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Effects of Stable Insulin-like Growth Factor 1 Receptor Knockdown on Triple-Negative Breast Cancer Cells

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2011

Abstract

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Next to non-melanomatous skin cancer, breast cancer is the most common cancer diagnosed among American women today. It is estimated that yearly, more than 190,000 women are diagnosed with breast cancer and greater than 40,000 succumb to the disease. Aggressive, metastatic disease is directly responsible for the majority of breast cancer-related deaths. Triple-negative (TN) breast cancers, which lack estrogen receptor, progesterone receptor and HER2/neu overexpression, lead to poorer survival outcomes compared to all other breast cancer patients, partly because of a lack of therapeutic targets. Insulin-like growth factor 1 receptor (IGF-1R) is overexpressed in 50% of primary breast tumors compared with normal tissues and 36% of TN breast cancers express IGF-1R. This tyrosine kinase receptor plays a role in proliferation, apoptosis, adhesion, and invasion, suggesting that breast cancers have enhanced responses to the mitogenic and anti-apoptotic effects of IGF-I. The objective of this project was to determine the significance of differential IGF-1R signaling in the aggressive properties of TN breast tumors. Stable lentiviral IGF-1R knockdown was performed in two morphologically distinct TN breast cancer cell lines, MDA-MB-468 and MDA-MB-231. Knockdown of IGF-1R led to down regulation of AKT signaling as well as lack of IGF-I-induced IGF-1R up-regulation in both IGF-1R (-/-) cell lines compared to empty vector control cell lines. Interestingly, each TN cell line underwent distinct morphological changes in response to IGF-1R silencing. As evidenced by confocal microscopy and Western blot analyses, MDA-MB-468 (epithelial) cells appeared to undergo epithelial to mesenchymal transition (EMT) while MDA-MB-231 (mesenchymal) cells

underwent mesenchymal to epithelial transition (MET). Epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (vimentin and fibronectin) were also differentially expressed in each IGF-1R (-/-) cell line. Combinatorial inhibition of IGF-1R, EGFR, and/or mTOR decreased cell survival more efficiently than single inhibition. These results suggest that IGF-1R inhibition, in combination with EGFR and/or mTOR down-regulation may provide clinical benefit in a subset of TN breast cancer patients, particularly those with mesenchymal-like tumor phenotypes.

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Acknowledgements

I would like to thank Dr. LaTonia Taliaferro-Smith for being an amazing mentor and teacher for the last three and a half years. I am proud to be the first Honors Student to graduate from her lab. I would also like to thank Dr. Ruth O'Regan for bringing in the clinical aspect and reminding us of the ultimate goal: curing patients. Dr. Tongrui Liu, for all her help and insight in the project. Dr. Erik Kline and Dr. Carrie Eggers, for their help and perspectives in this project. I would also like to thank my former mentors who taught me along the way in my path to becoming a scientist: Dr. Giovanni Bosco, Dr. Brandi Brandon Knight, and Dr. Ting Wu. Thanks to Dr. Arri Eisen and Dr. Gregg Orloff for reading this thesis and being a part of my committee. And thanks to my supportive family, friends, and Squinky.

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CHAPTER 1: Introduction

Breast Cancer Statistics

Breast cancer is the most frequently diagnosed cancer in American women and the second leading cause of cancer-related deaths. For women under the age of 40; breast cancer tends to affect a higher percentage of younger (pre-menopausal) African-American (AA) women compared to their Caucasian (CA) counterparts. The Carolina Breast Cancer Study (CBCS) is a population-based, case-control study started in 1993 in North Carolina to identify breast cancer risk factors [1]. The study deliberately oversampled AA and premenopausal women in order to better understand the disparities in breast cancer risk in these populations. The study combined population-based epidemiology and molecular biology to understand environmental and genetic factors involved in breast cancer [2, 3]. The age-adjusted mortality was found to be 28.3 deaths per 100,000 for CA women and 36.4 deaths per 100,000 in AA women [1]. Overall, African-American and younger women have a lower incidence but higher mortality rate from breast cancer than do women from any other racial/ethnic group [4]. Breast cancer in all AA women has a poorer overall prognosis, a more advanced stage at diagnosis, a more aggressive and metastatic phenotype, and a greater risk of recurrence [5].

Overview of Breast Cancer Subtypes

Over the years researchers have discovered that all breast cancers are not the same; rather the disease is composed of different subtypes, each with distinct characteristics, prognoses, and recommended treatment regiments. Breast cancer has been classified into four major groups based on the estrogen receptor (ER), progesterone receptor [6], and HER2/erbB2/neu receptor status: (1) luminal A (ER+ and/or PR+, HER2-) [7], (2) luminal B (ER+ and/or PR+, HER2+),

(3) HER2-positive (ER-, PR-) and (4) basal-like (ER-, PR-, HER2-). The latter, also referred to as triple-negative (TN) breast tumors, lack expression of all three receptor proteins and are associated with aggressive behavior and poor survival [1]. The basal-like subtype was named because it expresses cytokeratins often found in the basal epithelial cell layers. These boundaries for characterizing subtypes are not rigid, and some tumors may have phenotypes that overlap. For example, HER2+/ER-/PR- tumors overexpress HER2, often through gene amplification. However, in hierarchical analysis they cluster near the basal-like tumors [1].

Sixty-two percent of all breast cancers are ER+ [4]. ER+ tumors can be further classified as luminal A or luminal B. Luminal A tumors have higher ER expression than do luminal B tumors, and may also express PR, but are HER2 negative. Luminal A cancers have the best patient outcome, mainly due to the availability of a number of chemotherapeutic agents which target the ER. Luminal B tumors overexpress HER2, and often HER1 and/or cyclin E1 [1]. Approximately 25% of all tumors are typed as HER2+, and among those 60% are further defined as HER2-enriched. The HER2-enriched subtype makes up 15%-20% of all tumors and is defined by a specific gene expression signature, and not HER2 protein status. A tumor may be "HER2-enriched" even though it is HER2 negative, or HER2+ but not HER2 amplified. About 30-40% of HER2-enriched breast cancers are ER+, the rest are ER-, including triple-negative breast cancers [1]. The overexpression of ER and HER2 in many breast cancers has led to the development of drugs against those receptors. Luminal breast cancers (which express ER) can be treated with tamoxifen, a drug that blocks ER function, or with inhibitors of aromatase, an enzyme involved in the last step of estrogen synthesis. HER2 amplified and/or overexpressing tumors can be treated with trastuzumab/Herceptin®, which blocks that receptor [8].

Triple-Negative Breast Cancers

About one third of all invasive breast cancers in the U.S. are of the TN subtype. Because TN cancers do not express the hormone receptor targets (ER and HER2), they are not responsive to the treatments that target those receptors. TN cancers tend to have a higher grade, are diagnosed more frequently in younger women, and have a reduced survival rate as compared to ER+ or HER2 breast cancers [9]. AA women are twice as likely as CA women to be diagnosed with this subtype [10]. Even after controlling for age, disease stage, and access to healthcare, disparities in outcome persist between AA and CA TN breast cancer patients [11]. The increased prevalence of TN breast cancer in younger AA women could contribute to their higher incidence of mortality [1]. Identifying alternative molecular targets for the treatment of TN breast cancer is an area of critical concern that could lead to better patient outcome. TN breast cancers overlap with the other subtypes, including basal-like, HER-2-enriched, luminal A, luminal B, claudin-low, and normal-like [4]. In this study, the AA-derived MDA-MB-468 (468s, basal A) and the CA-derived MDA-MB-231 (231s, basal B) TN cell lines were used. In 2007 the MDA-MB-231 line was reclassified into a newly-recognized subtype, claudin-low [12]. This new subtype is still being defined, and many researchers still consider 231s to be basal B.

Basal-like Breast Cancers

Basal-like carcinomas (BLCs) make up 10-25% of all breast cancers in the general population. This classification is denoted because of the cells' expression of cytokeratins 5, 6, 14, or 17. These markers are usually expressed in the basal epithelial layer of the skin and airways. Clinically, these tumors are highly proliferative, genetically unstable, poorly differentiated, and often high grade (III-IV) carcinomas. They also have a tendency to metastasize, primarily to the brain and lungs. Genetically, basal-like cancers are often

retinoblastoma (RB) protein deficient and p53 mutated, which contributes to high proliferation rates [4]. In comparison, BLCs have more p53 mutations (44%) than do luminal A cancers (15%) [1]. Basal-like breast cancers have their highest prevalence, approximately 27%-39% in premenopausal African-American women [4, 13]. One way that BLCs achieve malignancy is through activation of the phosphatidylinositol 3-kinase (PI3K) pathway. It is up-regulated in BLCs compared HER+ tumors. Extracellular signals induce PI3K, which then phosphorylates phosphatidylinositol-4,5-bisphosphate, thus generating phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 binds and activates the serine/threonine kinase AKT, leading to cell survival, proliferation, motility and migration [13].

Phosphatase and tensin homologue (PTEN/MMAC1/TEP) is a tumor suppressor protein which dephosphorylates (and deactivates) PIP3. Thus PTEN protein and AKT activity are negatively correlated. Many BLCs have low/no PTEN expression and PI3K's downstream targets, AKT and mTOR, are significantly increased [13]. Mutations in PTEN are common in advanced cancers such as breast, glioblastoma multiforme, endometrial carcinoma, malignant melanoma, bladder carcinoma, small cell lung cancer, and endometrioid ovarian cancer [14]. The basal A MDA-MB-468 cell line contains a PTEN deletion at codon 70, and does not express PTEN protein. It has been verified that there is also a homozygous substitution at codon 253 in the PTEN gene [15]. It is not clear whether the deletion at codon 70 is heterozygous or homozygous. Evidently, there was some event leading to loss of heterozygosity (LOH) and absence of PTEN protein. The second allele, presumably still wild-type, may have been silenced through promoter methylation or another silencing mechanism. Induction of PTEN expression through adenovirus transfection of MDA-MB-468 cells resulted in decreased levels of pAKT, p70S6 kinase, BAD, and GSK3α protein expression [14]. In a study of four human BLC cell

lines, 468s were the most sensitive to treatments with the PI3K inhibitor LY294002 as well as rapamycin. Single treatment with either drug caused a decrease in proliferation and treatment with LY294002 (but not rapamycin) resulted in apoptosis [13].

Claudin-low Breast Cancers

The most-recently identified breast cancer subtype is the claudin-low (CL) subtype [16]. It accounts for 7-14% of all breast tumors [6, 17]. The exact percentage is not known because this subtype was only recently identified. Perou's group identified the CL subtype in 2007 while analyzing the relevance of murine models for human mammary cancers. This subtype was identified in both the human and mouse tumor datasets. It is characterized by low expression of the tight junction proteins claudins 3, 4, and 7. It also has low expression of genes involved in cell-cell adhesion, such as E-cadherin, a calcium-dependent adhesion glycoprotein often used as a marker for epithelial cells. Claudin-low tumors are distinct from other subtypes because they have low levels of luminal genes, no apparent pattern for basal genes, and high levels of lymphocyte and endothelial cell markers. In Perou's study, 12 of the 13 human tumors identified as CL were also ER-negative. All 13 were grade II or III infiltrating ductal carcinomas, and expressed mesenchymal genes. Microarray analyses of gene expression found statistically significant differences, indicating that this was indeed a new subtype [12].

In 2010 Perou's group further characterized claudin-low tumors using an updated human tumor database. They were categorized as a new subtype of TNs distinct from basal-like breast cancers [4]. They clustered next to the basal-like cancers; however they showed inconsistent expression patterns for basal keratins (keratins 5, 6, 14 and 17). They also grow more slowly and show lower expression of proliferation genes (such as the cell cycle gene Ki67) as compared to basal-like tumors. They are not considered luminal because they have low expression for luminal

markers (ER, PR, GATA3, keratins 18 and 19). They also found statistically relevant differences in gene expression: 1,308 genes upregulated and 359 genes downregulated in claudin-low as compared to other tumor subtypes. They have high levels of epithelial-to-mesenchymal transition (EMT) markers, such as vimentin and Twist [17]. CL tumors also have high levels of immune response genes, cell communication genes, extracellular matrix, differentiation, migration, and angiogenesis. The claudin-low subtype is stem-cell like in nature and highly resembles the mammary epithelial stem cell. This subtype shows many features of cancer stem cells and is enriched for gene expression patterns (such as high expression of ALDH1A1) found in tumor initiating cells (TICs). Clinically, claudin-low tumors show a lower pathologic complete response (pCR) rate than basal-like tumors and much higher than that of luminal tumors. Their response to standard chemotherapy (before surgery) is between that of the basal-like and luminal tumors. Despite partial sensitivity to chemotherapy they still have poor overall survival outcomes. Most are poor-prognosis TN invasive ductal carcinomas [6].

Triple-Negative Breast Cancers- Mechanisms of Malignancy and Role of IGF-1R

The TN subtype has been associated with differential protein expression beyond just the lack of the three hormone receptors. TN cancers have high expression of p16, p53 and Cyclin E, and low expression of Bcl-2 and Cyclin D1 [10]. Thirty-six percent of all TN breast cancers express insulin-like growth factor 1 receptor (IGF-1R), a transmembrane receptor tyrosine-kinase (RTK) that is involved in proliferation, apoptosis, angiogenesis and invasion. The IGF-1R gene is present in a single copy on chromosome 15, bands q25-26. Its 21 exons are spread over more than 100 kb of DNA, and the organization of the exons and introns is very similar to that of the insulin receptor (IR). However, IGF-1R does not contain the alternatively spliced exon 11 found in the IR gene [18]. IGF-1R protein is homologous to the IR, especially in the cytoplasmic

domain. Both IR and IGF-1R are ubiquitously expressed, though there is more IR expression and no IGF-1R in metabolic target organs, such as the liver.

The IGF-1R protein is comprised of two heterodimers; the α-subunits make up the extracellular ligand binding domains and the β subunits are the transmembrane and tyrosine kinase domains. Upon insulin-like growth factor (IGF) ligand binding, the receptor undergoes an activating conformational change [9, 19]. Both IGF-I and IGF-II can activate IGF-1R, though IGF-I has a slightly higher affinity for the receptor [18]. Breast tumors from AA women contain higher levels of IGF-II as compared to tumors from CA patients [20]. This differential expression may contribute to the different outcomes between these two groups.

After ligand-induced conformational change, adaptor proteins (i.e., SRC homology 2 domain-containing (Shc) and insulin-receptor substrates (IRS1-4)) are recruited to the phosphorylation sites in the cytoplasmic domain of IGF-1R. This leads to activation of the phosphatidylinositol-3-kinase (PI3-K)/AKT and mitogen-activated protein kinase (MAP-K) pathways (Figure 1). The end results of IGF-1R signaling include cell proliferation and differentiation, inhibition of apoptosis, malignant transformation, and changes in cell-adhesive properties [9, 19]. Mouse-derived fibroblasts lacking IGF-1R were resistant to tumorigenic transformation by many oncogenes, implicating IGF-1R as an important tumorigenic initiator [3, 20]. IGF-2R is a monomeric transmembrane protein that functions as a tumor suppressor by targeting IGF-II to the lysosome for degradation. Thus, there is little/no free IGF-II to bind and activate IGF-1R. Many human tumors have mutations or loss of function in the IGF-2R gene [3]. Overexpression of a constitutively active IGF-1R in immortalized mammary epithelial cells has been shown to lead to malignant transformation. A screen of 438 primary tumor tissues found

that activated IGF-1R and insulin receptor (IR) are predictors of poor patient outcome, while total levels of IGF-1R were not [8].

Transcriptional Control of IGF-1R

IGF-1R transcription is negatively regulated by three tumor suppressor genes: p53, breast cancer gene-1 (BRCA1), and the Wilms' tumor protein-1 (WT-1) [3]. p53 is the most well studied tumor suppressor gene and the most frequently mutated gene in many human cancers. It controls transcription of genes involved in cell-cycle progression, differentiation, DNA repair, apoptosis, and senescence. The p53 locus is most frequently altered with missense mutations, leading to a loss of function of the DNA-binding domain in the protein. These mutant proteins are therefore unable to complete their normal functions as transcription factors and tumor suppressors. The MDA-MB-468 cell line contains a homozygous missense mutation in p53 and expresses high levels of this mutant p53 protein [15, 21]. The MDA-MB-231 cell line is also homozygous mutant for p53 but still expresses an altered protein product [15, 22].

The BRCA1 tumor suppressor works by sequestering one of IGF-1R's transcriptional activators, thus preventing gene expression. p53 also prevents IGF-1R transcription and cooperates with BRCA1 in an additive manner. Mutant versions of p53 have been found to enhance IGF-1R expression. In the presence of a mutant p53, BRCA1 was unable to inhibit IGF-1R transcription and promoter activity. Interestingly, AA women have higher levels of BRCA1 mutations than do CA women [18]. WT-1 functions as a tumor suppressor by inhibiting IGF-1R promoter activity. However, in the presence of mutant p53, WT-1 loses its suppressive ability [18]. The two cell lines used in this study, MDA-MB-231 and MDA-MB-468 have been tested and no mutations were found in the BRCA1 or WT-1 genes [15]. However, the mutant p53 in

these cell lines could disrupt the function of the wild-type tumor suppressors. This may contribute to their higher levels of IGF-1R protein activity.

Epithelial to Mesenchymal Transition (EMT)

The morphology of cancer cells is variable, as cancer cells are genetically unstable. Epithelial to mesenchymal transition (EMT) is an important step in a solid cancer cell's path to metastasis [23]. Cells undergoing EMT acquire alterations that allow them to disrupt their normal adhesions and interactions in favor of invading to form metastases. During this process, cubodial-shaped epithelial cells become more spindle-like. EMT also occurs in development, but that is a more permanent transition. EMT in cancer cells is not permanent, because the distant metastases EMT facilitates often no longer have these mesenchymal characteristics and revert back to the more epithelial morphology. There is also evidence that cells undergoing EMT become more stem-like and like tumor initiating cells (TICs) [17].

Tumor Initiating Cells (TICs)

It has been hypothesized that some tumors contain a small subpopulation of TICs, also known as cancer stem cells. TICs have the ability to self-renew and also differentiate to form all cell types of the tumor. They are defined by a unique protein expression signature (ranging from 172 to 906 genes), including high expression of CD44 and low/no expression of CD24. These cells also have the ability to form mammospheres *in vitro*. TICs are resistant to typical treatment, and remain in high numbers after such treatment kills off the bulk of the tumor. The existence of TICs could explain why some patients relapse after standard chemotherapy. There is evidence that cells of the claudin-low subtype may be derived from these TICs. Analysis of breast cancer tumor databases with the TIC gene expression signature revealed that claudin-low tumors

expressed many of these same genes. Because TICs survive after conventional chemotherapy, it is essential to find ways to target and kill these TICs [17].

Oncogene Addiction and Targeted Therapies

Cancers that overexpress and depend on signaling from certain proteins can be said to be "addicted" to these oncogenes. Cancer cells depend very heavily on such pathways, so termination of signaling in those pathways can force cancer cells to cease proliferating. This provides a good therapeutic target using specific antibodies or small molecule inhibitors against these molecules. Normal cells are not so heavily dependent on these pathways and thus should not be negatively affected by such drugs. Some breast cancers are "addicted" to the HER2 and/or ER pathways. About 25-30% of breast cancers are HER2 amplified. The drugs trastuzumab/Herceptin® and pertuzumab are antibodies that bind and inhibit the HER2 receptor and have shown great success in patients with HER2 amplification [24]. Finding genes such as these, which are expressed at higher levels in TN breast cancer tissues, is critical for developing new therapies. Several tyrosine kinase inhibitors have been developed to inhibit several signaling pathways. For example, imatinib/Gleevec® targets multiple kinases in chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST). One weakness of targeting these addicting oncogenes is that patients soon acquire drug resistance and these mechanisms remain the focus of many intense studies. Perhaps acquired mutations may prevent the drugs from binding while preserving the kinase function of the protein. Alternatively, the cancer cells may instead upregulate another oncogenic pathway. Therefore, a combination of drugs may be the best approach for treating oncogene addiction in breast tumors.

EGFR and mTOR signaling in Triple-Negative Breast Cancer

Genetic and immunohistochemical analyses demonstrate that 50% of basal-like breast cancers, which account for about three quarters of TN breast cancers, express epidermal growth factor receptor (EGFR) [25] and that EGFR expression has been associated with poor prognosis [26]. However, the use of tyrosine kinase inhibitors directed towards EGFR in patients with unselected metastatic breast cancers produced little efficacy [27, 28]. More recently, the use of single agent cetuximab (an EGFR monoclonal antibody) in metastatic TN breast cancers patients resulted in a response rate of only 6% and a clinical benefit rate of 20% [29]. The addition of chemotherapy to cetuximab marginally increased the response rate to 17% [29]. Given these disappointing results, it appears that EGFR inhibition alone will not prove to be an effective therapeutic approach for patients with TN breast cancers.

The mTOR inhibitors, temsirolimus and everolimus, are currently approved for the treatment of metastatic renal carcinoma. The use of single agent mTOR inhibitors in patients with unselected metastatic breast cancers has not demonstrated encouraging results [30]. The suboptimal outcomes obtained from the use of single agent mTOR inhibitors, like rapamycin and its analogues (or rapalogues), in the treatment of metastatic solid tumors is thought to be due partly to an increase in phosphorylated AKT levels following exposure to these rapalogues [31]. mTOR inhibitor-induced AKT activation can be abrogated by the inhibition of upstream growth factors such as insulin-like growth-factor 1 receptors [31, 32].

Our Strategy: Targeting IGF-1R in TN Breast Cancer

The differential expression of IGF-1R in different tumor types marks it as a potentially excellent drug target for breast cancer patients whose tumors show increased IGF-1R expression. We proposed that some TN breast cancers with high levels of IGF-1R protein may be addicted to

this pathway and that targeting this receptor will suppress the aggressive and metastatic behavior of TN tumor cells. To test this hypothesis, stable shRNA IGF-1R knockdown cells were created and used to study the effects of IGF-1R knockdown on these cells compared to parental and empty vector control cell lines. We hypothesized that knockdown of IGF-1R signaling would decrease levels of the downstream pathways involved in survival, mitogenesis, differentiation, cell motility and adhesion in TN breast cancer cells. Because gene therapy is currently not a treatment option for patients, we also examined the effect of IGF-1R inhibition in TN breast cancer cells using an IGF-1R-specific monoclonal antibody.

Acquired drug resistance is always a concern in targeted therapies, so this drug was studied in combination with other inhibitors, including erlotinib (an EGFR inhibitor), lapatinib (an EGFR and HER2 dual inhibitor) and rapamycin (an mTOR inhibitor). Targeting multiple signaling molecules simultaneously may be the best option for treatment of aggressive TN breast cancer. Dual inhibition of EGFR and IGF-1R has been shown to provide further benefit and antineoplastic effects than inhibition of only one of these pathways. This combination of dual RTK inhibition has been studied in hepatocellular carcinoma, but not in TN breast cancer. Treatment of hepatocellular carcinoma cells (HCCS) with an anti-IGF-1R antibody led to moderate decreases in cell viability. It was found that cells were compensating for loss of IGF-1R through activation of the EGFR/HER3/AKT pathway. Combination treatments using this anti-IGF-1R antibody with a small molecule EGFR inhibitor or rapamycin showed greater effects through inhibition of AKT signaling, leading to cell cycle blockage. Combination treatments showed synergism and further reduced viability than treatments with single agents [33]. Thus, the anti-proliferative effects caused by loss of IGF-1R can be further enhanced through inhibition of EGFR and/or mTOR signaling.

Both IGF-1R and IGF-II expression levels have been previously found to be upregulated in AA normal tissue as compared to CA normal tissue [3]. Additionally, there is a positive correlation between circulating IGF-I levels and relative risk of breast cancer [18]. IGF-1R, IRS1 and Shc phosphorylation were significantly higher in AA tumor samples. The tumor suppressor IGF-2R was found to be upregulated in CA tumor samples as compared to AA tumor samples [3, 20]. Thus IGF-1R targeting may provide benefit to TN breast cancer patients, particularly AA women.

As noted earlier, addicting oncogenes are usually not critical to the survival of surrounding normal tissues. Under normal conditions, IGF-1R and IGF-2R are involved in development. The highest levels of IGF-1R and IGF-I signaling are found during embryogenesis; these levels decrease in adults [18]. IGF-2R shows its highest levels of expression during fetal developmental stages and organogenesis. These genes are important in early development and may not be as critical day to day in the adult mammal. Thus we speculate that abrogation of this pathway in adult patients should not negatively affect normal tissues. Progression of breast cancer is accompanied by reduced IGF-1R expression. Early tumors select for cells with high IGF-1R signaling, leading to increased proliferation. This rapid proliferation also increases the probability of creating mutations that favor tumorigenesis. IGF-I also plays a role in differentiation, so more advanced tumors select for cells with lower IGF-1R expression to avoid this fate. Metastases also tend to have lower IGF-1R levels as well [18]. IGF-1R's dual roles in differentiation and proliferation make the project more complex; however our strategy aims to inhibit proliferation.

Hypothesis and Specific Aims

Aberrant activation of growth factor signaling cascades promotes aggressive breast cancer phenotypes. Overexpression of a number of growth factor receptors, including IGF-1R, has been implicated in invasive tumor behavior. The purpose of this study is to examine the significance of IGF-1R signaling in the metastatic properties of TN breast cancers. We hypothesize that the worse survival of patients with TN breast cancers is partly due to differential growth factor expression and signaling through IGF-1R, and that inhibition of IGF-1R can provide a novel therapeutic approach that may be specifically effective in TN breast cancer patients. Furthermore, the combination of IGF-1R inhibition with EGFR and/or mTOR inhibition may improve the efficacy of IGF-1R inhibition in TN breast cancers and warrant further preclinical evaluation. Our specific aims were as follows:

Specific Aim 1: Create stable IGF-1R knockdown triple negative breast cancer cell lines. By creating stable lentiviral IGF-1R knockdown TN breast cancer cell lines, we will be able to generate models to investigate the contribution of the IGF-1R signaling pathway to the aggressive and metastatic behavior of TN tumors.

Specific Aim 2: Compare IGF-1R knockdown to control cells' response to IGF-I stimulation. We will also study key processes involved in cancer progression (including cell growth and survival) and determine which downstream signaling molecules play key roles in IGF-1R-mediated cancer progression in these cell line models.

Specific Aim 3: Examine the effects of IGF-1R knockdown in combination with mTOR and/or EGFR inhibition on cellular growth and signaling in TN breast cancer cell lines.

CHAPTER 2: Generation of Triple Negative Cell lines with A Stably Transfected IGF-1R (-/-) Knockdown Lentivirus

Specific Aim 1: Create stable IGF-1R knockdown triple negative breast cancer cell lines. By creating stable lentiviral IGF-1R knockdown TN breast cancer cell lines, we will be able to generate models to investigate the contribution of the IGF-1R signaling pathway to the aggressive and metastatic behavior of TN tumors.

Methods:

Cell lines, Antibodies and Reagents: The MDA-MB-231 breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA). The breast cancer cell lines HCC1806 and MDA-MB-468 were generously provided by Drs. Sean Kimbro and Paula Vertino, respectively, at the Winship Cancer Institute, Emory University. The HEK-293T packaging cell line was generously provided by Dr. Rita Nahta, at the Winship Cancer Institute, Emory University. All four cell lines were routinely maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2μM L-glutamine (Invitrogen, Carlsbad, CA, USA), and 1% each of: Pen-Strep Solution, Antibiotic-Antimycotic Solution. The antibodies against AKT, EGFR, IGF-1Rβ, p-AKT (S473), p-ERK (Thr202/Tyr204), p-IGF-1Rβ (Tyr1135), and PTEN were obtained from Cell Signaling Technology, Inc (Beverly, MA). Antibodies against β-catenin, p-EGFR (Tyr1173), and p-IRS1/2 (Ser270) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). E-Cadherin mouse IgG, and Fibronectin were from BD Biosciences. Antibodies against β-actin and Vimentin were obtained from Sigma (Saint Louis, MO). Secondary mouse and rabbit antibodies were from GE Healthcare (Piscataway, NJ). For the immunocytochemistry assay, primary

antibodies were used against E-Cadherin (1:1000, BD Biosciences), IGF-1R (1:500, Santa Cruz), and Vimentin (1:1000, Sigma). Secondary antibodies used were from the Invitrogen AlexaFluor series. IGF-1R α/β short hairpin (shRNA) plasmids and pLKO.1 empty vector (negative control) were obtained from Open Biosystems (Huntsville, AL, USA). The packaging and envelope helper plasmids (pCMV-dR8.2 dvpr and pCMV-VSV-G, respectively) were generously provided by Dr. Rita Nahta. Rapamycin (Rapa) was purchased from LC Laboratories (Woburn, MA). Figitumumab was from Pfizer (New York, NY). Erlotinib (Tarceva®, Genentech, San Francisco, CA) and lapatinib (Tykerb, GlaxoSmithKline, Research Triangle, NC) are commercially available. Protease inhibitor cocktail was purchased from Sigma (Saint Louis, MO) and puromycin was purchased from (Clonotech, Catalog # 8052-2).

Western Blotting: Total cell were harvested and prepared in NP-40 lysis buffer containing protease and phosphatase inhibitors (Sigma, Saint Louis, MO). Protein lysates were quantified using the Bradford Protein Assay. Thirty micrograms of whole cell protein lysate were separated by SDS-PAGE on 10% acrylamide gels. Proteins were transferred to PVDF membrane overnight at 4˚C. Membranes were blocked for 1h at room temperature in 5% nonfat dry milk in 1x-TBS. Membranes were subjected to Western blot analysis with antibodies following procedures described in manufacturer's instruction. Signals were detected by Enhanced Chemiluminescence (ECL) reagents (GE-Amersham, Piscataway, NJ), exposed on Hyblot CL autoradiography films (Denville Scientific, Metuchen, NJ) and developed using Konica SRX-101A medical film processor (Konica Medical & Graphic Corporation, Wayne, NJ).

RNA preparation and RT-PCR: Cells were grown to 70-80% confluency and total RNA was extracted using Trizol Reagent (Invitrogen, Cat. No. 15596-018, Carlsbad, CA). cDNA was synthesized from MDA-MB-468 and MDA-MB-231 TN cell lines using the iScript

cDNA synthesis kit (BioRad, Cat. No. 170-8890, Hercules, CA). cDNAs were used for PCR analysis using oligonucleotide primers specific for IGF-1R, IGF-2R, IRS-1, IRS-2, and GAPDH as a loading control (see Table 1 for primer pair sequences). The PCR conditions were as follows: 95 º C for 5 minutes, then 40 cycles at 95º C for 1 minute, annealed at 52-60º C for 1.5 min, extended at 72º C for 1 min and a final extension at 72º C for 15 min. PCR reactions were run on a 2% UltraPure agarose gel (Invitrogen). Bands were visualized under UV illumination.

Lentivirus preparation: The IGF-1R α/β shRNA plasmid and the pLKO.1 empty vector (EV) plasmid contained a puromycin resistance gene for selection. A puromycin kill curve was performed on all three cell lines to determine the concentration to be used for selection. 5 $x10^5$ cells/well (MDA-MB-468, MDA-MB-231, and HCC1806) were plated in 24-well plates in complete culture media. After 48 hours, media was changed to DMEM containing 0, 0.5, 1.0, 2.0, 5.0 or 10 µg/ml puromycin. Cells were then checked daily for five days to monitor cell death and determine the lowest concentration of antibiotic that kills 100% of cells.

Glycerol stocks of bacteria cells containing IGF-1R α/β shRNA (Open Biosystems, clones 422, 424, 425, and 426) and pLKO.1 plasmids were streaked and grown overnight on LB Agar plates containing 25 µg/ml kanamycin. Single colonies were selected, incubated in LB media containing 25 µg/ml kanamycin, and grown overnight in a shaker at 37˚ C. Plasmids were harvested using mini-prep reagents according to the manufacturer's protocol (Qiagen, Hilden, Germany). Plasmid DNA concentrations were measured by spectrophotometry. For production of each lentivirus, 1.5$x10^6$ HEK-293T cells were plated in 100 mm dishes and co-transfected with 3 µg shRNA constructs (either IGF-1R or pLKO.1), 3 µg pCMV-dR8.2, and 0.3 µg pCMV-VSV-G helper constructs following the TransIT-LT-1 Transfection manufacturer's protocol (Mirus Bio Corp., Madison, WI). Forty-eight hours after transfections, viral stocks were

harvested from culture media by centrifugation to remove cells. The viral supernatant was sterilized in a 0.22 micron filter and stored at -80º C until use. MDA-MB-231 and MDA-MB-468 cell lines were plated at subconfluent densities and infected with lentiviruses (1:20 dilution) in fresh culture media. Culture media was replaced with media containing 2 μg/ml puromycin 48 hours after lentivirus infection to select for breast cancer cells stably expressing the shRNA constructs. Stably-infected cells were harvested for use and cryopreservation.

***RESULTS*:**

ER, HER2, and IGF-1R expression levels were previously determined in our laboratory by Western blotting in a panel of triple negative (MDA-MB-468, HCC1806, CRL-2315, MDA-MB-231, Hs578t, BT-549), HER2+ (BT474), and ER+ (MCF-7) breast cancer cell lines (Figures 2A and 2B). ER and HER2 expression levels were undetectable in all the TN cell lines compared to ER+ MCF-7 and HER2+ BT-474 cell lines (Figure 2A). MDA-MB-468, MDA-MB-231, and BT-549 cells showed strong expression of IGF-1R, compared to the other TN cell lines, which expressed moderate to undetectable levels of IGF-1R (Figure 2B). Given the differences in IGF-1R expression levels, we chose the MDA-MB-231 and MDA-MB-468 cell lines for further evaluation. Expression of IGF-1R, IGF-2R, IRS-1, and IRS-2 mRNA transcripts were detected in both MDA-MB-468 and MDA-MB-231 TN breast cancer cells lines (Figure 2C). These data confirm that these two cell lines would make good models for knocking down IGF-1R expression, since the key signaling molecules in this pathway are present in these cell lines.

To generate TN breast cancer cell lines that lack expression of IGF-1R, we used vector-based shRNAs in a lentiviral system to ensure specific and stable gene silencing. Optimal puromycin concentration was determined to be 2 μg/ml for 231 and 468 cell lines. The HCC1806 (1806) cells were resistant to puromycin, even at 10 μg/ml puromycin concentration. We tested

several lentiviral clones and found that clone 424 (Cat. No. RHS3979-9568794) was able to specifically and efficiently knock down IGF-1R expression (Figures 3A and 3B). These stable cell lines were used for subsequent experiments. The empty vector pLKO.1 cells were verified by Western blotting to be similar to the parental cell line (Figure 3A), so they were used as control lines in the absence of parental lines for all studies. Even with multiple shRNA clones, we could not successfully generate stable IGF-1R knockdown HCC1806 cell lines without all of the cells dying. Most of the 1806 cells did not survive the transfection and did not proliferate even at the lowest concentration of puromycin. We were, however, able to generate and select for stable empty vector transfected 1806 cells.

CHAPTER 3: Effects of Stable Knockdown of IGF-1R on Triple Negative Breast Cancer Cells

Specific Aim 2: Compare responses of IGF-1R knockdown and control cells after IGF-I stimulation. We will also study key processes involved in cancer progression (including cell growth and survival) and determine which downstream signaling molecules play key roles in IGF-1R-mediated cancer progression in these cell line models.

Methods:

IGF-I stimulation and Western blots: Cells were plated in 6-well plates. Upon reaching 80-90% confluency, the cells were stimulated with 100 nM IGF-I for 15 min. The cells were harvested and their lysates were prepared and subjected to immunoblot assays as described above.

Colony formation assay: To perform colony formation assays we plated MDA-MB-468 and MDA-MB-231 breast cancer cells (single cell-suspension) in 12-well plates at a density of 250 cells per well overnight. After a 10-day growth period, the medium was removed and cell colonies were stained with crystal violet (0.1% in 20% methanol) for 1 hour and excess dye was washed off. Pictures were taken using a digital camera. All experiments were performed at least three times.

Immunocytochemistry: MDA-MB-468 and MDA-MB-231 TN breast cancer cells were plated in 4 well chamber-slides at a density of 50,000 cells/well and allowed to grow overnight in complete media. The media was aspirated off and cells were rinsed three times with 1X-PBS for 5 minutes each wash. Cells were fixed for 10 min at room temperature with 0.5 ml PHEMO fixative per well. Slides were washed three times in 1X-PBS for 5 minutes per wash and blocked with 3.0% BSA in 1X-PBS for one hour at room temperature. Primary antibodies were made up

in 3% BSA (E-Cadherin (1:1000, BD Biosciences), IGF-1R (1:500, Santa Cruz), and Vimentin (1:1000, Sigma)). Secondary antibodies used were from the Invitrogen AlexaFluor series. Diluted primary antibodies were applied to each well and incubated at 4˚C overnight in a humidified chamber. The next day, wells were rinsed 3x with 1X-PBS at room temperature, 5 minutes per wash. Secondary antibodies (Invitrogen, AlexaFluor) were made up in 3.0% BSA at a 1:500 dilution. Cells were incubated with secondary antibodies for one hour at room temperature. Cells were rinsed 3x with 1X-PBS at room temperature, 5 minutes per wash, stained with 350 nM Hoechst solution for 5 minutes at room temperature, and washed 3x with 1X-PBS as above. Cells were stained with Phalloidin (1:50) in 1X-PBS for 20 minutes and rinsed 3x as described above. Chambers were removed from the chamber-slides and coverslips were mounted onto slides using of mounting medium containing antifade agents (Biomedia Corp, GSS). Slides with coverslips were placed in dark at room temperature overnight and fixed and fluorescently stained cells were imaged using a Zeiss LSM510 laser scanning confocal system (Zeiss, Thornwood, NY, USA). All experiments were performed at using at least two independent biological replicates.

RESULTS:

Upon stimulation with IGF-I ligand, parental and empty vector cells upregulated IGF-1R expression. However, IGF-I failed to upregulate IGF-1R in IGF-1R (-/-) cell lines (Figure 3C). Collectively, these results verified the successful knockdown of IGF-1R protein expression in two morphologically distinct TN breast cancer cell lines. These lines are considered good models to examine the role of IGF-1R in TN tumor cell behavior. To test the time-dependent upregulation of IGF-1R in response to ligand, MDA-MB-231 parent, empty vector (EV) and two different knockdown (KD) cell lines (clones 423 and 424) were treated with 100 nM IGF-I for

either 15 minutes or 2 hours. Control cells were left untreated. At indicated times, cells were lysed and proteins were harvested and subjected to immunoblotting analysis. The parent and EV lines upregulated total IGF1-R with ligand stimulation. As expected, the KD (clone 424) cells still had no IGF1-R protein, even in the presence of the IGF-1 ligand (Figures 3B and 3C). Because IGF-I's effects are immediate, the 2 hour treatment was eliminated and all further ligand stimulation treatments were performed at 15 minutes.

To test the hypothesis that IGF-1R inhibition may alter anchorage-dependent growth of TN breast cancer cells, we subjected our knockdown models to colony formation assays. 468 KD cells formed significantly fewer colonies than controls. 231 KD cells formed fewer colonies than the controls, but this affect was less dramatic than in the 468s (Figure 4A). Hence, IGF-1R signaling in these cells is important for anchorage-dependent growth.

Next, IGF-1R-mediated proteins in the downstream PI3K survival pathway by were analyzed by Western blot (Figure 4B). In both 468 and 231, total AKT protein remained constant in both the EV and KD cells. In the 468 KD cells, p-AKT was dramatically reduced compared to the EV cells. The 231 EV and KD cells both showed low, equal levels of active pAKT, which is consistent with current literature which demonstrates that basal AKT levels are almost undetectable in 231 cells. PTEN tumor suppressor protein is normally absent in wild-type 468 cells (as described above), but its expression was induced by loss of IGF-1R. PTEN is present in wild-type 231 cells but expression appears to be lost in the 231 KD cells. These experiments are currently being repeated to confirm these results.

There is heterogeneity within the TN subtype in terms of morphology [22]. The two TN cell lines used in this study have different morphologies and associated gene expression patterns. The MDA-MB-468 cell line is classified as grape-like, and cells in this class express low levels

of epithelial marker, E-cadherin, and have poor cell-cell adhesion. MDA-MB-231 cells are stellate with an elongated shape and pointed ends that lack E-cadherin and are highly invasive. 468s have high levels of pAKT and do not express PTEN, as described above. Wild type 231s have very low levels of pAKT, and thus may not be as dependent on this pathway [22]. Because 231s belong to the claudin-low subtype they would be expected to express low levels of tight junction proteins and cell-cell adhesion proteins (including E-cadherin) [12].

An interesting phenotypic change was noticed in the IGF-1R KD cells. 468 KD cells appeared more stellate compared to the more cubodial 468 EV control cells; they appear to have undergone an epithelial to mesenchymal transition (EMT). Unlike the 468 KDs, the 231 KD cells changed from mesenchymal morphologies to more epithelial features, with the cells appearing to be more rounded (Figure 5A). It was concluded that the 231 KD cells were undergoing mesenchymal to epithelial transition (MET) and thus becoming less pathogenic and invasive.

To confirm our observations that differential EMT was taking place in our models, several epithelial and mesenchymal markers were analyzed by Western blot in the EV controls and KD cells (Figure 5B). We confirmed that the EV 231s lack and 468s express E-cadherin; however, IGF-1R KD in 231s induced expression of E-cadherin and IGF-1R KD in 468s caused a loss of E-cadherin expression. Another epithelial marker, β-catenin, was analyzed. Levels were consistent in 468 EV and KD cells, but expression was lost in 231 KD. Next, mesenchymal markers vimentin and fibronectin were analyzed. 468 EV cells did not express either of these proteins, but expression of both vimentin and fibronectin was induced in the 468 IGF-1R KD cells. 231 EV expressed high levels of vimentin, and this level was decreased in the 231 KD cells in the absence of IGF-1R. Neither 231 EV or KD cells expressed fibronectin.

To further illustrate the possible EMT in our EV and KD cells, immunocytochemistry (ICC) was performed (Figure 6). We assessed IGF-1R (data not shown), E-cadherin and vimentin expression levels and locations in 468 and 231 EV and KD cells. Nuclei were stained with Hoechst solution, and actin filaments were stained with Phalloidin. E-cadherin (which is critical for adherens junctions in epithelial cells) showed punctate staining in the cytoplasm of the 468 EV cells. In the 468 KD cells, E-cadherin appears ubiquitously. Vimentin, a component of the intermediate filaments of mesenchymal cells, is increased in the 468 KD cells compared to EV controls. In the 231 cell line, E-cadherin expression appears unaffected by IGF-1R knockdown, as levels remained unchanged in the EV and KD cells. Vimentin was slightly decreased in the 231 KD cells compared to the 231 EVs.

CHAPTER 4: Effects of IGF-1R, EGFR, and mTOR Inhibition on Triple-Negative Cell lines

Specific Aim 3: Examine the effects of IGF-1R inhibition in combination with mTOR and/or EGFR inhibition on cellular growth and signaling in TN breast cancer cell lines.

Methods:

Combination treatments and Western Blot Analyses: Combination treatments were performed with erlotinib, figitumumab (a monoclonal IGF-1R antibody), lapatinib, and/or rapamycin at indicated doses and times. Total protein lysates were collected and subjected to Western blotting as described above.

Cell survival assay: MDA-MB-231 and MDA-MB-468 cells were seeded at a density of 5000 cells/well in 96-well plates and grown overnight prior to treatments with various concentrations of erlotinib, lapatinib, or figitumumab alone or in combination for 72 hours. Cell viability was assessed by the sulforhodamine B (SRB) assay following procedures described previously.

RESULTS:

231 and 468 parental cell lines were treated with 20 μg/ml figitumumab for varying time points (0-24 hours) (Figure 7A). 468s showed high levels of IGF-1R protein, but it was significantly decreased with figitumumab treatment by 6 and 24 hours. 231s also responded well to figitumumab and IGF-1R levels were barely detectable by 6 and 24 hours. 231 and 468 cells were then treated with 20 μg/ml figitumumab, 10 μM lapatinib, and 10 μM erlotinib alone or in combination (Figure 7B). In the 231 cell line, the combination of figitumumab and erlotinib was

the most potent, with complete loss of both IGF-1R and EGFR observed. In the 468 cell line, erlotinib was more effective than lapatinib in inhibiting EGFR.

The 1806 cell line (in which IGF-1R stable knockdown was not possible) was treated with various concentrations of figitumumab (0-20 μM) for 24 hours (Figure 8A) followed by immunoblotting to determine the effects of IGF-1R inhibition on downstream signaling molecules. Figitumumab reduced IGF-1R protein levels in a dose-dependent and time-dependent manner (Figures 8A and 8B). A decrease in total IGF-1R was seen even at 0.625 μg/ml, the lowest concentration of figitumumab used (Figure 8A). A decrease in IGF-1R was observed starting at 3 hours post-treatment, and p-IRS1/2 was decreased at 1 hour. Total AKT remained unchanged, but levels of activated AKT decreased over time. Cells were treated with a 2-fold serial dilution of rapamycin alone (from 0.78nM to 100nM), figitumumab alone (from 0 to 20 μg/ml), and the agents in combination for 72 hours and cell survival was analyzed using SRB assays (Figure 8C). Treatment with figitumumab or rapamycin alone had little effect on cell survival. Figitumumab only showed a significant decrease in cell survival by the 2.5 μg/mL concentration. Treatment with increasing concentrations of rapamycin led to an approximately 20 % decrease in survival. The combination treatment resulted in a significant decrease in cell survival rate in a dose-dependent manner. The highest concentrations of drugs used (20 μg/mL figitumumab and 100 nM rapamycin) showed a 60% decrease in cell survival compared to the lowest concentrations (0.156 μg/mL figitumumab and .78 nM rapamycin). Combination treatments with figitumumab and lapatinib showed the most potent decrease in EGFR protein in this cell line (Figure 8D).

We analyzed the combined effects of rapamycin with erlotinib or lapatinib and/or figitumumab on IGF-1R protein in both 468 and 231 parental cell lines (Figure 9A) and MDA-

MB-468 empty vector and IGF-1R (-/-) cells (Figure 9B). The combined inhibition of mTOR, IGF-1R, and EGFR showed the greatest effects on IGF-1R reduction in both parental cell lines compared to single treatments with each inhibitor. We next compared the combined effects of rapamycin with erlotinib or lapatinib on activated EGFR (p-EGFR) protein levels in MDA-MB-468 EV and IGF-1R KD cells. Lapatinib alone and in combination with rapamycin decreased expression of activated EGFR more effectively in both cell lines, compared to erlotinib alone or in combination with rapamycin (Figure 9B). The fact that the TN cells express no or extremely low levels of HER2 suggests that the growth inhibitory effects of lapatinib are mediated through EGFR and not HER2.

To assess the effects of targeting multiple signaling pathways on TN cell survival, MDA-MB-231 and MDA-MB-468 cells were treated with 2-fold serial dilution of rapamycin alone (from 0.78nM to 100nM), figitumumab alone (from 0 to 20 μg/ml), and the agents in combination for 72 hours and cell survival was analyzed using SRB assays. For both cell lines, neither agent alone had a significant impact on cell survival, but combination treatments greatly reduced cell survival (Figure 10). In the 468 cell line, treatment with figitumumab alone showed a statistically significant decrease in survival only at the 2.5 and 5 μg/ml concentrations. In the 231 cell line, treatment with figitumumab alone did not show statistically significant changes in cell survival at any concentration. Treatment with rapamycin alone showed a statistically significant decrease in survival starting at the 0.3125 μg/ml concentration in the 468s and at the 5 μg/ml concentration in the 231s. In the 468s, a statistically significant decrease in survival was observed starting at the 0.625 μg/ml concentration figitumumab and 3.125 nM rapamycin combination treatments. In the 231s, a statistically significant decrease in survival was observed starting at the 0.3125 μg/ml concentration figitumumab and 1.56 nM rapamycin combination

treatments. For all three cell lines the baseline of survival was lower with the combination as compared to single treatments.

Next, 231 and 468 EV and KD cells were treated with varying doses of erlotinib (0-20 μM) for 72 hours and cell survival was analyzed via SRB (Figure 11). KD cells show slightly reduced initial survival as compared to the EV cells (1.0 vs 1.2 for the 468s and 1.02 vs 1.12 for the 231s). Erlotinib reduced survival in a very similar dose-dependent manner between the EV and KD cells.

CHAPTER 5: Discussion and Conclusions

Specific Aim 1:

Because of their similar expression levels of IGF-1R and other genes in the pathway, the 231 and 468 cell lines were selected for IGF-1R knockdown. The literature also stated that both cell lines contained mutant p53 and wild-type BRCA1. BRCA1 was reported to be unable to fulfill its normal task of inhibiting IGF-1R transcription when in the presence of mutant p53. This could help explain why both cell lines had approximately equal expression levels of IGF-1R protein. However, there are differences in the 231 and 468 cell lines. The 231 line is subtyped as basal B and claudin-low. The 468 line is basal A. They differ in PTEN expression; the 231s express a wild-type protein while the 468s do not show PTEN expression. PTEN inhibits PIP3 protein from phosphorylating and activating AKT. This could explain the observation that 468s highly express p-AKT while 231s have very low levels (due to the presence of PTEN). Their morphological differences are significant. The 468 cell line is more epithelial and less devolved into the stem-like cancer phenotype. The 231 cells appear more stem-like and less well differentiated, and thus farther along in the tumor progression pathway.

These differences in p53 status, BRCA1 status, and IGF-1R expression levels indicated that these two cell lines would be excellent candidates for side by side comparison of AA and CA TN breast cancer. A major focus of our laboratory is to deduce the outcome disparities between AA and CA TN breast cancer patients. However, the candidate cell lines' differences, especially those of morphology, may be responsible for the different responses observed after IGF-1R knockdown.

Specific Aim 2:

The results from the IGF-I ligand stimulation experiment provide further proof that the knockdown worked; even in the presence of the ligand, the knockdown cells were unable to induce IGF-1R expression. IGF-1R has dual roles in proliferation and differentiation. As noted above, primary tumors may express high levels of IGF-1R to increase proliferation. The results of the colony formation assay support this idea; the KD cells showed fewer colonies than the EV control cells. However, the two KD cell lines showed different responses; the 468s had fewer colonies than did the 231s. These different responses may be due to the fact that the two cell types have different initial morphologies.

The effects of IGF-1R knockdown on the PI3K survival pathway are interesting. As described above, the 468 cell line does not express PTEN protein. Both alleles have a substitution at codon 253 (effect unknown), and there is a deletion at codon 70, but the zygosity of this mutation is not known. After IGF-1R knockdown, expression of PTEN protein was induced in the 468 cell line. This suggests that the other allele is wild-type at codon 70, and was silenced by promoter methylation or another reversible event. With loss of IGF-1R expression, the repression of this PTEN allele was abrogated, leading to re-expression of protein. Our data suggest that the substitution at codon 253 has no effect, and that this protein may retain wild-type activity. The induced, active PTEN protein could have then inhibited PIP3 from activating AKT to p-AKT, leading to the observed decrease in p-AKT expression.

The morphological changes induced by loss of IGF-1R expression are interesting. It is known that IGF-1R downstream signaling affects motility and cell-cell adhesive properties. The 231 cell line may have been addicted to IGF-1R for maintaining its mesenchymal phenotype, and without it the cells reverted to a more epithelial state. It has been found that primary tumors

overexpress IGF-1R and their metastases tend to decrease this expression to avoid differentiation. It appears that the 468 cells relied on IGF-1R to maintain their more differentiated state. Upon loss of this signaling, they dedifferentiated to a more stem-like state. However, the role of IGF-1R in differentiation is not consistent in the 231s, as knockdown appeared to make the cells more differentiated. There may be other pathways at work responsible for this observation. Knockdown of IGF-1R in the 231 cells seems to have caused the cells to undergo MET, which would be of value in the clinical setting.

IGF-1R plays a role in EMT. Its expression can lead to sequestration and subsequent degradation of E-cadherin and the translocation of β-catenin to the nucleus (Figure 5C). Loss of E-cadherin is associated with increased tumor invasiveness and passive dissemination of cancer cells [34]. The role of E-cadherin is to bind β-catenin and form adherens junctions which function in cell-cell adhesion and migration. β-catenin bound to E-cadherin in these junctions is unable to translocate to the nucleus to turn on target genes. Loss of E-cadherin disrupts cell-cell adhesion and leads to more β-catenin in the nucleus. In the cytosol, β-catenin is vulnerable to sequestration by an inhibitory complex containing GSK-3 which phosphorylates and marks β-catenin for degradation. Active AKT phosphorylates and deactivates GSK-3, thus protecting β-catenin. Once in the nucleus, β-catenin acts as a transcription factor with T cell factor (TCF) and is involved in stem cell self-renewal and maintenance [35]. Nuclear β-catenin upregulates cyclin D1, c-Myc, Snail and slug. Nuclear Snail causes further translocation of β-catenin to the nucleus and also represses expression of E-cadherin [34]. Nuclear β-catenin has also been correlated with EMT and expression of CD44, a marker of TICs. Thus, activation of AKT and β-catenin is a critical event in EMT; these proteins work together to promote EMT [35].

A gene expression signature of EMT has been characterized and was closely correlated to that of basal B cell lines (such as the 231s), but not the basal A (468s), or luminal cell lines [36]. In the Western blots of EMT markers the vimentin decrease and E-cadherin increase support our hypothesis that 231s are undergoing MET. The 468s appear to be undergoing EMT because E-cadherin was lost and vimentin was gained. Vimentin is a mesenchymal marker, the intermediate filaments of mesenchymal cells are mainly composed of vimentin [34]. These blots are from whole cell lysates. The subcellular location of β-catenin determines its function, so these blots do not provide much insight into what the β-catenin was doing. Fibronectin is another marker of mesenchymal cells [36]. The EV 468 cells did not express fibronectin, but this protein was induced in the KD cells, indicating EMT. The 231 EV cells do not express fibronectin, but it is slightly visible in the KD cells.

The ICC results do not correlate exactly with the results from the EMT western blot. The EV 468 cells show the expected membrane-bound E-cadherin protein, but this protein appears everywhere and inside the KD cells. Vimentin, a major component of intermediate filaments in mesenchymal cells, shows more staining in the 468 KD versus EV cells. E-cadherin shows less specific staining throughout the EV and KD 231 cells. Vimentin in these cells is higher in the EV as compared to KD cells.

Specific Aim 3:

The epidermal growth factor receptor (EGFR) can also be involved in EMT. Receptor activation, either by EGF, TGF-β, or other ligand binding can disrupt desmosomal and/or adherens junctions, which leads to free cancer cells. Inhibition of EGFR in inflammatory breast cancer cells (using the drug erlotinib) reversed the cells' mesenchymal phenotype [34]. Because the IGF-1R and IR share structural homology, hybrid receptors can be formed between one IGF-

1R α/β-chain and one IR α/β-chain. Other groups developed dual IGF-1R/IR small molecule inhibitors. Results *in vivo* and *in vitro* have indicated that such inhibitors significantly decrease growth. However, it was found that mice treated with BMS-754807 (one such IGF-1R/IR inhibitor) showed hyperinsulinemia [37]. These compounds can also cause hyperglycemia, which can be treated with metformin, a commonly used type II diabetes drug [8]. However, our study specifically looked at IGF-1R and not IR inhibition, which may avoid the pitfall of affecting blood glucose and insulin levels *in vivo.* Our approach of targeting IGF-1R in combination with EGFR and mTOR inhibition avoids this problem as well. Additionally, the close homology of these proteins may lead to cross reactivity of the antibodies. The Cell Signaling IGF-1Rβ antibody we used for western blots stated that it does not cross react with IR. However, because of the close conservation of phosphorylation sites between these two receptor proteins, the p-IGF-1Rβ does cross react with p-IR according to the manufacturer's data sheet.

The 1806 cell line (in which IGF-1R stable knockdown was not possible) was more vulnerable to IGF-1R inhibition using figitumumab than the other cell lines were. The 231 and 468 lines required 20 μg/ml figitumumab for at least six hours to show a decrease in IGF-1R protein. However, in the 1806 cell line, a decrease in total IGF-1R was seen at 0.625 μg/ml for 24 hours. Phosphorylated IGF-1R was also significantly decreased at this low concentration. In the 1806 cell line, single treatment with figitumumab or rapamycin had only modest effects, while a dual treatment dramatically decreased survival. It is interesting that a combination was more effective than single treatment. Though treatment with figitumumab alone may not provide much benefit, combination appears to provide synergism. In the 1806 cell line, combination with figitumumab and erlotinib showed the most potent decrease in EGFR protein.

468 and 231 cells were treated with figitumumab, rapamycin, lapatinib, and erlotinib in various combinations. In the 468 cell line, treatment with figitumumab appears to increase IGF-1R expression, which does not correlate because the drug is an IGF-1R inhibitory antibody. In the 231 cell line, figitumumab has the expected effect of a decrease in IGF-1R. In both cell lines, the combination of figitumumab, rapamycin, and erlotinib appears very potent and there is no detectable IGF-1R protein.

In the 468 EV cell line, lapatinib showed the best inhibition of p-EGFR, alone and in combination with rapamycin. In the 468 KD cell line, erlotinib showed the best inhibition of p-EGFR, alone and in combination with rapamycin. The fact that the TN cells express no or extremely low levels of HER2 suggests that the growth inhibitory effects of lapatinib are mediated through EGFR and not HER2. The 231 and 468 cell survival assays with figitumumab and rapamycin single and combination treatments showed the best results with the combination treatments. These results indicate that the figitumumab IGF-1R inhibitor has little effect on its own, but in combination with EGFR or mTOR inhibition may provide therapeutic benefit.

Conclusions

IGF-1R signaling plays a role in cellular growth, proliferation, survival, adhesion, and mitogenesis. It is upregulated in many TN breast cancers, making it a tempting molecular target for this disease. IGF-1R inhibition may provide added benefit for African-American patients, where this pathway is already highly expressed, and who more frequently have mutations in BRCA1, a transcriptional inhibitor of IGF-1R. To study the potential therapeutic effects of IGF-1R inhibition, we successfully created two stable IGF-1R lentiviral knockdown cell lines. The AA-derived MDA-MB-468 cell line and the CA-derived MDA-MB-231 cell line were used for this study. Knockdown of IGF-1R signaling in these cells was confirmed by lack of response to

IGF-I ligand stimulation. As expected, a decrease in proliferation was observed after knockdown. These two representative cell lines expressed similar levels of genes in the IGF-1R pathway, but had different morphologies. The 468 cell line has a grape-like, more epithelial morphology while the CA-derived 231 cell line has a stellate, mesenchymal phenotype. After IGF-1R knockdown, the 468 cell line appeared to be undergoing epithelial to mesenchymal transition (EMT). The 231 cell line appeared to be undergoing the opposite process, mesenchymal to epithelial transition (MET). To explore this hypothesis, western blots and immunocytochemistry (ICC) were performed probing for EMT markers. There was some evidence that the knockdown cells were changing morphology, but it was not clear-cut. IGF-1R plays a role in morphology and adhesion, which may explain these transitions. Additionally, it was found that the 468 cell line, but not the 231 cell line, drastically upregulated EGFR signaling upon loss of IGF-1R expression. EGFR also can cause EMT to occur, providing further mechanisms for why the 468 cell line underwent this transition. Stable lentiviral knockdown was attempted multiple times, but ultimately not possible in the HCC1806 AA-derived cell line. This provides further support that IGF-1R signaling is a good target, because some tumors may be addicted to it. Overall, the results of the lentiviral knockdown confirmed that IGF-1R may be a good molecular target for the treatment of TN breast cancer.

Acquired resistance is always a concern for targeted therapies; cells often respond by upregulating another mitogenic pathway. Thus to address this weakness, combination treatments were performed with erlotinib (an EGFR inhibitor), figitumumab (an IGF-1R inhibitor), lapatinib (a dual EGFR/HER2 inhibitor) and rapamycin (an inhibitor of mTOR). Figitumumab, the specific anti-IGF-1R monoclonal antibody, was able to decrease IGF-1R expression in all three cell lines. This inhibition was only appreciable at 20 μg/ml of figitumumab in the 468 and 231

cell lines. Total loss of IGF-1R was observed at 0.625 μg/ml of figitumumab in the 1806 cell line, adding further evidence of its dependence and addiction to IGF-1R signaling. The effects of figitumumab alone on survival were very moderate, but enhanced greatly when combined with rapamycin treatment.

Overall, these data show that IGF-1R inhibition in combination with EGFR or mTOR inhibition decreases signaling in these pathways and lead to reduces survival in TN breast cancer cells. However, the different responses of EMT/MET also indicate that care should be taken in determining which patients should be given these treatments. Clinically, MET would provide benefit and make the cells less pathogenic and easier to target. EMT would make the tumor cells more metastatic, stem-like, and resistant to chemotherapies. These findings warrant further investigation into the therapeutic potential of IGF-1R inhibition alone and in combination with EGFR or mTOR inhibition.

Triple negative (TN) breast cancers, which lack estrogen receptor, progesterone receptor and HER2/neu overexpression, lead to poor survival outcomes in patients partly because of a lack of therapeutic targets. Thirty six percent of TN breast cancers express insulin-like growth factor 1 receptor (IGF-1R) which plays a role in proliferation, apoptosis, adhesion and invasion. Stable lentiviral IGF-1R knockdown was performed in two morphologically distinct cell lines, the epithelial basal A MDA-MB-468 cell line and the mesenchymal basal B MDA-MB-231 cell line. Knockdown led to down regulation of AKT signaling in both cell lines, but different responses in terms of morphology and cell-adhesive properties. The 468 cell line appeared to undergo MET (mesenchymal to epithelial transition) and the 231s underwent the opposite process, EMT. Combination treatments with IGF-1R, EGFR, and mTOR inhibitors decreased cell survival further than IGF-1R inhibition alone. These results suggest that IGF-1R inhibition,

in combination with EGFR and/or mTOR inhibition, may provide clinical benefit in patients, particularly those with mesenchymal-like tumors and may warrant further studies examining combinatorial inhibition *in vitro* and eventually *in vivo*.

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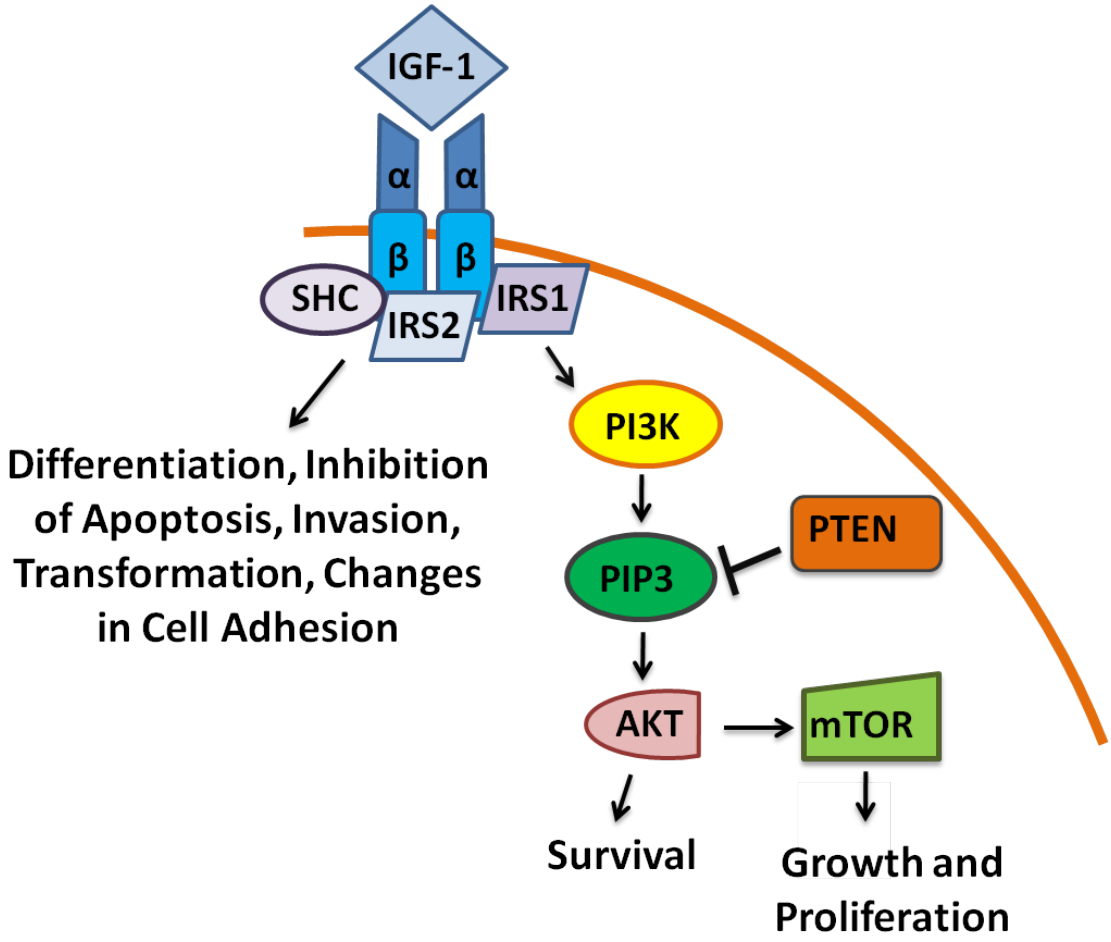


Figure 1: Insulin-like growth factor 1 signaling.

Table 1: Primers sequences used for RT-PCR.

Gene	Product Length (bp)	Sequence 5'-3'	Length	TM
IGF-1R Forward	314	TAAAAATGGCCAGAACCTGAG	21	50
IGF-1R Reverse		ATTATAACCAAGCCTCCCAC	20	48
IGF-2R Forward	144	TACAACTTCCGGTGGTACACCA	22	55
IGF-2R Reverse		CATGGCATACCAGTTTCCTCCA	22	54
IRS1 Forward	551	GCAGCCCCACCTGCCTCGAAAGGTAGACAC	30	68
IRS1 Reverse		CAGCAATGCCTGTCCGCATGTCAGCATAGC	30	66
IRS2 Forward	950	GAAGACAGTGGGTACATGCGAATG	24	58
IRS2 Reverse		CCTCATGGAGGAAGGCACTGCTG	23	62
GAPDH Forward	129	ACAACTTTGGTATCGTGGAAGGAC	24	57
GAPDH Reverse		CAGGGATGATGTTCTGGAGAGC	22	57

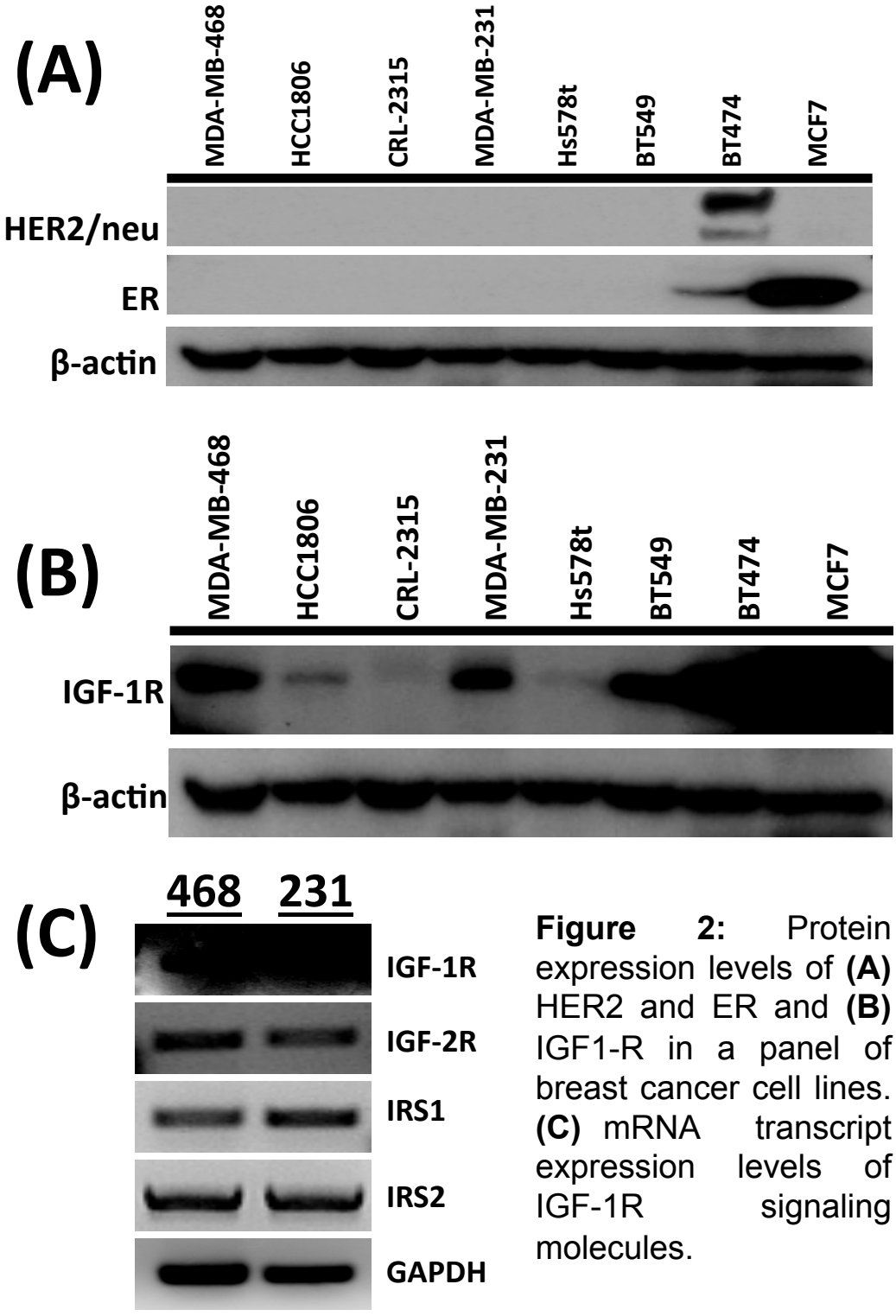


Figure 2: Protein expression levels of **(A)** HER2 and ER and **(B)** IGF1-R in a panel of breast cancer cell lines. **(C)** mRNA transcript expression levels of IGF-1R signaling molecules.

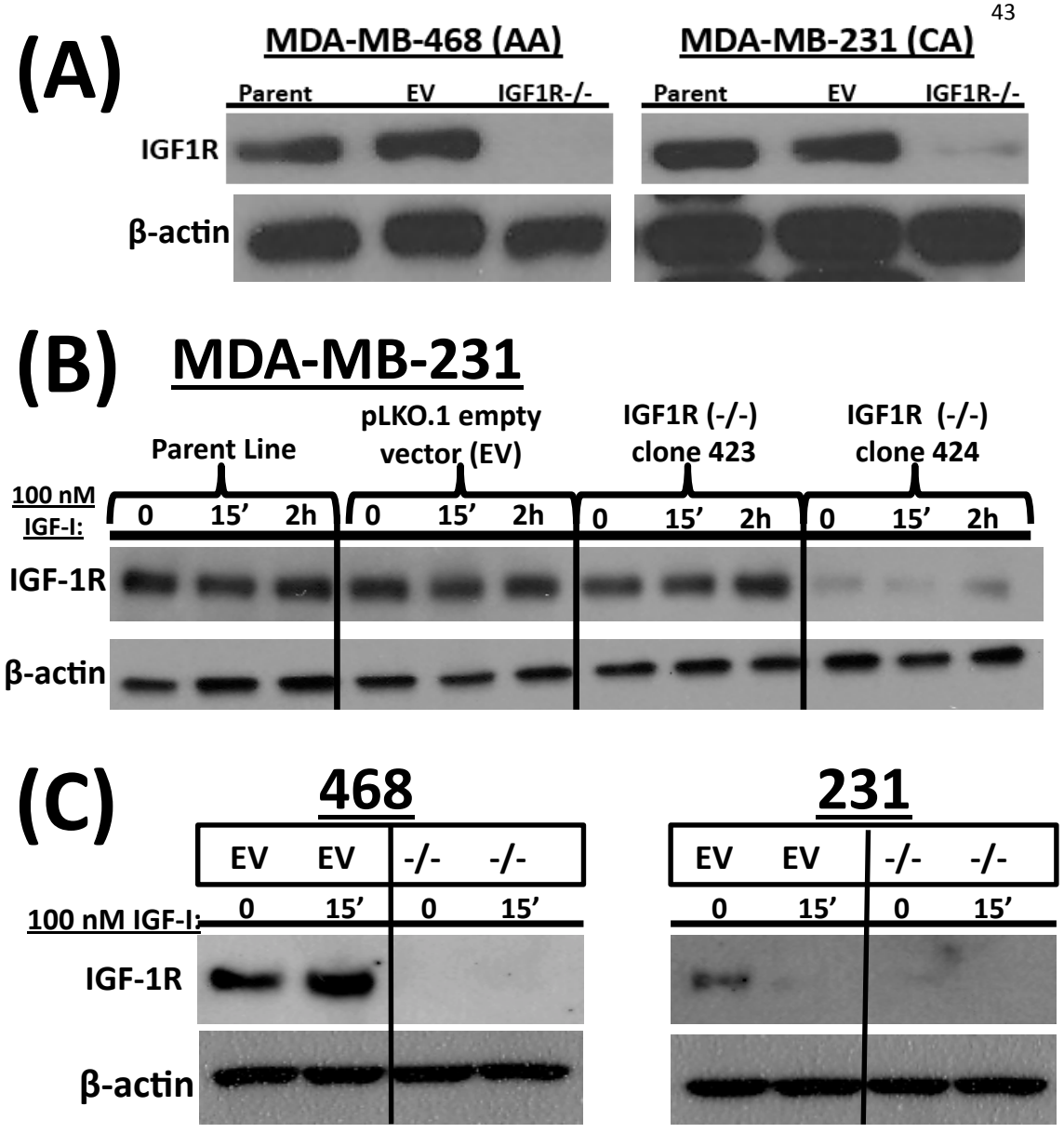


Figure 3: ***Stable knockdown of IGF-1R in triple-negative breast cancer cell lines.*** **(A)** Loss of expression of IGF-1R in triple-negative breast cancer cells using IGF-1R(-/-) clone 424. **(B)** Selection of IGF-1R lentiviral clone for stable knockdown studies. **(C)** IGF-I stimulated empty vector control and IGF-1R-negative cells.

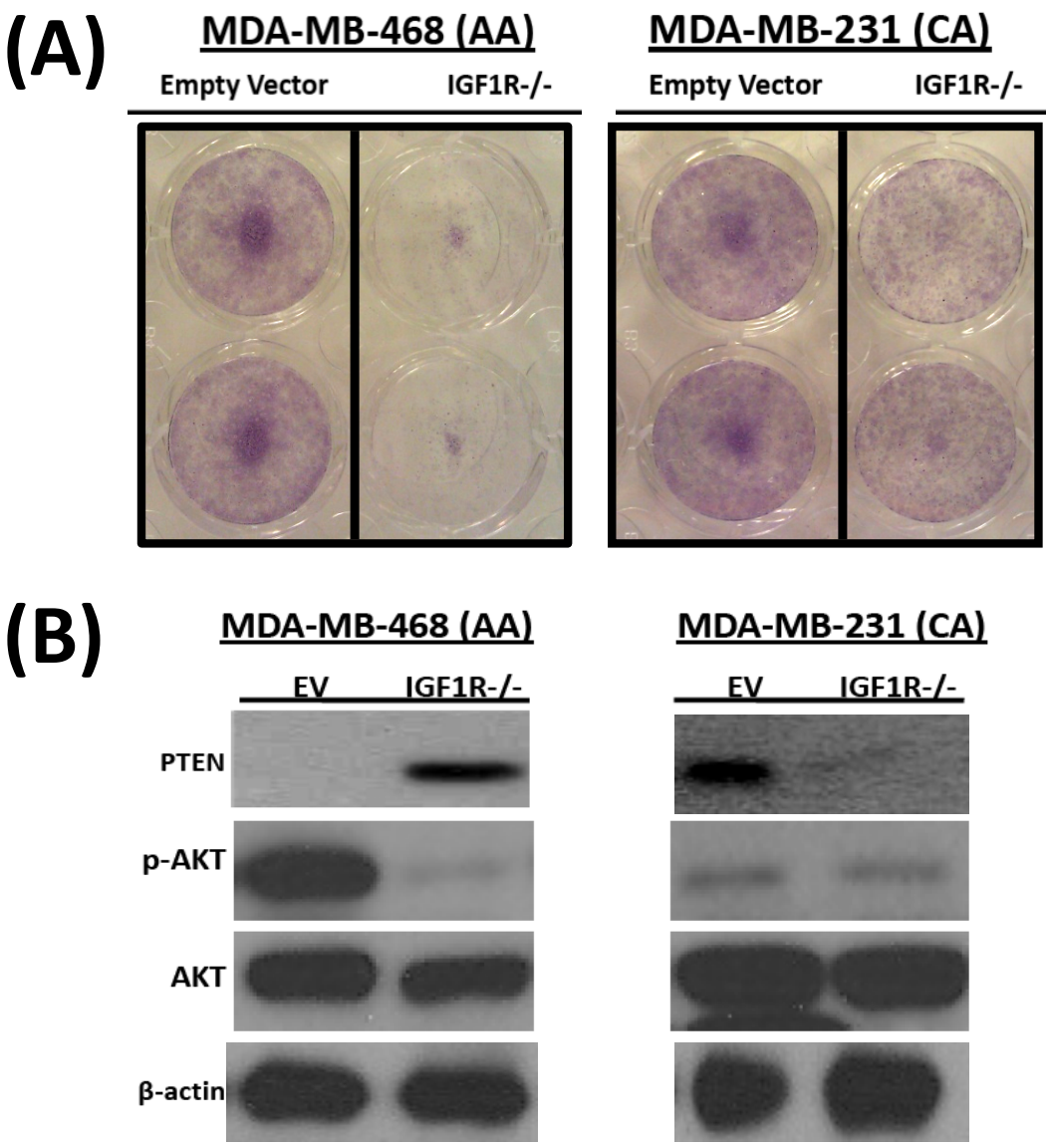


Figure 4: IGF-1R knockdown differentially affects **(A)** colony formation and **(B)** AKT signaling in triple-negative breast cancer cell lines.

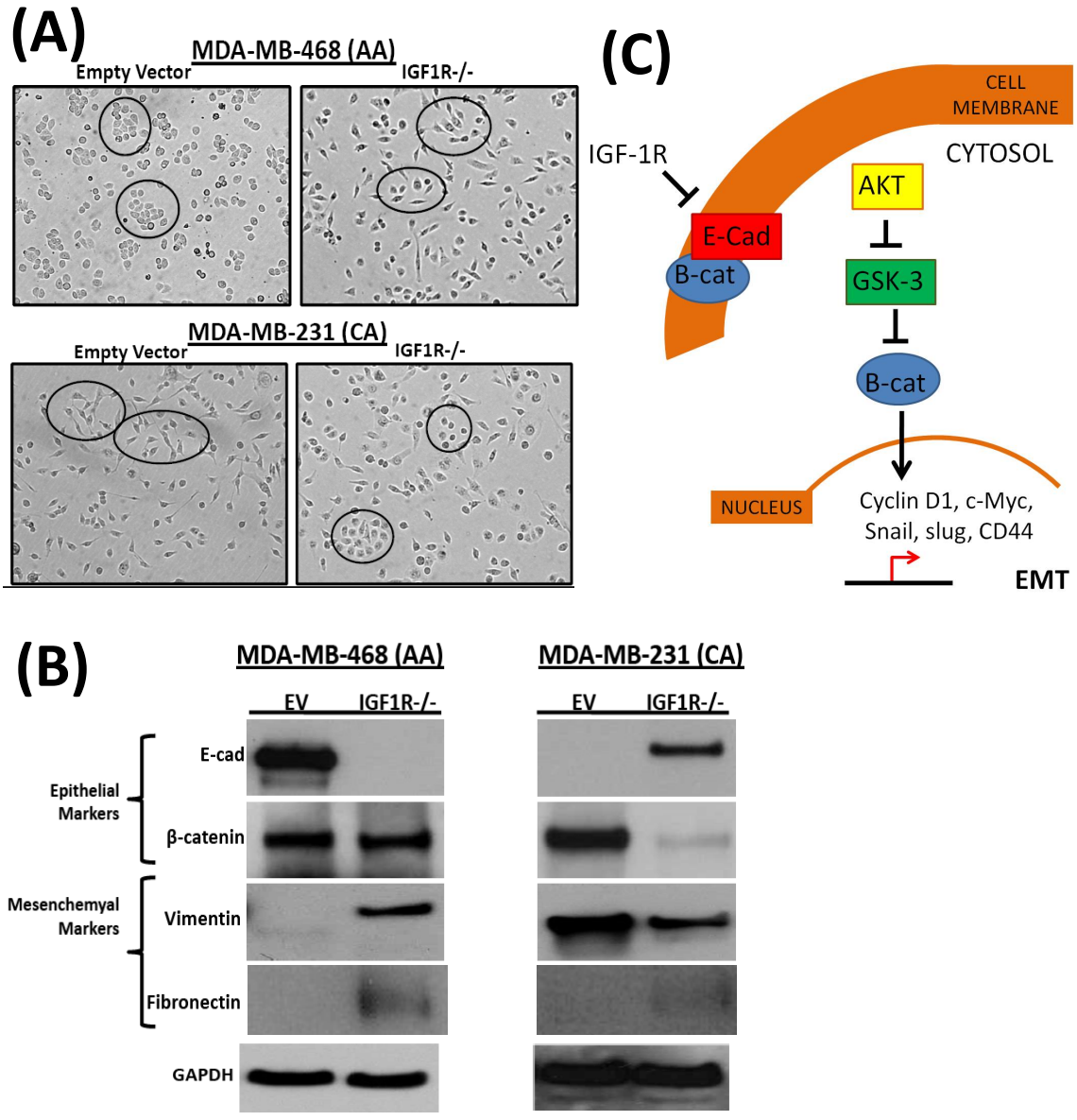


Figure 5: ***Assessment of EMT in triple-negative breast cancer cells*****. (A)** IGF-1R inhibition differentially modulates epithelial-to-mesenchymal transition in TN breast cancer cells. **(B)** Expression of E-cadherin (E-cad), beta-catenin (β-catenin), vimentin, fibronectin, and GAPDH in empty vector and IGF-1R knockdown TN cell lines. (**C)** The IGF-1R/β-catenin signaling mechanism in EMT.

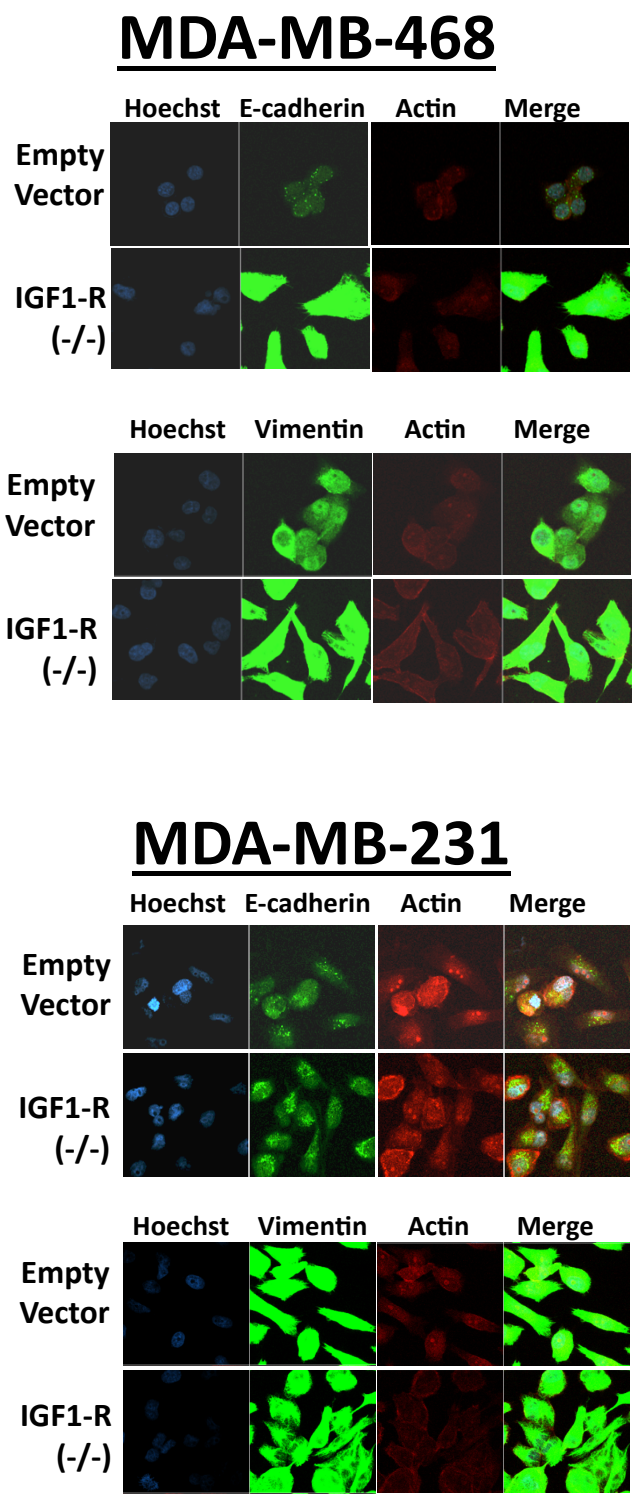


Figure 6: ***Assessment of EMT markers in human triple-negative (TN) breast cancer cells*****.**
The expression of E-cadherin, vimentin, and actin fibers were analyzed by immunocytochemical staining in triple-negative breast cancer cell lines.

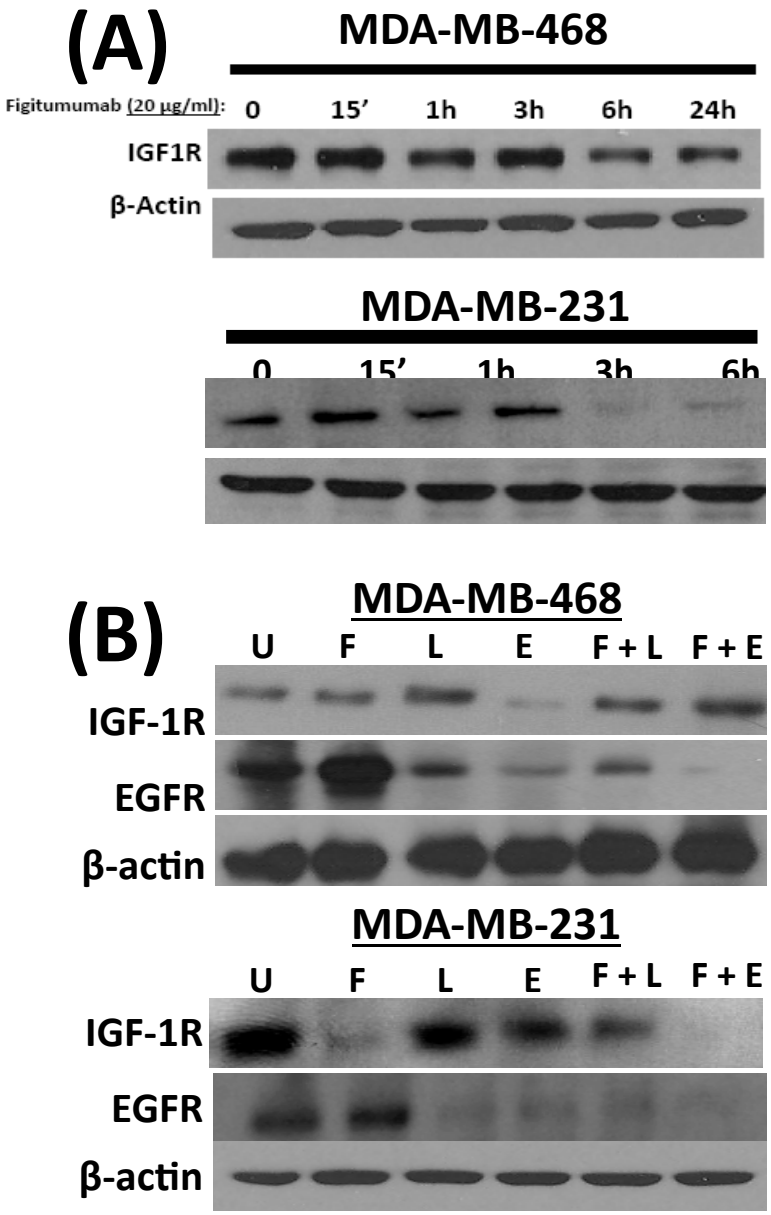
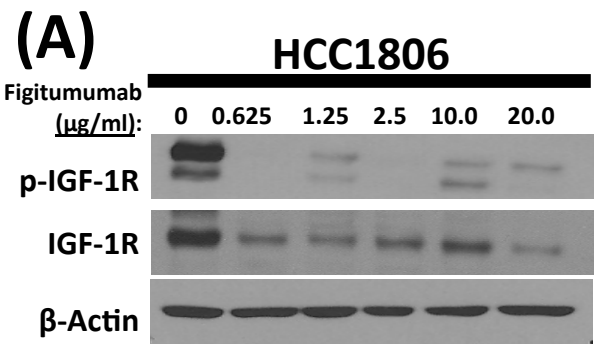
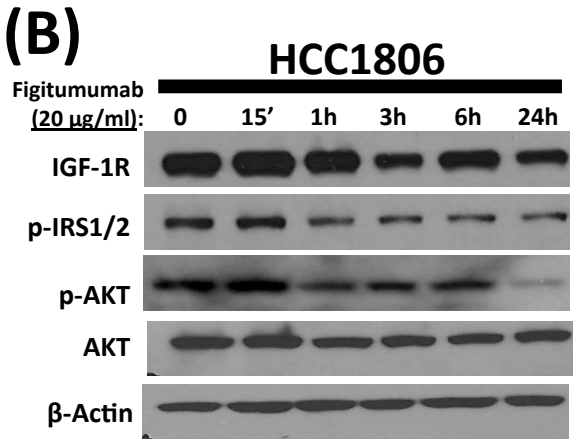


Figure 7: ***Effects of human IGF-1R and EGFR monoclonal antibodies on receptor expression levels in TN breast cancer cell lines.*** **(A)** IGF-1R inhibition with figitumumab decreases IGF-1R expression in triple-negative breast cancer cells. **(B)** Combination IGF-1R/EGFR inhibition effectively reduces receptor expression levels better than single inhibition. Untreated (U), Figitumumab (F), Lapatinib (L), Eroltinib (E).





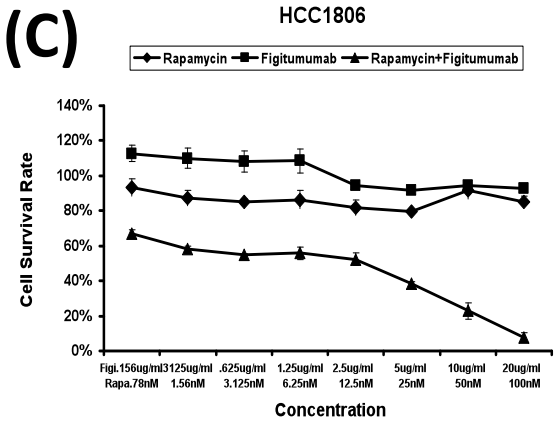
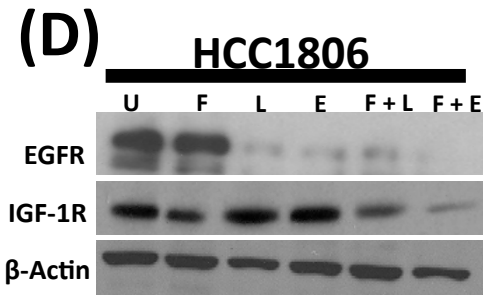
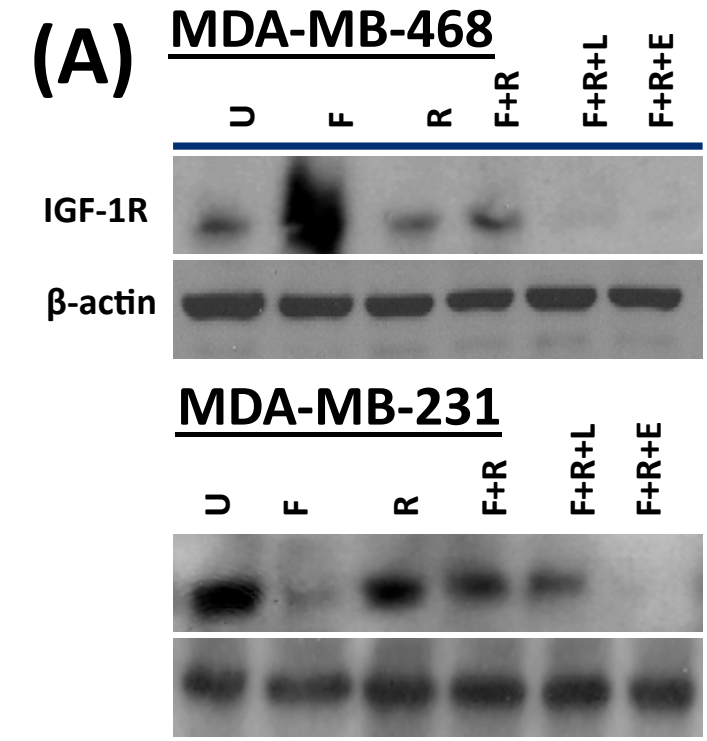


Figure 8: ***Combined inhibition effectively reduces IGF-1R signaling and survival in HCC1806 TN breast cancer cells.*** Treatment with figitumumab decreases IGF-1R signaling in HCC1806 cells in a dose-dependent **(A)** and time-dependent **(B)** manner. **(C)** IGF-1R/ mTOR combined inhibition reduces survival in a dose-dependent manner. **(D)** Combination treatments with figitumumab (F), lapatinib (L), and erlotinib (E) decrease EGFR and IGF-1R. Treatment with rapamycin and figitumumab decreases survival in a dose-dependent manner.





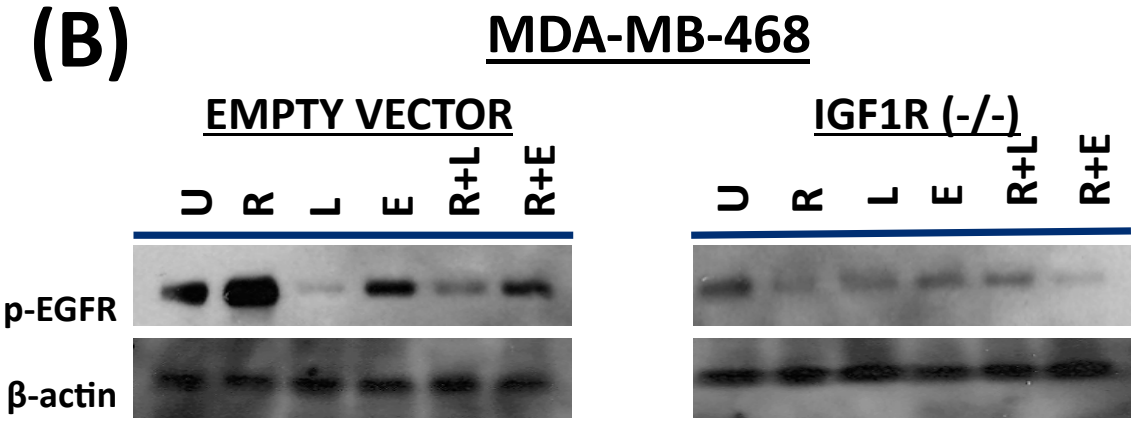


Figure 9: ***Effects of combination inhibitors on IGF-1R and EGFR expression in TN breast cancer cells.*** **(A)** Figitumumab (F), rapamycin (R), lapatinib (L), and erlotinib (E) together reduce IGF-1R levels in triple negative breast cancer cells. Untreated control cells are denoted (U). **(B)** Knockdown of IGF-1R makes 468 cells more sensitive to EGFR inhibitors.

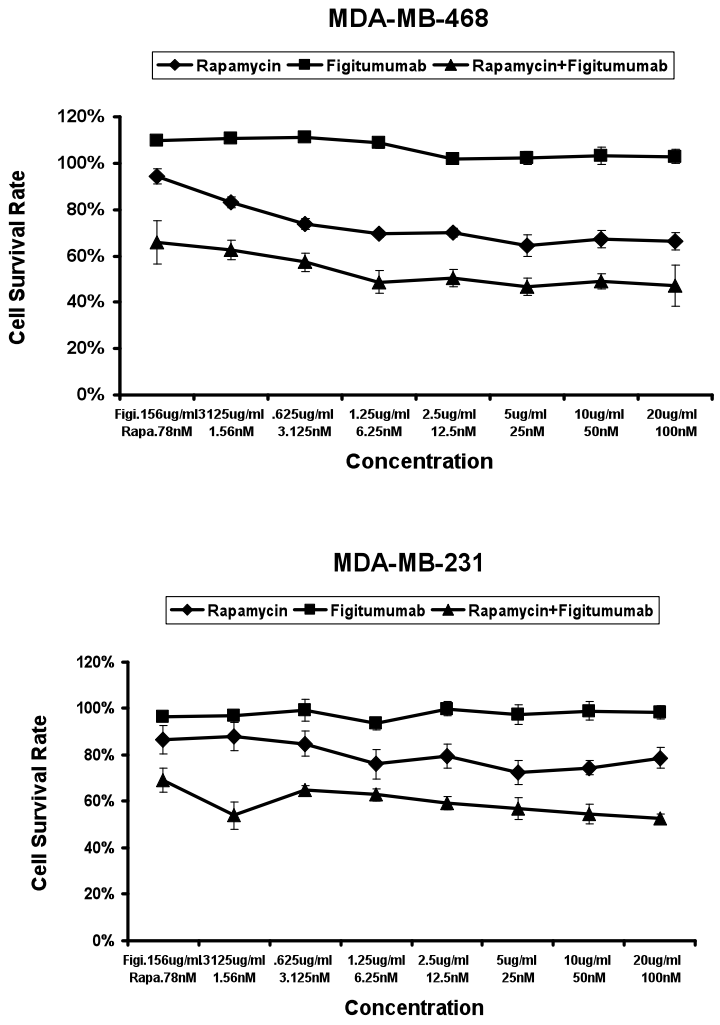


Figure 10: ***Effects of combined inhibition on TN breast cancer cell survival.*** Treatment of triple-negative breast cancer cells with rapamycin and figitumumab together reduces cell survival in 468 and 231 cells.

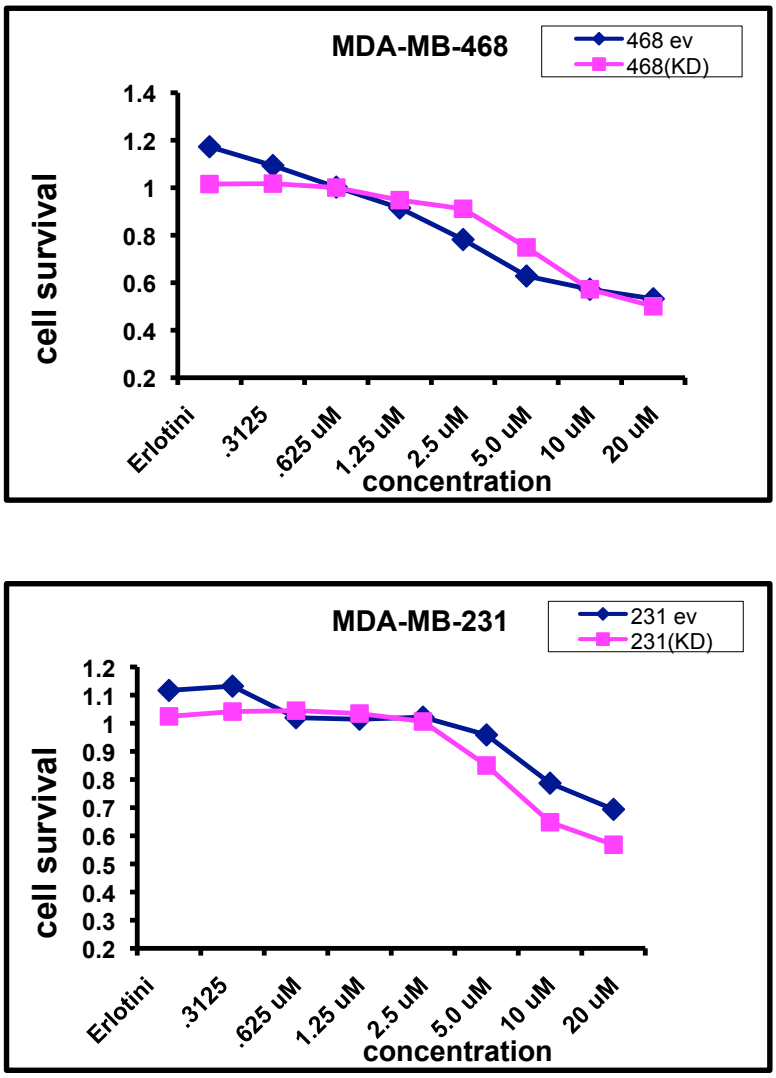


Figure 11: ***Effects of combined inhibition on TN IGF-1R (-/-) breast cancer cell survival.***
Empty vector (EV) and knockdown (KD) triple-negative breast cancer cells treated with increasing doses of erlotinib.