib and U0126) but not cytotoxic chemotherapy (doxorubicin).*

To determine if the FP-mediated induction of JUNB extended to therapeutic doses of small molecule inhibitors targeting kinases not directly associated with transcription we assessed JUNB expression in MCF10A<sup>met</sup> cells treated with sorafenib (multi-kinase inhibitor) and U0126 (MEK1 inhibitor). Both sorafenib (10µM) and U0126 (50 µM) treatments induced an initial decline in JUNB mRNA levels, followed by an induction in JUNB mRNA levels after 24 hours (Figure 10AB). To determine if the induction of JUNB mRNA levels is an obligatory component of the response of MCF10A<sup>met</sup> cells to chemotherapy treatments, MCF10A<sup>met</sup> cells were treated with the anthracycline, doxorubicin. Doxorubicin is commonly used in the treatment a number of breast cancers [151]. The mechanism of action of doxorubicin involves DNA intercalation and inhibition of topoisomerase 2 [152]. The results demonstrated that doxorubicin treatment (1µM) does not induce JUNB (Figure 10C). These results suggest that the delayed induction of JUNB is specific to small molecule kinase inhibitors and does not extend to cytotoxic drugs like doxorubicin.


3.4 Discussion

In this report we demonstrate the delayed induction of JUNB in response to FP, a CDK9 inhibitor. The unique induction of JUNB expression under conditions in which Pol IIser2p levels are reduced and gene expression levels are significantly diminished suggests that transcription complex components retain the capacity to associate with the JUNB proximal promoter in FP treated cells. Transcription of the JUNB gene is regulated by a “paused” Pol II control mechanism in which the Pol II transcription complex “pauses” ~50 nucleotides downstream from the transcription start site in a complex that includes two “pause factors”, DSIF (DRB Sensitivity Inhibitory factor) and NELF (Negative Elongation Factor) [119, 153]. Recent studies indicate that HSP90 stabilizes NELF within the transcription complex in a gene specific manner [154]. This suggests that HSP90 could regulate the selective expression of JUNB by influencing NELF stability and pausing function [155]. In addition, as a member of the AP-I family of transcription factors, JUNB induces the expression of Cyclophilin40 (CYP40), an HSP90 co-chaperone protein [156]. Co-chaperones promote the interaction between chaperone proteins and their targets [155]. The delayed induction of JUNB that occurs at ~4 hours after the initiation of FP treatment and continues for 24 hours (last time point taken) could result from the delayed release of the HSP90 stabilized NELF/“paused” Pol II complex. The kinase responsible for the phosphorylation events that result in the delayed induction of JUNB gene expression has not been identified but could include kinases
capable of phosphorylating the Pol II CTD on serine 2, including Brd4 or CDK12 [141, 157, 158].

JUNB plays a key role in the pathogenesis of anaplastic large cell lymphoma but a functional role for JUNB in breast cancer has not been previously reported [159]. This study reveals a novel profile of JUNB induction that is delayed by several hours from the putative initiating event (small molecule kinase inhibitor treatment) and is also prolonged (~24 hours). A delayed, “biphasic” induction of JUNB in response to cytokine and FP treatments has been previously reported but the biological significance of this novel JUNB expression pattern was not investigated [149, 160]. Functional studies reported herein demonstrate that JUNB plays a pro-survival role in breast cancer cells in response to a lethal dose of FP. This is plausible as previous reports demonstrated that JUNB activates the transcription of survivin (BIRC5) an “inhibitor of apoptosis protein” that promotes cell survival [36]. In addition, JUNB protects pancreatic beta cells from cytotoxic fatty acid and cytokine treatments by inducing the expression of pro-survival target genes including Bcl-xl, activating transcription factor3 (ATF3), CEBPD and X-box protein1 (XBP1) [85, 94]. The results from this study are consistent with a role for JUNB mediated activation of an anti-apoptotic gene program that promotes cell survival in response to kinase inhibitor treatment.
In addition to inducing the expression of anti-apoptosis related genes, JUNB has also been associated with autophagy. Limited reports suggest that JUNB inhibits autophagy in response to non-physiological serum and growth factor starvation in vitro [95]. An extensive literature has accumulated in support of autophagy as a protective, pro-survival mechanism that promotes chemotherapy resistance [96]. Recent studies in chronic lymphocytic leukemia (CLL) cell lines and CLL patients demonstrate that FP induces a complex response that includes ER stress and a protective induction of autophagy that is associated with chemotherapy resistance [97]. In addition, direct evidence linking JUNB with chemotherapy resistance was reported in a global screening study in which JUNB was among a limited subset of 170 out of 15,906 human cDNAs tested for their ability to confer resistance to kinase inhibitor therapy [98]. JUNB transcriptional activity is activated by Jun N-terminal Kinase (JNK) phosphorylation, suggesting that combination therapy that includes the JNK inhibitor SP600125 may reduce the development of chemotherapy resistance [161, 162]. In breast cancer patients, treatment resistance underlies disease recurrence, metastases development and a poor prognosis [163]. The results from this study demonstrate the novel induction of JUNB as an early event in the progression to treatment resistance (Figure 11).
Figure 7. JUNB is induced by flavopiridol (FP) treatment.

(A) MCF10A\textsuperscript{met} cells were treated with FP (300 nM) for 1, 2, or 4 hours. JUNB, CEBPD, MYC and MCL1 mRNA levels were assessed by Real time PCR. GAPDH was used as the loading control. (B) MCF10A\textsuperscript{met} cells were treated with FP (300 nM) without or with Actinomycin D (10 µg/mL). JUNB mRNA levels were assessed at 0, 0.5, 1, 2 and 4 hours by Real Time PCR. β-actin was used as the RNA loading control. (C) MCF10A\textsuperscript{met} cells were treated with FP (300 nM) for 0, 1, 2, 4 and 8 hours. JUNB protein levels were assessed Western blot. β-actin was used as the loading control.
Figure 8. JUNB is induced in multiple human breast cancer cell lines in response to flavopiridol treatment.

(A) MCF10A<sub>met</sub> cells were treated with FP (300 nM), a highly selective CDK9 inhibitor. Pol IIser2p and JUNB protein levels were assessed at 0-24 hours by Western blot. β-actin was used as the loading control. (B-E) JUNB and MYC mRNA levels were assessed at 0-24 hours after FP (300 nM) treatment by Real Time PCR. Data is presented as fold change in mRNA levels in FP treated cells compared to pretreatment (0 time) mRNA levels.
Figure 9. JUNB plays a pro-survival role in flavopiridol treated MCF10A<sup>met</sup> cells.

(A) MCF10A<sup>met</sup> cells were nucleofected with JUNB siRNA, Non-coding siRNA (NC-siRNA) or no siRNA and cultured in complete growth media for 24 hours. JUNB and Cyclin D<sub>1</sub> protein levels were assessed by Western blot. β-actin was used as the loading control. (B) MCF10A<sup>met</sup> cells were treated with vehicle or FP (300nM) for the designated times. Cleaved Caspase 3 protein levels were assessed by Western blot. β-actin was used as the loading control. (C) MCF10A<sup>met</sup> cells were nucleofected with NC-siRNA or JUNB siRNA, and then treated with vehicle or FP (300nM) for 12 hours. Cleaved Caspase 3 and JUNB protein levels were assessed by Western blot. Tubulin was used as the loading control. (D). Cells were stained with crystal violet and photographed.
Figure 10. JUNB gene expression is induced by treatment with sorafenib and U0126, but not doxorubicin.

(A-C) MCF10A<sup>met</sup> cells were treated with Sorafenib (10 µM), U0126 (50 µM) or Doxorubicin (1 µM). JUNB and MYC mRNA levels were assessed at selected time points (0-24 hours) after drug treatments by real time PCR. Data is presented as fold change in mRNA levels in drug treated cells compared to pretreatment (0 time) mRNA levels.
Figure 11. JUNB plays a role in survival of breast cancer cells treated with kinase inhibitors.

Breast cancer cells treated with the kinase inhibitors sorafenib, U0126 or flavopiridol, have an increase in JUNB production which functions to protect breast cancer cells from dying. This may be an early event in chemotherapy resistance.
Chapter 4: Neoadjuvant dual HER2-targeted therapy with lapatinib and trastuzumab improves pathologic complete response in patients with early stage HER2-positive breast cancer. A meta-analysis of randomized prospective clinical trials

4.1 Introduction

HER-2/neu is a 185 kilodalton trans-membrane protein that belongs to the human epidermal growth factor receptor (HER) family. When activated by homodimerization or by hetero dimerization with other HER family proteins (HER-1, HER-3 and HER-4) it signals via RAS and phosphoinositide 3 kinase (PI3K) transduction pathways leading to transcription of multiple genes responsible for cell proliferation, survival, invasion, metastasis and angiogenesis [164-166]. HER-2/neu is over-expressed by HER-2 gene amplification on chromosome 17 in approximately 18-25% of all breast cancer patients [167]. Historically, HER-2/neu amplified breast cancer has been associated with higher risk of disease recurrence, worse disease free and overall survival and resistance to endocrine therapy [164, 168, 169]. With the development of trastuzumab, a chimeric monoclonal antibody to the extracellular region of HER-2/neu protein, the outcome of patients with HER-2/neu positive breast cancer improved dramatically. Objective response rates of 50-60% were observed in patients with untreated metastatic, HER-2/neu positive breast cancer. These responses translated to improvement in progression free and
overall survival to about 9-12 months and 25-30 months respectively [170, 171]. Improvement in disease free and overall survival by about 35%-65% and 33%-50% respectively was seen in randomized, phase III clinical trials that tested addition of trastuzumab to adjuvant chemotherapy for HER-2/neu positive operable breast cancer [172-175].

In the recent years, multiple other inhibitors of HER-2/neu protein have been developed including small molecule tyrosine kinase inhibitors, other monoclonal antibodies and antibody-drug conjugates [176]. Lapatinib is an orally bioavailable dual inhibitor of intracellular portion of HER-2/neu protein and epidermal growth factor receptor (EGFR) and has been perhaps the best studied of these in clinical trials to date. Lapatinib has been shown to result in improved progression free survival in patient with previously treated metastatic breast cancer when added to standard palliative chemotherapy (capecitabine) from 4.4 to 8.2 months [177]. When combined with trastuzumab, lapatinib was found to prolong both median progression free survival (from 9.5 to 14 months) compared to treatment with lapatinib alone in heavily pre-treated patients with metastatic, HER-2/neu over-expressing breast cancer [178].

Given the promising results of clinical trials in metastatic, HER-2/neu positive breast cancer, multiple studies have been conducted to evaluate the clinical benefit of adding lapatinib to standard, trastuzumab containing chemotherapy for operable HER-2/neu
positive breast cancer in both the neo-adjuvant and adjuvant setting. Given that pathological complete response (pCR) is generally considered to be a predictor of improved disease specific survival in breast cancer patients (especially in patients with HER-2/neu positive and triple negative breast cancer), multiple neo-adjuvant trials were conducted to study the degree of improvement in pCR rate with the addition of lapatinib to standard, trastuzumab containing neoadjuvant chemotherapy (NAC) [179-181]. Unfortunately, these studies revealed mixed results. The Neo-ALTTO trial tested the addition of lapatinib to standard weekly paclitaxel with trastuzumab. It showed a near doubling of pathologic complete response rate from 29.5% to 51.3% (p<0.0001) [182]. However, other large randomized neo-adjuvant trials only showed a trend towards improvement in pCR rate when the combination of NAC with trastuzumab and lapatinib was compared to standard NAC with trastuzumab alone which did not reach statistical significance [183, 184].

Given the mixed results of the above clinical trials, we have conducted a meta-analysis of published or presented RCTs that compared pCR in patients with HER-2/neu positive breast cancer treated with NAC and trastuzumab alone or NAC and trastuzumab plus lapatinib. The main objective of the analysis was to evaluate whether the combination of lapatinib plus trastuzumab and NAC leads to a statistically significant improvement in pCR rate as defined by absence of residual invasive breast cancer in the breast and lymph nodes. As a secondary outcome we also used an alternative definition of pCR (no residual
invasive breast cancer in the breast only) and as a subgroup analysis we assessed the
degree of improvement in pCR rates in patients with HR+ vs. HR- breast cancer.

4.2 Methods

Literature search strategy and study criterion

To review the relevant literature, we searched Pubmed using clinical trials published in
English with the key words “HER2”, “breast cancer” and “neoadjuvant” between January
1998 and January 2014. Abstracts from San Antonio Breast Cancer Symposium (SABCS,
www.sabcs.org) and American Society of Clinical Oncology (ASCO, www.asco.org)
annual meetings between 2009 and 2013 were also queried. This search was performed
independently by 3 authors (SF, RW and EM) and any disagreement in the selected
studies was resolved by consensus. Efforts were made to contact investigators when
relevant data was unclear [183, 185].

Data extraction, quality control, and risk of bias

Data extracted from relevant studies was conducted independently by three investigators
(MA, MH, EM) according to the Preferred Reporting Items for Systematic Reviews and
Meta-Analysis statement [186], any discrepancies were resolved by consensus. For each
study we collected the following information: first author’s name, year of publication,
trial phase, definition of pCR (no invasive cancer in the breast only vs. no invasive breast
cancer in breast and axillary lymph nodes), hormone receptor status, stage of breast
cancer included, number of enrolled patients, chemotherapy backbone, treatment arms, and duration and schedule of neo-adjuvant chemotherapy plus trastuzumab with or without lapatinib. To assess the validity of the studies included, we examined the randomization procedure, sample size, blinding procedure, loss to follow-up, drop-out, and the intention to treat. Jadad scores were used to assess the quality of each study [187].

**Outcome Definition**

The primary objective of our study was to compare the rate of pCR following NAC plus trastuzumab with or without lapatinib. Treatment with lapatinib alone was excluded from the analysis due to toxicities and sufficient evidence that single agent lapatinib plus NAC has inferior efficacy compared to NAC with trastuzumab or trastuzumab and lapatinib combination [182-184]. The definition of pCR varied between the studies. Some studies defined pCR as no invasive disease in breast tissue alone while others defined pCR as no invasive disease in both breast tissue and axillary lymph nodes at the time of surgery. For the purpose of our analysis, we used pCR in the breast and lymph nodes as our primary endpoint. We also performed secondary analysis using different pCR definition (no invasive breast cancer in the breast) and difference in pCR rate in patients with hormone receptor positive (HR+) vs. hormone receptor negative (HR-) breast cancer. Given incomplete information on DCIS, it was not used as part of the definition of pCR. We
feel that this is acceptable given that residual DCIS was not accounted for in pCR definition in multiple published studies [188-190].

Statistical analysis

For the calculation of pCR incidence, the number of patients achieving pCR and the total number of patients in each treatment group were extracted from the selected studies for both definitions of pCR respectively. The proportion of patients achieving pCR and the 95% confidence intervals (CI) were derived for each treatment group for both definitions of pCR from each trial. We calculated summary pCR incidence rates and confidence intervals for each treatment group and pCR definition respectively using random-effects meta-analysis modeling utilizing the DerSimonian and Laird method that considers both within-study and between-study variations [191].

The pooled estimates for the main effect of adding lapatinib to trastuzumab containing NAC for both definitions of pCR and the subgroup analysis were calculated from random-effects meta-analysis models using the DerSimonian and Laird method. Results were reported as pooled odds ratios and 95% confidence intervals with the patients receiving trastuzumab alone as the control group. Hypothesis tests comparing the difference in treatment groups for each definition of the outcome were conducted and two-tailed p-values < 0.05 were considered statistically significant. We assessed
statistical heterogeneity among trials included in the meta-analysis using the $I^2$ statistic [192] that estimates the percentage of total variation across studies due to heterogeneity rather than chance. We considered an $I^2$ value of greater than 50% as indicative of substantial heterogeneity.

A pre-specified subgroup analysis was performed to determine if the effect of adding lapatinib to trastuzumab containing NAC differed between hormone receptor positive versus hormone receptor negative patients. The difference in the odds ratios for the two subgroups was tested and a two-tailed p-value <0.05 was considered statistically significant.

Finally, publication bias was evaluated through funnel plots, and with Begg’s and Egger’s tests [193, 194]. Statistical analyses were performed using RevMan 5.2, and Stata/SE version 11.0 software (Stata Corp, College Station, TX) (Review Manager (RevMan) [Computer program]. Version 5.2. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012).

4.3 Results

Population Characteristics

The original search yielded a total of 1804 potentially relevant HER-2/neu neoadjuvant breast cancer studies: 706 abstracts from PubMed and 1098 from ASCO and SABCS.
meetings. The detailed selection process is presented in Figure 12. After evaluating each study for eligibility, we initially excluded 1773 studies. The remaining 31 studies were carefully screened and an additional 17 were excluded as duplicates. Two studies were excluded as they did not report pCR as their primary endpoint [195, 196], 2 studies were excluded because they had no outcome data [197, 198] and 5 studies were excluded because they did not contain a combination lapatinib and trastuzumab arm [199-203]. Five eligible randomized trials were retrieved, four were peer-reviewed reports [182, 184, 185, 204] and one was an abstract from ASCO [183]. Of the 5 trials, two were phase II trials [185, 204] and three were phase III trials [182, 184, 204]. For the CALGB 40601 study [183], number of patients who achieved pCR was assumed from the percent of patients who achieved a pCR relative to the number of total patients.

For the primary outcome of pCR (breast and lymph nodes), 767 patients were available for the analysis (384 for the dual therapy arm and 383 for trastuzumab alone arm). For the secondary outcome of pCR (breast only), 887 patients were available for the analysis (441 for the dual therapy arm and 446 for trastuzumab alone arm). For studies that reported both pCR definitions, pCR (breast and lymph nodes) were a subset of the pCR (breast only) patients. Six hundred and forty nine patients were available for subgroup analysis of pCR (breast only) based on hormone receptor status. Baseline characteristics of each trial are presented in Table 1. All trials included HER-2/neu positive breast cancer patients, with a diagnosis of operable breast cancer (no distant metastatic disease).
In all these trials, patients were randomly assigned to a control group (neoadjuvant chemotherapy plus trastuzumab) or to the treatment group (neoadjuvant chemotherapy plus trastuzumab and lapatinib). Treatments arms within trials were similar in terms of mean/median patient ages, hormone receptor status, nodal status and stage of breast cancer. Patients were required to have adequate baseline organ function and good performance status. They also had normal systolic heart function as assessed by baseline echocardiogram or multi-gated acquisition scan (MUGA scan). Chemotherapy agents given with lapatinib and trastuzumab included taxanes [182-185, 204], anthracyclines [184, 185, 204], Cytoxan [185, 204] and Fluorouracil [185, 204].

Trastuzumab was administered at a 4 mg/kg loading dose followed by 2 mg/kg in every study. All studies initially administered lapatinib at a dose of 1500 mg orally (PO) once daily. Three studies reduced the lapatinib dose from 1500mg to 1250 mg to limit toxicities (diarrhea, neutropenia, transaminase elevation, and rash) in the lapatinib alone arm [184, 185, 204]. When lapatinib was used in combination with trastuzumab, it was initially administered at 1,000 mg in 4 studies then reduced to 750mg due to unacceptable toxicities [182, 184, 185, 204]. One study reported 750 mg lapatinib from the time of study onset for the combination anti-HER-2/neu therapy [183]. Two studies reported using the intent to treat population in their analysis [182, 183], while 2 studies reported different populations because patients were lost due to not having breast surgery [184], protocol violations or withdrawal of consent [204]. In studies where large proportion of
patients did not undergo breast surgery analysis was performed only on a selection of patients who underwent the surgery and received at least 75% of chemotherapy [185].

Among the five trials included in this analysis, 2 studies included only a pCR definition of no invasive disease in the breast and lymph nodes (our primary objective) [185, 204], 1 study included only a pCR definition of no invasive breast cancer in the breast only (our secondary objective) [183], and two studies included both definitions of pCR and a subgroup of pCR definition of no invasive disease in the breast only using hormone receptor status (subgroup analysis) [182, 184]. Because only two studies reported a pCR definition of no invasive disease in the breast and lymph nodes by hormone receptor status, one reported useable numbers [184], and one reported only percentages [185], a subgroup analysis could not be done based on that definition of pCR.

**Primary Outcome: Incidence of pCR in Breast and Lymph Nodes**

Among the five trials included in this analysis, a total of 767 patients (384 patients in lapatinib plus trastuzumab arm and 383 patients in trastuzumab arm) from four studies were analyzed for the effect of adding lapatinib to trastuzumab containing NAC on a pCR rate defined as no invasive breast cancer in the breast and lymph nodes [182, 184, 185, 204]. One study was excluded because only percentages of pCR, not absolute numbers were included [183]. The absolute pCR rate was estimated to be 38.36% (95% CI: 23.85% - 52.88%) in the trastuzumab arm and 55.76% (95% CI: 45.19% - 66.33%) in the
combination arm using random effect meta-analysis modeling. The odds of pCR in the breast and lymph nodes was 1.94 times higher for the combination arm than the trastuzumab arm (95% CI 1.44-2.60, p<0.0001; heterogeneity test: $p=0.55$; $I^2 = 0$%; Figure 13).

**Secondary Outcome: Incidence of pCR in Breast Only**

Data was available for pCR definition of no invasive disease in the breast for the comparison of trastuzumab versus the combination of trastuzumab and lapatinib in 3 trials (887 patients; 446 patients treated with trastuzumab and 441 patients treated with lapatinib plus trastuzumab) [182-184]. Two studies were excluded because pCR in the breast only was not evaluated [185, 204]. The absolute pCR rate was 40.70% (95% CI: 26.87% - 54.53%) in the trastuzumab arm in comparison with 55.01% (95% CI: 47.55% - 62.46%) in the combination arm. The probability to achieve pCR in the breast was higher for the combination arm than the trastuzumab arm (OR 1.78, 95% CI 1.27-2.50, $p=0.0007$; heterogeneity test: $p=0.22$; $I^2=35$%; Figure 14).

**Subgroup Analysis: Incidence of pCR on Hormone Receptor Expression**

Two trials [182, 184] (649 patients; 326 patients treated with trastuzumab and 323 patients treated with lapatinib plus trastuzumab) also reported the effect of hormone receptor status on pCR rates (defined as no residual invasive cancer in breast only) for the comparison of trastuzumab versus the combination of trastuzumab and lapatinib. One
study was excluded as it did not examine HR status [204] and two studies were excluded as they reported HR+ and HR- subgroups as percentage pCR with no absolute numbers [183, 185]. The pCR rate for HR+ patients was 34.76% (95% CI: 11.18% - 58.33%) in the trastuzumab arm in comparison with 48.87% (95% CI: 35.16% - 62.57%) in the combination arm. The absolute pCR rate for HR- patients was 50.80% (95% CI: 22.42% - 79.219) in the trastuzumab arm in comparison with 67.19% (95% CI: 55.74% - 78.64%) in the combination arm. The odds of achieving pCR in the breast are 1.76 times higher for the combination arm than the trastuzumab arm in the hormone receptor positive subgroup (OR 1.76, 95% CI 1.06-2.93, p=0.03; heterogeneity test: p=0.23; I²=29%; Figure 15). The odds of achieving pCR in the breast was 2.06 times higher for the combination arm than the trastuzumab arm in the hormone receptor negative subgroup (OR 2.06, 95% CI 1.08-3.91, p=0.03; heterogeneity test: p=0.21; I²=37%; Figure 15). The odds ratios for the HR+ and HR- subgroups did not differ significantly (p=0.71).

Quality of the Study and Publication Bias

All trials included in this meta-analysis were randomized multicenter, open-label phase II/III trials. No evidence of publication bias was detected for the odds of pCR in breast and lymph nodes by treatment regimen using Egger’s test (p=0.365) Begg’s test (p=0.99). Additionally, no evidence of publication bias was detected for the odds of pCR in breast only by treatment regimen using Egger’s test (p=0.856) or Begg’s test (p=0.99).
4.4 Discussion

To our knowledge, this is the largest and most current report to show a significant increase in the pCR rate with the use of dual-HER-2/neu targeted therapy with lapatinib in combination with trastuzumab in early stage HER-2/neu amplified breast cancer. The incidence of pCR (breast and lymph nodes) with lapatinib plus trastuzumab was 55.76% (95% CI 45.19% - 66.33%) compared to 38.36 % (95% CI 23.85% - 52.88%) in the trastuzumab only arms. Similar efficacy was seen in studies examining the pCR rate in the breast only with lapatinib plus trastuzumab. Using this definition, pCR rates were 55.01% (95% CI 47.55% - 62.46%) in the lapatinib and trastuzumab arm compared to 40.70% (95% CI 26.87% - 54.53%) in the trastuzumab only arm. There is an almost two fold increase overall in the odds of becoming free of invasive breast cancer in lapatinib plus trastuzumab-treated patients compared with controls.

Similar results were found regardless of pCR definitions, and the effect did not differ between hormone receptor positive and hormone receptor negative breast cancer subtypes. This remains a hypothesis-generating analysis, and caution should be used when interpreting these subgroups because of the limited sample size. Additionally, as expected, the degree of benefit from neo-adjuvant chemotherapy plus trastuzumab or the combination of trastuzumab and lapatinib appears to be higher in patients with hormone receptor negative breast cancer (pCR rate of 50.80% (95% CI: 22.42 – 79.19) in the trastuzumab compared to 67.19% (95% CI: 55.74 – 78.64) in the dual therapy compared
to patients with hormone receptor positive breast cancer pCR rate of 34.76% (95% CI: 11.18 – 58.33) in trastuzumab arm and 48.87% (95% CI: 35.16 – 62.57) in the dual therapy arm. While not statistically significant, this result is consistent with multiple reports that showed overall higher sensitivity of chemotherapy in tumors with low or absent hormone receptor expression.

Inhibiting the function of HER-2/neu via multiple mechanisms is a well-known avenue of achieving durable responses in cancers driven by HER-2/neu amplification [176]. Valachis et al [205] reported an improvement in pCR when lapatinib was combined to trastuzumab in patients receiving neoadjuvant chemotherapy. All trials [182, 184, 185, 204] that used combination therapy with lapatinib and trastuzumab that were analyzed by Valachis et al were included in our meta-analysis. However, our study analyzed additional data from the phase III CALGB 40601 trial [183] which was not included in the publication by Valachis et al. Compared to our results, the investigators reported a pCR rate of 53% for the combination of lapatinib and trastuzumab [205]. By increasing the number of patients who received dual-HER-2/neu therapy with the addition of CALGB 40601 in our analysis, the pCR of combination therapy remained above 50% and significantly better than in the arm of patients with trastuzumab alone.

Dual HER-2/neu inhibition with pertuzumab and trastuzumab plus chemotherapy in the neoadjuvant setting is FDA approved based on the NEOSPHERE and TRYPHAENA trials [206, 207]. Likewise, blocking the HER-2/neu protein at multiple loci has been
shown to improve both progression-free and overall survival in the metastatic setting [208]. Despite our analysis and the data from multiple randomized clinical trials [182-185, 204], lapatinib plus trastuzumab has not received the same accolades leading to FDA approval as its pertuzumab counterpart [206, 207]. Reasons for this observation may be secondary to the modest efficacy data in conjunction with the increase in toxicity seen with the addition of lapatinib [182-185, 204, 209].

Most recently, the results of the ALLTO trial [210] where lapatinib was administered in combination with trastuzumab post-operatively for one year did not show an improvement in disease free and overall survival for patients with early stage HER-2/neu-amplified breast cancer. Importantly, the number of recurrence events from this trial was significantly smaller than anticipated. The data and results of the APT trial [211] suggests that the recurrence risk in the post-trastuzumab era may be extraordinarily small and that clinical trials attempting to improve on these already outstanding outcomes may be grossly underpowered. While the breast cancer community has rapidly adopted a dual-HER-2/neu targeted approach as superior to single-agent targeted therapy [176] we may be over treating a large number of patients with early stage, low risk breast cancer who would derive sufficient benefit from trastuzumab monotherapy alone in the adjuvant setting. The final disease-free and overall survival results of the neoadjuvant trials that utilized dual HER-2/neu inhibition may help elucidate the final role of this approach in early stage disease.
Despite the size of our analysis, there are several limitations to this study. Data were abstracted from published clinical trial results. Therefore, individual patient information was not available. Clinical verification of the amount of residual disease at the time of definitive surgery was not possible in this analysis. The studies included were conducted by multiple investigators at different institutions; therefore, pCR rates may not have been consistently reported across studies because of subjectivity and disparities in investigator interpretation. Similarly, the definition of pCR was not consistent through the studies, with 2 [185, 204] studies examining both the breast and lymph node status after surgery and one study [183] including only breast in the final definition of pCR, and two studies [182, 184] included both definitions of pCR. Lack of a standard definition of pCR rate could make cross-trial comparisons more difficult to interpret.

4.5 Conclusion

Patients with early stage HER-2/neu-positive breast cancer have a statistically significant increase in the odds of achieving pathologic complete response rate with the addition of lapatinib to trastuzumab when given with preoperative chemotherapy. While the difference in pCR between single agent trastuzumab versus lapatinib and trastuzumab is modest, the odds ratio is significant with an increase in the number of patients without disease at the time of their definitive surgery after exposure to dual HER2-targeted therapy. This is clinically relevant as we continue to learn more about the impact of pCR
on long term disease free survival. Although lapatinib combined with trastuzumab may be an important treatment strategy, the risk of increased toxicity [205] should be weighed against the potential of overall benefit. Long term disease-free and overall survival outcomes from these large randomized studies will be crucial to understanding the overall impact of this combination on early stage HER-2neu-positive breast cancer.
Figure 12. Selection process for the randomized controlled trials included in the meta-analysis.
<table>
<thead>
<tr>
<th>Source (Trial Name)</th>
<th>Phase</th>
<th>Chemotherapy Backbone</th>
<th>Duration (weeks)</th>
<th>Anthracycline Containing</th>
<th>Number of Participants Enrolled (n)</th>
<th>Number of Participants for Analysis (n)</th>
<th>Quality of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carey et al, 2013 (CALGB 40601) (^{109})</td>
<td>III</td>
<td>P [80 mg/m(^2)]</td>
<td>16</td>
<td>No</td>
<td>120</td>
<td>120</td>
<td>1</td>
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<td>118</td>
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<td>67</td>
<td>67</td>
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<tr>
<td>Robidoux et al, 2013 (NSABP B-41) (^{109})</td>
<td>III</td>
<td>AC [80 mg/m(^2), 600 mg/m(^2)] P [80 mg/m(^2)]</td>
<td>4 cycles chemo then 4 cycles P +AHT</td>
<td>Yes</td>
<td>181</td>
<td>171</td>
<td>3</td>
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<td></td>
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<td>174</td>
<td>171</td>
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<tr>
<td>Guarnieri et al, 2008 (CHERLOC) (^{109})</td>
<td>III</td>
<td>P [80 mg/m(^2)] than FEC [600 mg/m(^2)], 75 mg/m(^2), and 600 mg/m(^2)]</td>
<td>12 weeks then 4 courses</td>
<td>Yes</td>
<td>36</td>
<td>36</td>
<td>2</td>
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<td>38</td>
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<tr>
<td>Baselga et al, 2012 (NeoALTTO) (^{109})</td>
<td>III</td>
<td>P [80mg/m(^2)]</td>
<td>6 week run-in then 12 weeks AHT and P</td>
<td>No</td>
<td>149</td>
<td>149</td>
<td>3</td>
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<td>154</td>
<td>154</td>
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</tr>
<tr>
<td>Holmes et al, 2013 (^{109})</td>
<td>III</td>
<td>FEC75 [500 mg/m(^2), 75 mg/m(^2), 500 mg/m(^2)] then P [80mg/m(^2)]</td>
<td>AHT 2 week run-in then with 4 courses then p 12 courses</td>
<td>Yes</td>
<td>33</td>
<td>23</td>
<td>3</td>
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</tbody>
</table>

\(^{1}\) The lapatinib arm was closed when negative efficacy and toxicity data emerged from preliminary analysis of ALTTO.

\(^{2}\) On June 10, 2008 they reduced the starting dose of lapatinib to 1250 mg in the lapatinib group and 750 mg in the combination group because of the excessive diarrhea reported in other trials using the initial doses.

\(^{3}\) Lapatinib doses were reduced because of the occurrence of grade 2 diarrhea in 20% of patients in the lapatinib alone and 41% of patients in the combination group.

\(^{4}\) Lapatinib dose reduced from 1,000 mg to 750 mg when P was added to reduce the occurrence of diarrhea.

\(^{5}\) Lapatinib dose was reduced to reduce diarrhea after a safety review of the first 45 patients enrolled.

\(^{6}\) Study quality was assessed on the 7-item Jadad score, with a score range of 0 to 5.\(^{10}\)

\(^{7}\) Patients analyzed differed from intent to treat because they either-withdraw consent from study or did not have surgery.

\(^{8}\) Patients analyzed differed from intention to treat population due to selection of patients who received surgery and >75% chemotherapy.

Abbreviations: P=weekly paclitaxel, AC=doxorubicin and cyclophosphamide, FEC=5-fluorouracil, epirubicin, cyclophosphamide, AHT=anti-HER2 therapy.

Table 1. Characteristics of the Randomized Control Trials Included in the Meta-Analysis.
### Table 2. Incidence and Odds Ratio (OR) of pCR Stratified by Definition of pCR and Hormonal Status.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Trials</th>
<th>No. Patients achieved pCR</th>
<th>Total No. Patients</th>
<th>% pCR</th>
<th>95% CI</th>
<th>No. Patients achieved pCR</th>
<th>Total No. Patients</th>
<th>%pCR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Definition of pCR</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Breast and Lymph Nodes</td>
<td>4</td>
<td>209</td>
<td>384</td>
<td>55.7</td>
<td>6</td>
<td>45.19-66.53</td>
<td>150</td>
<td>383</td>
<td>38.36</td>
<td>23.85-52.88</td>
<td>1.94</td>
</tr>
<tr>
<td>Breast only</td>
<td>3&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>244</td>
<td>441</td>
<td>55.0</td>
<td>1</td>
<td>47.55-62.46</td>
<td>185</td>
<td>446</td>
<td>40.70</td>
<td>26.87-54.53</td>
<td>1.78</td>
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<tr>
<td><strong>Hormonal status</strong></td>
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<tr>
<td>Hormone Receptor Positive</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>92</td>
<td>185</td>
<td>48.87</td>
<td>35.16-62.57</td>
<td>74</td>
<td>197</td>
<td>34.76</td>
<td>11.18-58.33</td>
<td>1.76</td>
<td>1.06-2.93</td>
</tr>
<tr>
<td>Hormone Receptor Negative</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>92</td>
<td>138</td>
<td>67.19</td>
<td>55.74-78.64</td>
<td>63</td>
<td>129</td>
<td>50.80</td>
<td>22.42-79.19</td>
<td>2.06</td>
<td>1.08-3.91</td>
</tr>
</tbody>
</table>

*NOTE: The p value for the OR by Breast and Lymph Nodes =<0.001; heterogeneity test: p=0.55; I²=0%.
The p value for OR by Breast Only =0.0007; heterogeneity test: p=0.22; I²=35%.
The p value for the OR among the Hormone Receptor Positive sub group =0.03; heterogeneity test: p=0.23; I²=29%.
The p value for the OR among the Hormone Receptor Negative sub group =0.03; heterogeneity test: p=0.21; I²=37%.
There was no difference of effect of the dual therapy between Hormone Receptor Positive and Hormone Receptor Negative subgroups (p=0.71).
* Hormone receptor status was subcategorized in the breast only definition of pCR.
*<sup>b</sup> 1<sup>182</sup> studies reported breast only pCR, 2<sup>182,184</sup> studies reported only breast and lymph node pCR, and 2<sup>182,184</sup> studies reported both breast only and breast and lymph node path pCR rate.
Abbreviations: pCR, pathological complete response, breast only: no invasive disease remaining in the breast. Breast and lymph nodes: no remaining invasive disease in breast or lymph nodes,
Figure 13. Odds ratio of incidence of pathological complete response definition of no invasive disease in the breast and lymph nodes.

Abbreviation: T+L, trastuzumab plus lapatinib, T, trastuzumab, CI, confidence interval.
Figure 14. Odds ratio of incidence of pathological complete response definition of no invasive disease in the breast.

Abbreviation: T+L, trastuzumab plus lapatinib, T, trastuzumab, CI, confidence interval.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
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<th>Odds Ratio IV, Random, 95% CI</th>
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<td>Total events</td>
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Figure 15. Odds ratio of incidence of pathological complete response definition of no invasive disease in the breast based on hormone receptor status.

Abbreviation: T+L, trastuzumab plus lapatinib, T, trastuzumab, HR+, hormone receptor positive, HR-, hormone receptor negative, CI, confidence interval.

<table>
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<th>Study or Subgroup</th>
<th>T+L</th>
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90
Chapter 5: Characterization of Trametinib Resistance in Triple Negative Breast Cancer

5.1 Introduction

Due to advances in molecular profiling, breast cancer can now be split into four subtypes [53]. Each of these subtypes has unique characteristics and can be used to determine prognosis and outcome of patients [212]. TNBC is a basal-like breast cancer subtype that is characterized by the absence of expression of the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor (HER2) [213, 214]. TNBC patients lack the ER, PR or HER2 making them unresponsive to available targeted breast cancer therapies such as tamoxifen and trastuzumab [214]. TNBC is more prevalent in African American women and women of a younger age [215]. In addition, TNBC is often diagnosed at an advanced disease stage and as a result TNBC is associated with a poor clinical outcome [216]. Because of the poor prognosis, TNBC accounts for a disproportionately higher number of metastatic cases and breast cancer deaths. Less than 30% of TNBC patients live beyond 5 years [217].

Chemotherapy research has shifted in recent years from cytotoxic agents to targeted therapy. Targeted therapy is directed toward cells addicted to a particular oncogene in an attempt to inactive the oncogene’s effects and kill the cancer [218]. This therapeutic
approach reduces the impact of the treatment on “normal” cells that do not harbor the activated oncogene. Since TNBC patients are not candidates for targeted therapies, such as trastuzumab and tamoxifen, the primary treatment modality remains cytotoxic chemotherapy, resulting in a limited durable clinical response and almost uniform disease progression; thus underlying the significant need for TNBC targeted therapies [219].

The Cancer Genome Atlas (TCGA) analysis of TNBC tumors have identified that the majority of TNBC tumors have amplifications in the EGFR-RAS-RAF-MEK-ERK signaling pathway [220]. Unfortunately, in a clinical trial, cetuximab, a monoclonal antibody against EGFR/HER1, was found to be ineffective in TNBC patients [213]. Other EGFR targeted therapies have also been a disappointment in clinical trials, likely due to activation of downstream signaling pathways [221]. Interestingly, Hoeflich et al determined that TNBC cells with an activated RAS profile (downstream of EGFR) are more sensitive to treatment with a MEK inhibitor than other types of breast cancer [222]. In addition, treatment with the MEK inhibitor AZD6244 caused reduced tumor growth in TNBC cell lines and tumor xenografts [222].

As of November 2013 there are twenty-five kinase inhibitor drugs that are Federal Drug Administration (FDA) approved [223]. One of these, trametinib was originally developed by Japan Tobacco for the treatment of inflammation [224]. Trametinib, the only FDA approved MEK inhibitor, is a potent oral allosteric inhibitor of MEK1/2 [225, 226]. In the
first published phase III trial of trametinib, there was 81% overall survival after 6 months in advanced melanoma patients [227]. Trametinib was well tolerated with preliminary activity in solid tumors with 21 of 206 (10%) of patients having an objective response [228]. A patient with triple negative breast cancer had a complete response that was maintained for >2.5 years with the treatment of trametinib and an AKT inhibitor [75]. Trametinib is currently in phase I & II trials for use in pancreatic cancer, non-small cell lung cancer, colon cancer, and refractory leukemia [224].

As with most small molecule inhibitors, drug resistant tumors will eventually emerge and lead to disease progression. Given the heterogeneity of responses to kinase inhibitors, it is important to understand the mechanisms by which the tumors become drug resistant to design new therapeutic strategies to prevent or overcome these resistance mechanisms. Resistance mechanisms uncovered in clinical trials have been successfully modeled in drug-treated cell lines [229, 230]. This indicates that cell lines are an appropriate surrogate to study clinical drug resistance.

To date, no pre-clinical data for resistance to trametinib exists in TNBC. To investigate possible mechanisms of resistance to trametinib we have established drug-resistant cell lines derived from human triple negative breast cancer tumors that differ in PTEN status; wild type MDA-MB-231 and PTEN null MDA-MB-468 cells. Initially, the PTEN wild type cells (MDA-MB-231) were sensitive to trametinib with an IC$_{50}$ of ~25 nM. In
contrast, the PTEN null (MDA-MB-468) cells did not achieve an IC$_{50}$ up to 200 nM trametinib. Because of this 200 nM trametinib was used as the “resistance mark” and MDA-MB-468 were determined to be a near intrinsically resistant cell line, while the MDA-MB-231 developed resistance over a period of 6 months indicating acquired resistance. Trametinib resistant cells have an altered kinome profile with PRAS40 being the most activated kinase in both the intrinsic and acquired resistant lines and ERK1/2 being the most repressed kinase in the resistant lines. These results represent the first experiments to understand resistance to the MEK inhibitor trametinib in TNBC.

5.2 Methods

Cell culture/reagents

MDA-MB-231 and MDA-MB-468 were purchased from American Type Cell Culture Collection (ATCC, Manassas, VA). Both lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% Gibco Fetal Bovine Serum (FBS, Invitrogen) 100U/ml penicillin, 100μg/ml streptomycin and 500ng/ml Fungizone, at 37°C and 5% CO2. Trametinib was purchased from Active BioChem (CAT#: A-1258, Maplewood, NJ), suspended in DMSO and stored at -20°C in the dark.

Generation of trametinib-resistant TNBC cell lines

MDA-MB-231 and MDA-MB-468 cells were plated at 60% confluence in 10 cm dishes overnight. Cells were treated with 25, 50, 100 or 200 nM trametinib initially. Media and
drug were changed every other day for 6 months. Cells were defined as resistant after they could be continuously cultured in 200 nM trametinib, and their proliferation patterns were similar to that of the parental cell lines. Resistant lines are denoted by an “R” (231R and 468R).

Cell Viability
MDA-MB-231 and MDA-MB-468 cells (750 cells/well and 3,000 cells/well respectively) were seeded in triplicate in 96-well plates overnight in 10% FBS supplemented medium then incubated with DMSO or increasing concentrations of trametinib for 24, 48, 72, 144 hours. The medium was removed and the plates were frozen at −80°C overnight before processing with the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Cell proliferation was calculated as a percentage of the DMSO-treated control wells. Each experiment was repeated 3 times.

Cell Proliferation
MDA-MB-231 and 231R or MDA-MB-468 and 468R cells were plated at 1X10^4 cells in a 6 well plate plates. Cells were counted with a hemocytometer every day for 5 days. Proliferation index was calculated by cell count each day divided by original plating.
**Western Blotting**

Cells were rinsed with PBS, scraped from the plastic plate with a rubber policeman, and lysed in RIPA buffer containing protease, kinase and phosphatase inhibitors for 30 minutes at 4°C. Total cell lysates were isolated by centrifugation and the soluble supernatant was collected and protein levels quantified by BCA microprotein assay kit (Pierce). Protein lysates (30µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P PVDF membrane (Millipore), and membranes were blocked for 1hr with PBS containing 10% non-fat dry milk and 0.5% Tween 20. Membranes were incubated with PBST containing 5% non-fat dry milk and the following primary antibodies pERK (1:1,000, cell Signaling), ERK (1:1000, rabbit, Cell Signaling), pAKT (Ser473) (1:1000, rabbit, Cell Signaling), AKT (1:1000, rabbit, Cell Signaling), PTEN (1:500, rabbit, Cell Signaling), or β-actin (1:2000, rabbit, Cell Signaling). After washing with PBS, membranes were probed with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:5,000, Cell Signaling) for 1 hr. Membranes were developed using ECL chemiluminescence detection reagent (Pierce). Results are representative of 3 independent experiments.

**Human Kinase Antibody Array Kits**

A human phospho-Kinase Antibody Array (R&D Systems) was used to simultaneously detect the phosphorylation status of kinases (n=43) and two associated total proteins.
under normal and 200 nM trametinib resistant conditions. After incubating on ice for 20 min, cell debris was pelleted at 4°C. Lysates (200 μg protein) were applied to membranes overnight. Washing and antibody steps were performed according to the manufacturer's instructions. Kinase array images were analyzed using ImageJ (version 1.46r; http://rsbweb.nih.gov/ij). Fold change was calculated by either dividing the parental average mean pixel density by the resistant average mean pixel density to show repressed phosphorylation of kinases or resistant average mean pixel density by the parental average mean pixel density to show activation of phosphorylation of kinases. Kinase phosphorylation repressed in the resistant cells (higher in the parental cells) were arbitrarily given a negative value and positive values are given to kinases activated in the resistant lines (repressed in the parental lines) [231].

5.3 Results

*PTEN status can accurately predict sensitivity to trametinib in TNBC cell lines.*

To determine if PTEN can predict sensitivity to trametinib in TNBC, we used two TNBC cell lines; MDA-MB-231 (PTEN wild type) and MDA-MB-468 (PTEN null). TNBC cells were treated with 50, 100 or 200 nM trametinib and after 24 hours, neither MDA-MB-231 nor MDA-MB-468 cells showed reduced cell viability (Figure 16A). This result is consistent with other studies using trametinib [218, 232]. After 72 hours cells the PTEN wild type MDA-MB-231 cells showed a ~80% decrease in cell viability. This is in contrast to the PTEN null MDA-MB-468 cells, in which there was no change in cell viability.
viability after 72 hours (Figure 16B). These results suggest that TNBC cells with intact PTEN are sensitive to trametinib. Conversely, TNBC cells with loss of PTEN are less responsive to the MEK inhibitor trametinib.

_Treatment with trametinib reduces pERK and increases pAKT levels in both MDA-MB-231 and MDA-MB-468 cells_

To determine if the PI3K pathway is involved in sensitivity to trametinib, Western blotting was performed to assess cell signaling. As expected, short term treatment with 200 nM trametinib (24 hours) significantly decreased the phosphorylation of ERK1/2 to undetectable levels in both the MDA-MB-231 and MDA-MB-468 cell lines (Figure 16C) Short term trametinib treatment caused an increase in phosphorylation/activation of AKT. Total PTEN levels were unaffected by trametinib treatment in MDA-MB-231 cells (Figure 16C). These results suggest that short term treatment with trametinib in PTEN wild type TNBC cells causes an increase in pAKT signaling that appears to be independent of total PTEN expression. Cells that lack PTEN (MDA-MB-468) show no change in pAKT levels.

_Generation of TNBC cell lines with acquired and near intrinsic resistance to trametinib._

Resistance to small molecule inhibitors is common in cancer patients and is associated with disease recurrence and a poor prognosis [138]. Preclinical models of drug resistance provide both critical insights in to the cellular mechanisms that underlie drug resistance
and model systems to test second line therapies to circumvent resistance mechanisms [138]. To develop trametinib resistant TNBC cell lines MDA-MB-231 and MDA-MB-468 cells were treated with 25, 50, 100 or 200 nM trametinib. Media and drug were changed every other day. After ~11 days significant cell survival was observed in the MDA-MB-231 cells cultured in 25 nM trametinib. Higher concentrations (50-200nM) of trametinib were associated ~100% lethality of MDA-MB-231 cells. In contrast, MDA-MB-468 cells survived culture in doses of trametinib up to 100 nM. Over a period of 6 months, the trametinib resistant MDA-MB-231 cells were developed by gradually increasing the dose of trametinib in cell cultures from 25nM to 50nM, then 50nM to 100nM then finally to 200nM (Figure 17A). MDA-MB-468 could initially withstand 200 nM of trametinib until dying at day 20 (data not shown), therefore MDA-MB-468 cells were cultured in 100nM initially, and then increased to 200nM after 10 days (Figure 17A). This result suggests the MDA-MB-468 PTEN mutant cells exhibit a high degree of intrinsic resistance to trametinib. To confirm the development of trametinib resistant cell cultures, cells were continuously cultured in trametinib for more than 6 months until they could proliferate in the presence of 200nM trametinib in a similar manner to that of the parental cell line (Figure 17B/C). These results suggest that PTEN wild type cells (MDA-MB-231), while initially sensitive to trametinib, can develop resistance in response to dose escalation over a period of 6 months (231R). PTEN null cells (MDA-MB-468) tolerated a higher initial dose of trametinib and developed resistance to 200nM trametinib (468R) significantly faster than PTEN wild type cells.
Trametinib resistance is associated with activation of the pAKT in MDA-MB-231R cells. To investigate the influence of trametinib resistance on intracellular signaling pathways MAPK and PI3K pathway components were assessed in trametinib resistant TNBC cell lines. In contrast to results from studies with other MEK inhibitors, the TNBC cells resistant to 200 nM trametinib did not exhibit reactivation of phosphorylated ERK (pERK; Figure 17D) [233]. Naïve MDA-MB-231 cells treated with 200 nM trametinib for 24 hours did however, exhibit an increase in pAKT phosphorylation on Serine 473 that persisted in the cells resistant to trametinib (Figure 17D). Interestingly, PTEN levels were relatively unaffected by short term trametinib treatment, but decreased by trametinib resistance (231R; Figure 17D). As the MDA-MB-231 and MDA-MB-468 cell lines differ in their PTEN status they also differed in their ability to acquire resistance, indicating a possible difference in alterations of their proteomic and genomic signaling. Neither cell line re-activates ERK, suggesting growth/proliferation is not caused by re-activation of the MAPK pathway.

Trametinib resistant cells can withstand 32 times the initial MDA-MB-231 IC_{50} dose. To further characterize the degree of resistance the cells have developed against trametinib, TNBC cells were subjected to increasing doses (up to 32X the MDA-MB-231 original IC_{50} dose) of trametinib. MDA-MB-231 parental cells and 231R (Figure 17E) or MDA-MB-468 and 468R (Figure 17F) were treated with 0, 25, 50, 100, 200, 800nM
trametinib for 6 days. Parental MDA-MB-231 cells were sensitive to trametinib with an IC$_{50}$ value of ~25nM while the 231R resistant cells could withstand 800nM without effectively achieving 50% killing (Figure 17E). Neither MDA-MB-468 parental or 468R cells were responsive to any dose of trametinib after 6 days (Figure 17F). These results indicate the high level of resistance exhibited by these cells as cells resistant to 200 nM trametinib are also resistant to a 32 fold dose increase of trametinib.

*Trametinib resistance causes an altered kinome profile that is different between the intrinsically resistant TNBC cells and the TNBC cells that developed resistance over time.*

MDA-MB-231 and 231R (Figure 18A) or MDA-MB-468 and 468R (Figure 18B) TNBC cells were assessed using the Phospho-Kinase Antibody Array to determine if there is an altered signaling profile with trametinib resistance. Compared to parental MDA-MB-231 cells, 231R cells showed a 16-fold repression of pERK and a 4-fold repression of pCREB, a downstream target of MAPK signaling (Figure 18). In addition, 231R cells exhibited a 13-fold increase in phospho-PRAS40 levels and marked increases in HSP60 levels as well as increased, pAKT, pc-JUN and pWNK1. These differences were muted in MDA-MB-468R cells as the only major different was a 5-fold repression of pERK. These results indicate an alteration in kinase signaling between trametinib resistant and parental cells.
5.4 Discussion

The RAS-RAF-MEK-ERK pathway is the most altered pathway in cancer and an attractive pathway for therapeutic intervention [16]. TCGA analysis of TNBC tumors has determined that the majority have amplification in the EGFR-RAS-RAF-MEK-ERK signaling [212]. As a potent and specific inhibitor of MEK 1/2 trametinib has shown efficacy in clinical trials in patients with RAS/RAF mutant solid tumors [218]. In a phase I study of trametinib, a TNBC patient had a complete response when given trametinib in combination with an AKT inhibitor for more than 2.5 years [228]. In a study examining trametinib in cell lines, 9 out of 11 TNBC cell lines tested responded to trametinib [213]. To date, no pre-clinical or clinical studies have examined trametinib resistance.

A common mechanism of drug resistance is mutations in the target oncogene [234]. Naturally occurring mutations in MEK have not been found in human cancers, making MEK an attractive therapeutic target [235]. The first MEK inhibitor, CI-1040, entered phase I clinical trials in 2000. Since then, more than 10 MEK inhibitors have entered clinical trials, but only one MEK inhibitor, trametinib, has been approved for clinical use [223, 236].

Compared to other MEK inhibitors, trametinib has low inter-patient variability, prolonged half-life and small peak to trough ratios which allow trametinib to overcome
the narrow therapeutic index normally associated with MEK inhibition [228]. Typically MEK inhibitors cause an accumulation of phosphorylated MEK and increasing pMEK levels is associated with ERK reactivation upon drug removal [237, 238]. Duncan et al also demonstrated reactivation of ERK, showing that treatment with the MEK inhibitor AZD6244 resulted in aberrant activation of a number of receptor tyrosine kinases (140) and reactivation of ERK by a mechanism that bypassed MEK inhibition [233]. Previous studies in MDA-MB-231 cells using the MEK inhibitor PD325901 led to the discovery of a MEK mutation following drug treatment [239]. The presence of the MEK1L115P mutation prevented PD325901 from binding to its target on the MEK1 protein and allowed for continued phosphorylation of ERK [239]. Trametinib differs from these MEK inhibitors in that it binds to non-phosphorylated MEK and prevents phosphorylation (activation) of MEK [237, 240]. Results from this study confirm this mechanism of action as we found no evidence of ERK reactivation in our studies (see Figure17D/18A). These results indicate that the mechanism of trametinib resistance developed in TNBC cells in this study differs from previously characterized mechanisms.

It has been proposed that at least four genes can predict sensitivity to MEK inhibitors. In the context clinical medicine, further validation of these genes individually may be useful as biomarkers of susceptibility or resistance to a specific therapy. Mutations in KRAS, PIK3CA, BRAF, or PTEN have all been shown by several groups to confer resistance to the MEK inhibitors AZD6244, CI-1040, and PD0925301 [11, 16, 241]. This is consistent
at least in part with our results in that PTEN status can predict trametinib sensitivity in TNBC. It is, however, possible that a KRAS$^{G13D}$ mutation in addition to wild type PTEN may play a role in the sensitivity of the MDA-MB-231 cells [16]. Further experiments are needed to understand the role of KRAS and PTEN in trametinib resistance.

The present study is the first to characterize TNBC cells resistant to the MEK inhibitor trametinib. The MAPK and PI3K pathways are known to compensate for each other to promote cell proliferation, survival, and migration [242]. Because a) EGFR primarily signals through the MAPK pathway but can also activate the PI3K pathway to compensate for MEK inhibition [243], b) 30% of TNBC tumors have a loss of PTEN[244, 245] and c) loss of PTEN has been found to activate PI3K/AKT [10, 222, 235], the PI3K/AKT pathway was hypothesized to be the mechanism of resistance in these cells. This is consistent with reports that PTEN loss is associated with innate resistance mechanisms in cancer cells [79]. In our study, TNBC cells with wild type PTEN were more sensitive to trametinib than PTEN null cells. Although pAKT was up regulated in PTEN wild type cells after short term treatment, PTEN levels were unaffected indicating that short term treatment of trametinib caused an activation of AKT that was independent of PTEN’s ability to inhibit the PI3K/AKT pathway (Figure 16C). This is consistent with Halilovic et al, who found elevated pAkt levels in MEK inhibitor resistant colorectal, pancreas and lung cancer lines in the absence of a PIK3CA or PTEN mutation [241]. This suggests there are alternative ways to activate AKT that are
independent of PTEN. In the trametinib resistant cells, pAKT levels persisted, while PTEN levels seemed to decrease, indicating trametinib resistance may affect PTEN levels in the MDA-MB-231 cells.

Interestingly, in our broader analysis of signaling kinases, pAKT was the third most activated kinase. pPRAS40 and HSP60 were found to be most activated in the acquired resistance TNBC cell line followed by pAKT and p-c-JUN. Proline-rich AKT substrate of 40 kDa (PRAS40) is a cytosolic protein ubiquitously expressed [246]. This protein is known to be an integral intersection of the AKT and mTOR signaling pathways [247]. Association with 14-3-3 protein is crucial for insulin to stimulate mTOR via PRAS40 [248] as pretreatment of cells with PI3K -inhibitors prevented the insulin induced phosphorylation of PRAS40 and the ability of -PRAS40 to bind to 14-3-3 protein, causing insulin resistance [249]. Conversely, the MEK inhibitor PD98056 had no effect on PRAS40 [249, 250]. This indicates that PI3K, not MAPK, may be an important regulator of insulin sensitivity and therefore a target for insulin resistance. This result may be extended to chemotherapy resistance, as PRAS40 was significantly up-regulated in both trametinib resistant cell lines (Figure 18A/B). Overexpression of pPRAS40 attenuated apoptosis in neuronal cells after ischemia [251]. Inhibition of AKT or PRAS40 caused reduced tumor growth in mouse models [246]. PRAS40 has been shown to play a role in resistance to apoptosis inducing agents in melanoma cells [246]. To our surprise, AKT was not highly activated in our resistant cells (Figure 18A/B). Proto-oncogene
PIM1 has also been found to phosphorylate pPRAS40 and PIM1 mediated hyper phosphorylation has been reported in radiation resistant non-small cell lung cancers [252, 253]. These results combined with our trametinib resistant cells indicate that PRAS40 may be a viable target to overcome trametinib resistance in TNBC.

HSP60 was also found to be highly activated in the TNBC resistance MDA-MB-231R cells. HSP60 is a 60kD member of the heat shock protein family of chaperones. Normally involved in responding to a sudden increases in temperature, environmental and other stresses, HSP family members are involved with protein folding and assembly, transport between subcellular compartments and signaling pathways leading to transcription [254, 255]. Family member HSP90 is commonly associated with HER2 shuttling in HER2-positive breast cancer and inhibitors are in clinical trials [256]. HSP70, another family member, has also been implicated in imatinib resistance in chronic myeloid leukemia [257]. HSP60, however, seems to be a double edged sword in human disease. HSP60 has been implicated in the pathogenesis of various diseases ranging from prostate and breast cancer to Crohn’s disease, myocardial ischemia and autoimmune diabetes [256]. High HSP60 expression in pretreated esophageal adenocarcinoma tumor biopsies was correlated to a positive response to platin/5FU based neoadjuvant chemotherapy [258]. HSP60 levels are also elevated in malignant breast cancers [259]. Conversely, expression of HSP60 in cervical cancer and glioblastoma multiform seems to correlate with a good prognosis [260, 261]. HSP60 also appears to have both a pro-survival function and pro-
death functions via its association with procaspase3, BAX and BIRC5 [262-265]. Overexpression of HSP60 in cardiac myocytes increased their survival against ischemic injury suggesting a pro survival function [266]. HSP60 is also involved in defense against free radical oxygen and nitrogen species during aging [267]. In TNBC, HSP60 seems to play a survival role in trametinib resistance, and therefore these pre-clinical models may benefit from an HSP60 inhibitor.

Proto-oncogene c-Jun is a 3.1 kb intronless gene initially discovered as the human counterpart of the avian sarcoma virus 17 [268]. C-Jun is a member of the AP-1 complex which forms hetero-dimers and homo-dimers to regulate cell proliferation, growth and angiogenesis of solid squamous cell carcinomas [269]. In mammary epithelial cells, c-Jun overexpression led to increased mobility and invasiness [270]. In trametinib resistant TNBC cells, phospho-c-JUN was up-regulated and would be a rationale target for drug design.

Limitations of our study include alternative mechanisms of action of trametinib and the range of MAPK activation in TNBC. In biopsies from metastatic melanoma patients, increased infiltration of CD8+ T cells decreased immunosuppressive cytokines and increased markers of T-cell cytotoxicity were found [271]. This suggests that a possible immune mediated cytotoxicity may be induced in vivo with trametinib treatment and suggests a possible benefit of combining trametinib with immunotherapy. Future work in
vivo is needed to determine if immune mediated effects might be relevant in trametinib sensitivity in TNBC. It may also be possible that TNBC patients harbor a MAPK activating mutation only after chemotherapy treatment, as the RAF/MEK/ERK pathways has been implicated as a mechanism of resistance to cytotoxic chemotherapy in TNBC [272, 273]. Trametinib therapy may be most useful in patients with prior exposure to cytotoxic therapy.

It is known that prolonged treatment with targeted inhibitors is universally met with the development of drug resistance [274]. It is important to study the mechanisms of resistance to targeted therapies like trametinib to help prolong the disease free survival of cancer patients. A better understanding of the mechanisms of acquired resistance will be useful in designing rational combination therapies to treat patients that are candidates to develop resistance to inhibitor monotherapies [275]. Our results provide initial insights into the molecular mechanisms of resistance to single use MEK inhibitors and provide a rationale for dual MEK/AKT inhibition.
Figure 16. PTEN status can predict sensitivity to trametinib.

(A) Wild type PTEN MDA-MB-231 were plated at 750 cells/well in a 96 well plate then treated with increasing doses of trametinib (0 (DMSO), 50nM, 100nM, or 200nM) for 24, 48 and 72 hours. (B)PTEN null MDA-MB-468 triple negative breast cancer cells were plated at 2,000 cells/well in a 96 well plate then treated with increasing doses of trametinib (0 (DMSO), 50nM, 100nM, or 200nM) for 24, 48 and 72 hours. Cells were harvested, frozen overnight and a CyQuant Proliferation assay was performed according to manufacturer’s instructions. (C) MDA-MB-231 and MDA-MB-468 cells were treated with increasing doses of trametinib for 24 hours and protein levels were assessed via Western Blot. β-actin was used as a loading control.
Figure 17. PTEN wild type MDA-MB-231 TNBC cells develop an acquired resistance to trametinib, in contrast to the intrinsically resistant PTEN null MDA-MB-468 TNBC cells.

(A) Initially, MDA-MB-231 could only withstand 25nM of trametinib, so a step wise increase in dose over a period of 6 months was used to develop resistance to 200 nM trametinib. MDA-MB-468 were able to be cultured in 100 nM initially which was increased to 200 nM after 10 days. (B)(C) MDA-MB-231, 231R (>6 months cultured in trametinib), MDA-MB-468 and 468R (>6 months cultured in trametinib) TNBC cells were plated 1X10^4 cells per 6-well dish and treated with 200 nM trametinib. Cells were harvested and subjected to cell counting with a hemocytometer and graphed relative to Day 0 (Proliferation Index). (D) MDA-MB-231 (Parental), naïve MDA-MB-231 cells treated with 200 nM for 24 hours (Treated) and 231R (Resistant) and MDA-MB-468 cells (Parental), naïve MDA-MB-468 cells treated with 200 nM for 24 hours (Treated) and 468R (Resistant) were treated with 200nM of trametinib for 24 hours and protein levels were assessed via Western Blot. β-actin was used as a loading control. MDA-MB-231 and 231R cells (E) or MDA-MB-468 and 468R (F) were plated at 750 cells/well in a 96 well plate and treated with increasing doses of trametinib for 6 days. Cells were harvested, stored at -80°C overnight, and CyQuant was performed according to manufacturer’s instructions.
Figure 18. Trametinib resistant TNBC cells have an altered kinase profile relative to parental cells and intrinsically resistant cells have a different profile relative to TNBC cells that acquired resistance.

MDA-MB-231 and 231R (A) or MDA-MB-468 and 468R (B) cells were subjected to a Phospho-Kinase Antibody Array. Differential expression between resistant and parental cells are depicted as relative fold change, where negative values are repressed in the resistant cells (higher in the parental cells) and positive values are activated in the resistant lines (repressed in the parental lines).
Chapter 6: Discussion

6.1 Principal Findings

In this section the major findings from each chapter will be summarized.

6.1.1 Chapter 2

1. Breast cancer cell lines exhibit elevated JUNB expression compared to non-transformed mammary epithelial cells.
2. Treatment with Oncostatin M further augments JUNB expression
3. Oncostatin M regulation/activation of Pol II on the JUNB promoter relies primarily on MAPK signaling instead of the STAT3 signaling pathway.

6.1.2 Chapter 3

1. Flavopiridol (FP) normally inhibits global gene transcription in breast cancer cells. JUNB is able to escape FP-mediated transcriptional inhibition in breast cancer cells.
2. JUNB is induced by other kinase inhibitors (sorafenib, U0126) but not cytotoxic chemotherapy agents (doxorubicin).
3. JUNB protects against FP-mediated apoptosis in breast cancer cells.
4. JUNB plays a pro-survival role in kinase inhibitor treated breast cancer cells.

6.1.3 Chapter 4

1. The odds of pathological complete response defined as no invasive disease in the breast and lymph nodes was 1.94 times higher in the neoadjuvant chemotherapy and dual anti-HER2 therapy (lapatinib plus trastuzumab) compared to neoadjuvant chemotherapy and trastuzumab alone.

2. The odds of pathological complete response defined as no invasive disease in the breast only was 1.78 times higher in the neoadjuvant chemotherapy and dual anti-HER2 therapy (lapatinib plus trastuzumab) compared to neoadjuvant chemotherapy and trastuzumab alone.

3. The odds of achieving pathological complete response defined as no invasive disease in the breast only was 1.76 times higher in the combination arm compared to trastuzumab alone arm for hormone receptor positive patients. The odds of achieving pathological complete response defined as no invasive disease in the breast only was 2.06 times higher in the combination arm compared to trastuzumab alone arm for hormone receptor positive patients.

4. There was no difference of effect of combination treatment between the hormone receptor positive and hormone receptor negative subgroups.
6.1.4 Chapter 5

1. PTEN status can accurately predict trametinib sensitivity in triple negative breast cancer (TNBC) cell lines as cells without PTEN are inherently resistant to trametinib while PTEN wild type cells are sensitive to trametinib.

2. Short term trametinib treatment dramatically decreases expression of pERK in both PTEN wild type and PTEN null TNBC cell lines.

3. pAKT levels were induced in short term trametinib treatment that persisted in trametinib resistance PTEN wild type MDA-MB-231 cells, with a reduction in PTEN only seen after 6 months of trametinib treatment.

4. Phospho-kinase array analysis revealed PRAS40, HSP60 and AKT as possible mediators of resistance to trametinib treatment in TNBC cells.

6.2 Future Directions

In this section, future directions for each chapter will be described

6.2.1 Chapter 2&3

JUNB is well established as playing an oncogenic role in Hodgkin’s lymphoma and anaplastic large cell lymphoma [93]. Tight control of JUNB protein levels is critical as persistent JUNB expression during G2/M induces mitotic defects and genetic instability [84]. In addition, JUNB protects pancreatic beta cells from cytotoxic fatty acid and
cytokine treatments by inducing the expression of pro-survival target genes including Bcl-xl, Activating Transcription Factor3 (ATF3), CEBPD and X-box protein1 (XBP1) [85, 94]. Less is known, however about the role of JUNB in breast cancer, specifically chemotherapy resistance. We have shown dual functions for JUNB in breast cancer cells.

First we have shown that Oncostatin M (OSM) induces JUNB expression in a MAPK dependent manner. Both Oncostatin M and JUNB have been shown to play roles in breast cancer progression, but the functional role of JUNB in response to Oncostatin M has yet to be determined. JUNB targeting siRNA could be used to determine if JUNB plays a role in OSM induced migration/invasion/metastasis of human breast cancer cells. To enhance the translational impact of these observations it would be of interest to investigate the influence of OSM secreted from activated macrophages on JUNB expression and breast cancer cell biology. This could be done either by co-culturing macrophages and breast cancer cells or using a transwell insert, where macrophages and breast cancer cells can grow separately, but share media, including components such as OSM which would be secreted from the macrophages. It might also be important to study what causes macrophages to secrete OSM; are the breast cancer cells secreting a product that may cause the macrophages to produce OSM? This is important as patients with high circulating OSM were found to have a worse prognosis in breast cancer [107].
In chapter three we investigated the influence of Flavopiridol on JUNB expression. Flavopiridol is a CDK inhibitor, known to prevent Pol II activation via CDK9 [115, 116]. Unexpectedly, treatment with Flavopiridol for 24 hours caused an induction of JUNB. A similar results was found with experiments performed with a MEK inhibitor, U0126 (MAPK pathway), and a multi-kinase inhibitor Sorafenib (upstream MAPK). While OSM induction of JUNB is dependent on MAPK signaling, treatment with a lethal dose of several MAPK inhibitors causes an induction of JUNB that is independent of MAPK. The mechanism underlying the induction of JUNB in response to MAPK and CDK9 inhibitors has yet to be determined and warrants further investigation. It is possible that other mechanisms, independent of CDK9/MAPK may activate Pol II activation, such as BRD4 or CDK12 [141, 157, 158]. JUNB has been found to be regulated by a “paused” polymerase using a complex including DSIF and NELF [118, 119]. HSP90 has been shown to stabilize NELF [154] in a CDK9 independent manner, indicating that HSP90 could influence the expression of JUNB in breast cancer cells [155].

6.2.2 Chapter 4

Prior to the development of trastuzumab, patients with the HER2/neu amplification had a higher risk of disease recurrence, and a worse overall survival compared to non-HER2/neu amplified patients [164, 168, 169]. After the addition of trastuzumab, patients with the HER2/neu amplification have a 60% response rate and an improvement in overall survival [170, 171]. Multiple other HER2 inhibitors have entered clinical trials,
and pertuzumab in combination with trastuzumab has been Federal Drug Administration approved for use in breast cancer [207, 276]. Lapatinib has not received as much attention, as the addition of lapatinib increases toxicity in patients [206, 207]. As this meta-analysis only looks at pathological complete response, it will be important to follow these trials to evaluate long term disease free survival to determine if there is a benefit from the addition of lapatinib to trastuzumab and neoadjuvant chemotherapy.

6.2.3 Chapter 5

Compared to other MEK inhibitors, trametinib has low inter patient variability, prolonged half-life and small peak to trough ratios which allow trametinib to overcome the narrow therapeutic index normally associated with MEK inhibition [228]. Typically MEK inhibitors cause an accumulation of phosphorylated MEK and increasing pMEK levels is associated with ERK reactivation upon drug removal [237, 238]. Duncan et al also demonstrated reactivation of ERK, showing that treatment with the MEK inhibition AZD6244 resulted in aberrant activation of a number of receptor tyrosine kinases (140) and reactivation of ERK by a mechanism that bypassed MEK inhibition [233]. Previous studies in MDA-MB-231 cells using the MEK inhibitor PD325901 led to the discovery of a MEK mutation following drug treatment [239]. The presence of the MEK1L115P mutation prevented PD325901 from binding to its target on the MEK1 protein resulting in defective MEK inhibition and continued phosphorylation of ERK [239]. Trametinib differs from these MEK inhibitors in that it binds to un-phosphorylated MEK and
prevents phosphorylation (activation) of MEK [237, 240]. Results from this study are consistent with this mechanism of action as we found no evidence of ERK reactivation in our studies (see Figure17D/18A). These results indicate that the mechanism of trametinib resistance developed in TNBC cells in this study differs from previously characterized mechanisms.

Lehman, et al, has sub-classified TNBC into six categories based on gene expression profiles from 21 breast cancer data sets. The MDA-MB-231 cells fell into the mesenchymal stem cell like group and the MDA-MB-468 fell into the basal like 1 group [217]. Interestingly, the MDA-MB-231 cells were only sensitive to a PI3K inhibitor while the MDA-MB-468 cells were sensitive to a variety of treatments. In this study, we found that MDA-MB-231 cells were sensitive to trametinib, while the MDA-MB-468 cells were not. One interpretation of these results is that the unique mesenchymal stem cell group may be a subtype of TNBC that might benefit the most from trametinib treatment. The role of other factors that differentiate MDA-MB-231 cells and MDA-MB-468 cells, such as differential PTEN status, will be determined in future studies.

There are a number of future directions that may be pursued in this trametinib resistance project. It is first important to determine potential biomarkers of sensitivity in TNBC. Reliable biomarkers of sensitivity and/or resistance to trametinib would be useful in the clinical management of TNBC patients. Next we plan to further investigate the biology of
TNBC cell lines by doing knockdown/therapeutic targeting of the signaling components found to be activated in the resistant cells. These studies will advance our understanding of the cellular mechanisms that drive trametinib resistance in TNBC cells. In future studies we plan to include additional global gene expression and kinase analyses to provide a more comprehensive analysis of alterations in trametinib resistant TNBC cell lines. Once a list has been developed of potential therapeutic targets are identified using TNBC cell lines, it will be important to move to xenograft mouse models. Finally it will be important to move to patient data to confirm our in vitro findings in patients that are trametinib refractory.

**Trametinib sensitivity**

It has been proposed that as few as four genes can predict sensitivity to MEK inhibitors. Mutations in *KRAS, PIK3CA, BRAF,* or *PTEN* have all been shown by several groups to confer resistance to the MEK inhibitors AZD6244, CI-1040, and PD0925301 [11, 16, 241]. This is consistent at least in part with our results in that PTEN status can predict trametinib sensitivity in TNBC using MDA-MB-231 PTEN wild type cells and MDA-MB-468 PTEN null cells. It is however possible that a KRAS$^{G13D}$ mutation in addition to wild type PTEN may play a role in the sensitivity of the MDA-MB-231 cells [16]. Neither the MDA-MB-231 or MDA-MB-468 cells have a known mutation in either *BRAF* or *PIK3CA* mutation, but it would be important to investigate TNBC cell lines with these mutations to confirm sensitivity to trametinib [277]. To determine if PTEN
and KRAS play major roles in trametinib sensitivity or if they are merely bystanders, siRNA directed against PTEN or KRAS should be used in MDA-MB-231 cells treated with trametinib. If knocking down the expression of PTEN or KRAS causes the cells to become resistant to trametinib, this would be strong evidence that these signaling components play a major role in determining sensitivity to trametinib. If the cells do not become resistant to trametinib, other factors may be contributing to the trametinib sensitivity. Conversely, by overexpressing PTEN or KRAS in trametinib in the MDA-MB-468 cells, we can further validate their role in resistance as these cells should become sensitive to trametinib.

**Drivers of trametinib resistance**

It is important to understand which alterations are driving the resistance and which alterations have accumulated due to independent mechanisms. We can continue to understand the biology of cell lines by doing knockdown/therapeutic targeting of the signaling components found to be activated in the resistant cells. If these targets are driving the resistances, inhibiting them should cause the cells to become sensitive to trametinib, or the inhibitor will synergize with trametinib to kill the cells. From a translational standpoint, it might also be important to overexpress the targets that were repressed in the resistant lines. If overexpressing the repressed targets causes the cells to become sensitive to the drug, then it will be important to further investigate these targets as biomarkers for patients receiving trametinib. To further examine the role these targets
play in resistance, resistant cells with the siRNA/overexpression should be grown in soft agar (anchorage independent growth) and 3D-collagen based matrices. Increasing evidence indicates that 3-D models better represent in vivo cell biology, particularly tumor cell proliferation and invasion, than 2-D models [278]. It would be important to test the combination of trametinib resistance and siRNA/drug effects on apoptosis [233]. Several candidates have emerged as potential therapeutic targets for trametinib resistance in TNBC. By continuing to characterize resistance to MEK inhibitors, we can develop novel treatment strategies for patient-specific disease management to delay the onset of resistance and improve overall survival of these patients. To begin to prioritize the altered kinases uncovered in the kinase array, it is important to first understand a little more about each of the top targets.

**AKT**

The MAPK and PI3K pathways are known to compensate for each other to promote cell proliferation, survival, and migration [242]. These two pathways share a common input receptor tyrosine kinase (i.e. EGFR), and they both can be activated by Ras [235, 243]. Whole genome and whole transcriptome sequencing in TNBC patients reveal co-activation of the MEK and PI3K/AKT pathways [279]. Loss of function alterations in PTEN have also been demonstrated in 30% of TNBC tumors, causing activation of the PI3K/AKT pathway [244, 245, 280]. TNBC cell lines with loss of PTEN or high levels of AKT signaling were less responsive to the MEK inhibitor AZD6244 [222]. As the
literature suggests, AKT seems to be the most likely candidate for trametinib resistance. To determine the role of AKT in trametinib resistance, we used Ly294002 an inhibitor of both PI3Kα/δ/β, thus blocking the PI3K pathway [281]. Treatment with increasing doses of LY294002 for 6 days caused a dramatic loss of cell viability in the MDA-MB-231R (trametinib resistant) cells compared to the MDA-MB-231 parental cells (0-10μM; Figure 19). MDA-MB-231 parental cells did show a decrease in cell viability which is perplexing as MDA-MB-231 cells don’t have detectable levels of pAKT. Both MDA-MB-468 and MDA-MB-468R (trametinib resistant) both showed a dramatic decrease in cell viability when challenged with LY294002 (Figure 19). This data confirms our hypothesis that AKT plays a role in trametinib resistance in TNBC cell lines. Unfortunately, this drug is not bioavailable in patients; therefore other inhibitors must be investigated. It is possible that drugs targeting PI3K like BKM120 or BEZ235, may be used, or AKT inhibitors currently in clinical trials such as GSK690693 or GSK2141795.

**PRAS40**

Elevated pAkt levels have also been described in MEK inhibitor resistant colorectal, pancreas and lung cancer lines in the absence of a *PIK3CA* or *PTEN* mutation, suggesting that there are other genes possible to convey resistance [241]. This is consist with our MDA-MB-231 cells, as they are PTEN and PIK3CA wild type, yet are resistant to trametinib after continuous culture. (Figure 18A).
Proline-rich AKT substrate of 40 kDa (PRAS40) is a cytosolic protein that is ubiquitously expressed [246]. This protein is known to be an integral component of the AKT and mTOR signaling pathways [247]. Phosphorylation of PRAS40 by AKT and mTORC1 causes dissociation of PRAS40 from mTORC1 thus allowing mTORC1 to be activated, causing protein synthesis [247]. PRAS40 also forms a complex with 14-3-3 protein, a scaffold protein, with mTORC1 to mediate AKT signaling to mTOR complex [248]. Association with 14-3-3 protein is crucial for insulin to stimulate mTOR via PRAS40 [248], as pretreatment of cells with PI3K inhibitors prevented the insulin induced phosphorylation of PRAS40 and the ability of PRAS40 to bind to 14-3-3 protein [249]. The MEK inhibitor PD98056 had no effect on PRAS40 [249], indicating that PI3K, not MAPK, is an important regulator of insulin sensitivity and therefore a target for insulin resistance. This result may be extended to chemotherapy resistance, as overexpression of pPRAS40 attenuated apoptosis in neuronal cells after ischemia [251], and pPRAS40 was significantly up-regulated in both trametinib resistant cell lines (Figure 18A/B). Increased levels of pPRAS40 decreased sensitivity of melanoma cells to apoptotic stimuli and inhibition of PRAS40 via AKT inhibition caused increased cellular apoptosis, a decrease in anchorage-independent cell viability, and reduced tumor growth in mouse models, indicating PRAS40 as a possible resistance mechanism [246].

The most common way to inhibit PRAS40 is to target the PI3K pathway in a similar way to inhibit AKT activation [249]. Inhibitors of PI3K are important for the inhibition of
PRAS40, but can affect normal cells ability to metabolism insulin and glucose homeostasis [250]. To our surprise, AKT was not highly activated in our resistant cells. AKT seems to be the major player in PRAS40 activation but it is possible that other pathways can circumvent AKT signaling as leucine in the heart was found to promote pPRAS40 via a pathway that requires PI3K and PDK1 but was independent of AKT [282]. Alternatively, proto-oncogene PIM1 has been found to phosphorylate pPRAS40 in a kinase assay and PIM1 mediated hyper phosphorylation has been reported in radiation resistant non-small cell lung cancers [283]. Further investigation is needed to understand the role that PRAS40 plays in trametinib resistance.

HSP60

In addition to PRAS40, HSP60 was also activated in the trametinib resistant cells relative to the parental cells (Figure 18A/B). HSP60 is a 60kD member of the heat shock protein family of chaperones. Normally involved in sudden increases in temperature, environmental and other stresses, HSP family members are involved with protein folding and assembly, transport between sub-cellular compartments and signaling pathways leading to transcription [254-256]. HSP family member, HSP90 is commonly associated with HER2 shuttling in HER2-positive breast cancer and clinical trials using HSP90 inhibitor are currently ongoing [284]. Another family member, HSP70 has been implicated in imatinib resistance in chronic myeloid leukemia [257] and chemotherapy resistant breast cancers [285]. HSP60’s involvement with cancer however, seems to be
context and cell specific as HSP 60 has been implicated in various diseases ranging from prostate and breast cancer to Crohn’s disease, myocardial ischemia and autoimmune diabetes [256]. HSP60 was associated with poor prognosis and a poor clinical outcome in androgen resistant prostate cancer [258], but high HSP60 expression in pretreated esophageal adenocarcinoma tumor biopsies correlated with a positive response to platin/5FU based neoadjuvant chemotherapy [258]. HSP60 levels are also elevated in malignant breast cancers compared to normal tissue [259]. Conversely, expression of HSP60 in cervical cancer and glioblastoma multiform seems to correlate with a good prognosis [256, 260].

HSP60 also appears to have both a pro-survival function and a pro-death function which may depend on the presence of the MSS (N-terminal mitochondrial-targeting sequence) [262, 286]. The pro-apoptotic function of HSP60 involves the association with procaspase 3 and the involvement in its maturation upon being released from the mitochondria [262, 263], the sequestration of BAX [264] and/or p53 [265], and the stabilization of the BIRC5 (survivin) protein [265]. Overexpression of HSP60 in cardio myocytes increased their survival against ischemic injury suggesting an anti-apoptotic function [266]. HSP60 is also involved in defense against free radical oxygen and nitrogen species during aging [267].
Treatment with HSP inhibitors may however cause adverse events in patients due to the multiplicity of the defensive roles of HSP and their role in normal chaperone functions [256]. There are however, a number of HSP inhibitors in both pre-clinical and clinical studies. Mizoribine, epolactaene tert-butly ester, avrainbillamide are HSP60 inhibitors currently under investigation [286] and could be used to inhibit HSP60 function in the trametinib resistant TNBC cells.

c-JUN

While only mildly induced relative to the other activated substrates, future studies should also investigate c-JUN in trametinib resistant TNBC cells. Proto-oncogene c-Jun is a 3.1 kb intronless gene initially discovered as the human counterpart of the avian sarcoma virus 17 [268]. C-Jun is a member of the AP-1 complex which forms hetero and homodimers to regulate cell proliferation, growth and angiogenesis of solid squamous cell carcinomas [269]. In mammary epithelial cells, c-Jun overexpression is associated with increased mobility and invasiness [270]. c-JUN is a downstream target of the MAPK/ERK signaling pathway [287]. Results from the kinase array analysis indicated that c-Jun phosphorylation was increased in trametinib resistance, suggesting activation of alternate mechanisms of c-JUN phosphorylation may be activated in trametinib resistance cells. This is perplexing as MAPK via ERK is inhibited in our trametinib resistant TNBC cell lines, yet c-JUN is activated. c-Jun NH2-terminal Kinase (JNK) can also activate c-JUN and has been shown to play a role in resistance to DNA damaging
agents in breast cancer cells [288]. c-JUN was also found to be up regulated in chemotherapy resistant MCF7 breast cancer cells [289]. Selectively inhibiting c-JUN is difficult however, as c-JUN is a transcription factor. It is possible however to treat JNK (upstream of c-JUN) with the inhibitor SP 600125, which has been shown to reduce cell viability in doxorubicin resistant cells [290].

*Global analysis*

It is important to note the limitations of the trametinib study. We used a kinase array to investigate alterations in signaling in trametinib resistant TNBC cell lines. While this array only studies 45 proteins, many of the proteins on this array are key substrates in critical cellular signaling pathways. If none of the targets identified in this initial screen provide the necessary insights into mechanisms of trametinib resistance, it would be important to do a more global protein analysis. Reverse phase protein array (RPPA) is a high-throughput antibody-based technique currently available through MD Anderson [291]. This global method assesses total protein expression in addition to protein modifications such as phosphorylation, cleavage, and fatty acid modification in order to fully understand pathway alterations [292]. It might also be possible to use the elegant system from University of North Carolina, where altered kinases alterations were determined using multiplexed inhibitor beads (MIBs) [233]. This process determines targets for kinase alteration by first performing RNA-Seq to measure the expression and mutation spectra of the kinome at the transcript level. With this information MIBs are
generated against the expressed, altered kinases to affinity capture then and quantitative them using mass spectrometry. By using these two techniques, it will possible to understand the alterations that cause resistance to trametinib in our TNBC cells.

**In vivo models**

The next logical step in this project would be moving to a more relevant *in vivo* model using mouse xenograft models. By injecting the resistant cell lines into mice and continuing to treat them with trametinib and the inhibitors of interest from the *in vitro* screens, we will be able to translate our *in vitro* findings into a more clinically relevant *in vivo* system. This will elevate our data one step closer to clinical relevance. Patient derived xenografts could also be an important model to understand mechanisms of trametinib resistance. By xenografting patient tumors directly into mice, we can understand the biology of the tumor without having to develop cell lines. This is the most clinically relevant form of *in vivo* studies [293].

**Patient Data**

Finally, we will analyze the targets in patient data. There have only been 79 studies investigating trametinib according to clinicaltrials.gov (accessed 6/20/14); 40 are recruiting, 13 have not yet started, 7 are active, 15 are completed and 9 ended early (terminated, suspended or withdrawn). Three of the trials (NCT01324258, NCT00687622, NCT01476137, NCT01964924) plan to take pre and post biopsies to
measure genetic/protein profiles on tumor tissue. Interestingly, NCT01964924 is a clinical trial ongoing here at OSU investigating the use of trametinib in TNBC. It would be important to measure the level of expression of our top targets, filtered through mice xenograft models, on patient samples pre and post trametinib treatment. We can then correlate this data to objective response rate (complete response + partial response) and progression free survival, as measured in the study. This data would confirm our findings from our in vitro studies and provide rationale targets for future clinical trials and appropriate biomarkers to determine the development of trametinib resistance in TNBC patients.

Side project: Role of trametinib resistance on a metastatic phenotype

During the investigation of trametinib resistance, we noticed that the resistance cells appeared to look and act differently than the parental cells. MDA-MB-231 cells treated with trametinib for >6 months have an altered cell morphology and MDA-MB-468 cells after being treated with trametinib for >6 months no longer grow in a grape-like structures and tend to grow as individual cells (Figure 20). This led us to investigate E-cadherin, a marker for epithelial cells, in our trametinib resistant cells. Epithelial-to-mesenchymal transition (EMT) has been widely investigated as a mechanism of metastasis in epithelial tumors [294]. MDA-MB-231 cells do not normally express E-cadherin in either the parental, naïve treated, or trametinib resistant cells, which is consistent with their known aggressive, mesenchymal metastatic phenotype [217] (Figure
MDA-MB-468 normally express E-cadherin, which is unaffected by short term trametinib treatment, but it decreased in the trametinib resistant lines (Figure 21). These results suggest that long term treatment with trametinib may cause an EMT transition, but further work is needed to validate these findings.

The EMT transition has been implicated as a mechanism of metastasis in cancer [294]. This EMT transition is thought to be regulated by the TGFB, ALK WNT/β-catenin and RAC pathways [217]. It will be important to dissect these pathways in our trametinib resistant cells, particularly the MDA-MB-468R cells. This can be accomplished by examining genes known to be markers for an epithelial phenotype, such as CLDN1, DSP, OCLN, CDH1 and genes known to be markers for mesenchymal phenotype, such as MMP2, ACTA2, SNAI2, SPARC, TWIST1, ZEB1, ZEB2, COL3A1, FN1, VIM, S100A4 [217, 218].

It will also be important to further investigate the metastatic potential of trametinib resistant cells. This can be accomplished through scratch assays, Matrigel invasion kits, and using xenograft models of metastasis. These methods can be used to determine if trametinib resistant cells are more migratory, invasive and metastatic respectively than their parental controls.
In 2013 the Gap Analysis Working Group as part of the Breast Cancer Campaign described ten gaps in translational medicine [295]. They claim there are ten critical research gaps for the prevention/treatment of breast cancer. First they want more research to understand the function interactions of epigenetic changes in malignant transformation. They also want to know more about molecular drivers behind breast cancer subtypes. Finally they would do know molecular mechanisms of tumor heterogeneity, de novo or acquired resistance and how to target these changes. It is my hope that the work outlined in this dissertation will provide the foundation to provide clarity for these gaps in knowledge.
Figure 19. TNBC cells are sensitive to the AKT inhibit LY294002.
MDA-MB-231 (A) and MDA-MB-468 (B) cells were plated in a 96 well plate and treated with increasing doses of LY294002 (0 (DMSO), 5µM, 10µM, 20µM and 40µM) for 6 days. Cells were harvested, frozen overnight and a CyQuant Proliferation Assay was performed according to manufacturer’s instructions.
Figure 20. Cell Morphology changes with trametinib resistance.

Cells lines were plated at similar densities, allowed to adhere for 24 hours followed by staining with 0.5% crystal violet in 20% methanol for 3 minutes.
Figure 21. E-Cadherin is reduced in trametinib resistant MDA-MB-468 cells.

MDA-MB-231 and MDA-MB-468 cells were untreated (parental), naïve cells treated with 200 nM trametinib for 24 hours (treated), or continuously cultured in 200 nM trametinib (resistant). Protein levels were assessed via Western Blot with β-actin used as a loading control.
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140


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