EXAMINATION OF MOLECULAR CHANGES IN ACQUIRED TAMOXIFEN RESISTANCE AND SUBSEQUENT RESPONSE TO ANTI-IGF1R THERAPY

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Abstract

The type-I insulin like growth factor (IGF1R) contributes to the tumorigenicity, proliferation, survival, metastasis, and drug resistance of breast cancer cells. Disruption of type I insulin-like growth factor receptor (IGF1R) signaling alone or in combination with established therapies has emerged as an important strategy in cancer therapy. Several anti-IGF1R antibodies and tyrosine kinase inhibitors (TKI's) are being evaluated in phase I, II, or III clinical trials, often in endocrine resistant populations. Thus far, clinical trials have provided less than promising results. Although preclinical studies showed promising results, these studies were performed using endocrine sensitive cell models. Here, we sought to determine the efficacy of IGF1R inhibitors using an endocrine resistant human breast cancer cell model in vitro and in vivo.

Tamoxifen is widely used in all stages of breast cancer treatment; from prevention to advanced disease. Although many patients with estrogen receptor positive disease benefit from hormonal therapy, such as tamoxifen, resistance ultimately ensues. Historically, after exhausting hormonal therapy options, chemotherapy treatment was given. More recently, growth factor receptors have emerged as targets in breast cancer therapy. These receptors have also been implicated in endocrine resistance. Understanding the mechanisms of endocrine resistance, including the role of the estrogen receptor and its interplay with growth factor receptors is the key to developing and utilizing emerging therapies in this patient population.

The first section of this work highlights the creation and characterization of a tamoxifen-resistant cell line. We demonstrate in two estrogen receptor positive breast cancer cell lines that tamoxifen resistant cells maintain estrogen receptor expression.

Additionally, the estrogen receptor maintained its ability to become phosphorylated and activate transcription of genes. Although basal levels of known estrogen-regulated genes were lower in TamR cells, estrogen was able to stimulate the transcription of unique genes in TamR, but not parental cells. Levels of IGF1R, a known estrogen regulated gene, were greatly reduced in TamR cells and protein levels were undetectable. Further, signaling, proliferation, and anchorage independent growth through the receptor were abolished in TamR cells. Interestingly, signaling and growth through the closely related insulin receptor (a non-estrogen regulated gene) remained intact in TamR cells. Finally, we show that estrogen can stimulate the proliferation of both parental and TamR cells and that this proliferation can be inhibited with the pure antiestrogen fulvestrant. Thus, estrogen receptor expression is preserved in TamR cells and still plays a role in the growth of these cells. Our findings are validated by clinical studies that demonstrated a benefit to using aromatase inhibitors and pure antiestrogens in tamoxifen resistant patients.

The second part of this work utilizes TamR cells to examine the efficacy of IGF1R inhibitors in endocrine resistant and sensitive breast cancer cells. We show that the signaling, proliferation, and anchorage-independent growth of endocrine sensitive MCF-7 cells can be inhibited using a variety of IGF1R monoclonal antibodies. Further, estrogen stimulated xenograft growth can also be inhibited using an IGF1R monoclonal antibody. TamR cells, which lack IGF1R expression, are not affected by IGF1R antibody treatment. In contrast, tyrosine kinase inhibitors which can inhibit both IGF1R and IR

were able to inhibit the signaling, proliferation, and anchorage-independent growth of both TamR and parental cells.

Taken together, our data demonstrate that tamoxifen resistant cells and tamoxifen treated xenografts have reduced levels of IGF1R, making IGF1R antibody treatment ineffective. Our work highlights the importance of evaluating new therapies using a preclinical model that matches the patient population the therapy will be used in. Finally, our data suggest that inhibition of IR may be necessary to manage tamoxifen resistant breast cancer.

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Introduction

Introduction

Breast cancer accounts for nearly 1 in 3 cancers diagnosed in US women and is the second-leading cause of cancer deaths in women [1]. The treatment and detection of breast cancer has improved rapidly over the last two decades, leading to an overall decrease in breast cancer death rates. Although "traditional" therapies, such as endocrine therapy and chemotherapy have proven effective, resistance ultimately ensues. Reports regarding endocrine resistance indicate that nearly 50% of estrogen receptor positive breast cancers exhibit resistance to endocrine therapy [2]. Thus, identifying new therapies that alone or in combination with traditional therapies in the treatment of breast cancer is critical. Cancer cells often display enhanced growth and proliferative properties through the activation of growth factor pathways; targeting of these pathways may be successful where traditional therapies have failed. Trastuzumab, a monoclonal antibody directed against the human epidermal growth factor receptor-2, is an example of a successful growth factor receptor targeted therapy in breast cancer. However, not all breast cancers express the HER2 receptor, thus the need exists to identify and target other growth factor pathways. The insulin-like growth factor (IGF) pathway has been identified to regulate proliferation, growth, and metastasis in breast cancer and therapies to inhibit this pathway are currently being investigated in breast and other cancers [3-5].

The IGF System

The insulin-like growth factor (IGF) system is composed of two ligands (IGF-I and IGF-II); three cell-membrane receptors (IGF-I receptor [IGF1R], insulin receptor [IR], and IGF-II receptor [IGF2R]); and six high affinity binding proteins (IGFBP1-6).

IGF-I and IGF-II are single-chain polypeptides that share 62% homology in their amino acid sequences [6]. Circulating IGF-I is primarily produced by liver via growth hormone (GH) released from the pituitary [7]. IGF-I regulates long-bone growth during puberty and IGF-I deficiencies lead to growth deficiencies [8]. Growth hormone production is elevated during puberty, leading to increased activation of the GH/IGF-I axis [9]. In addition to its endocrine function, IGF-I also plays a role in prenatal growth. This was demonstrated in mice with a homozygous deletion of IGF-I, which demonstrated a decreased birth weight and reduced mortality when compared to wild-type littermates [10]. Additionally, IGF-I may be secreted in other organs and exert autocrine and paracrine functions. Unlike IGF-I, IGF-II expression is not regulated by GH; however, it does have proliferative and anti-apoptotic functions similar to IGF-I. IGF-II plays a more important role in early development rather than later in life. Mice with a homozygous deletion of IGF-II are smaller than their wild-type littermates [11]. Serum IGF-I levels decrease significantly after puberty; however, significant levels of IGF-II remain circulating in adult serum throughout life.

The IGF1R is a receptor tyrosine kinase that shares ~60% sequence homology with the IR. IGF1R is synthesized as a single chain pre-propeptide that is glycosylated, dimerized, and transported to the Golgi to be processed into α- and β-subunits [12]. The

subunits form tetramers through disulfide bonds and are transported to the membrane as dimers. The fully processed, cell membrane-bound IGF1R is composed of two 135-kDa α-chains and two 95-kDa β-chains linked through multiple disulfide bonds. The extracellular α-subunits form the ligand-binding pocket that binds one ligand molecule. Unlike other tyrosine kinase receptors, IGF1R/IR activation requires ligand binding. Upon ligand binding, the intracellular β-subunits trans-autophosphorylate and transduce the signal through their tyrosine kinase domains. Phosphorylated β-subunits can recruit adaptor proteins, such as IRS1, IRS2, and GRB2 through plextrin-homology-domains. These phosphorylated adaptor proteins can activate several downstream substrates in both the PI3K and MAPK pathways, ultimately resulting in protein synthesis, survival, growth, and proliferation.

The overall homology between IR and IGF1R is ~60%; however, homology in the tyrosine kinase domain is much higher (~80%) than in the extracellular α-subunit (~45-60%). This similarity has implications when trying to target the IGF1R, as the IR may also be targeted. Two isoforms of the IR exist; the exon-11 positive isoform IR-B and the exon-11 negative form IR-A, which is 12 amino acids shorter than IR-B. Both isoforms can bind insulin as well as IGF-II and both signal similar to the IGF1R, by recruiting adaptor proteins and activating the downstream MAPK and PI3K pathways. While IR-B is considered to play more of a metabolic role, IR-A is thought to have more of a mitogenic role.

The ligands IGF-I and IGF-II are highly (~60%) homologous and also share a 40% homology with insulin [13]. Although the ligands are homologous, they are slightly

different in their binding properties. While both IGF-I and IGF-II can bind the IGF1R, insulin at physiologic concentrations cannot. Interestingly, both insulin and IGF-II can bind the IR, but IGF-I is unable to bind IR at physiologic concentrations. In addition to IGF1R and IR, there are hybrid receptors composed of one hemi-dimer IGF1R and one hemi-dimer IR. Hybrid receptors are able to bind and signal in response to both IGF-I and IGF-II, but not insulin, similar to IGF1R receptors.

Several components of the IGF system may regulate the signaling pathway. The signaling inactive cation-independent mannose-6-phosphate IGF-II receptor (IGF2R) binds both IGF-I and IGF-II and serves as a "sink" [14]. Additionally, IGF ligands can be regulated through a family of six IGF binding proteins (IGFBP1-6). The IGFBPs can regulate the bioavailability of IGF-I and IGF-II in the circulation by binding to them, which can prolong their half-life as well as compete for their binding to receptors. Thus, IGFBPs can both positively and negatively regulate IGF-mediated signaling. The cell context determines whether IGFBPs act to inhibit or enhance IGF signaling. In MCF-7 cells, IGFBP-3 inhibits IGF-mediated signaling by activating a phosphotyrosine phosphatase that dephosphorylates IGF1R [15]. Conversely, in mammary epithelial cells, IGFBP-3 enhances the effect of IGF-I stimulated DNA synthesis [16].

Overlapping and Differential Roles of IR and IGF1R

The highly homologous IR and IGF1R evolved from a common gene and are highly conserved in both vertebrates and invertebrates. They are a part of a common system that works to coordinate metabolic and growth responses in response to nutrient

availability. These receptors, and this system, can also function aberrantly to drive cancer progression. A study performed in NIH-3T3 cells transfected with either IR or IGF1R showed that both receptors were equally potent in activation of ERK and subsequent DNA synthesis [17]. Although derived from a common gene, these receptors have evolved to serve different biological purposes. While the IR has acquired a role in glucose homeostasis, the IGF1R has become a regulator of long-bone growth under the influence of GH. In accordance with their differential functions, IR and IGF1R are distributed differently in tissues: in adult tissues, IR is expressed at high levels in adipose tissue, liver, and muscle; however, IGF1R is highly expressed throughout the body [18]. Further, multiple studies have addressed differential recruitment of intracellular substrates and adaptor proteins. Specifically, the adaptor protein Grb10 and the membrane protein CEACAM-2 have been shown to preferentially interact with IR [19, 20]. In contrast, other substrates such as CrkII and 14-3-3 proteins have been shown to preferentially interact with IGF1R [21, 22].

Micro-array analysis has demonstrated both overlapping and differential gene expression between IR and IGF1R. Work from at least two studies has found that most genes are similarly regulated by the two receptors; however, there are a subset of genes that can be differentially regulated by either IR or IGF1R activation alone [23, 24]. Insulin responsive genes fell in a broad spectrum, and included genes involved in metabolism; whereas IGF-I responsive genes were involved in the regulation of proliferation, adhesion, and differentiation. Overall, these receptors have both common

and distinct effects on proliferation, differentiation, and metabolism, all of which may be important in cancer biology and therapy.

Role of the IGF System in Malignancy

Compelling evidence for the role of the IGF system in cancer initiation, progression, and metastasis has been demonstrated in cell culture, animal, and epidemiological studies. Studies in mouse embryonic fibroblasts (MEFs) have shown IGF1R expression is required for cell transformation by multiple oncogenes [25]. More recently, gene expression analysis studies have demonstrated the importance of IGF1R in regulating genes that control cell survival, metastasis, motility, attachment, and cell cycle [26]. Mice which were engineered to express a human CD8α-IGF1R fusion protein under the control of the mouse mammary tumor virus promoter (MMTV) developed salivary and mammary adenocarcinomas [27]. Overexpression of IGF1R was observed in approximately 40% of breast tumors [28]. Additionally, IGF1R expression was found to be correlated with both estrogen receptor (ER) expression and disease-free survival [29].

Transgenic mouse models of cancer have established the role of IRS adaptor proteins in tumor growth and metastasis. *In vitro* experiments suggest activation of IRS-1 is associated with cell growth, whereas IRS-2 activation was associated with cell motility [30]. Mammary tumors obtained from mice lacking IRS-1, but expressing IRS-2 had enhanced metastasis [31]. Interestingly, an overexpression model showed that both IRS-1 and IRS-2 enhanced mammary tumor formation and metastasis [32]. In addition to

expression of IRS adaptor proteins, IRS phosphorylation has also been shown to play a role in cancer progression. McCampbell and colleagues showed that while both normal and hyperplastic endometrial tissues express IRS-1 and IRS-2, IRS phosphorylation on key serine residues was lost only in hyperplasia [33]. Serine phosphorylation normally results in degradation of IRS proteins, acting as a critical negative feedback mechanism; however, it appears in endometrial hyperplasia and cancer this mechanism may not be functioning properly. IRS-1 is overexpressed in some human primary breast tumors; furthermore, elevated IRS-1 levels are associated with decreased disease-free survival in a subset of tumor samples [34, 35].

The IGF ligands have also been implicated in the initiation, progression, and metastasis of cancer. In the transgenic adenocarcinoma of mouse prostate (TRAMP) model, overexpression of IGF-I in the prostate resulted in the development of prostate cancer [27]. Mice engineered to overexpress des(1,3) IGF-I demonstrated a 53% increase in the incidence of mammary tumor formation when compared to control animals [36]. Conversely, LID (liver-specific IGF-I deficiency) mice demonstrated a reduction in mammary tumor formation [37]. Additionally, LID mice have decreased metastases from colon and breast tumors. High circulating levels of serum IGF-I are associated with increased risk of numerous cancers, including colorectal, prostate, and breast [38, 39] [40]. In a study by Hankinson, *et. al.* breast cancer risk was increased in patients with elevated circulating IGF-I levels [41]. Overexpression of IGF-II due to loss of imprinting was first described in Wilms tumor; however, increased IGF-II levels have subsequently been observed in gastrointestinal, gynecological, and breast tumors [42-45].

The role of IGFBPs in cancer development and risk has also been examined in numerous studies. In mouse models of prostate cancer and in prostate cancer cells, IGFBP-3 has been shown to have a protective role [46]. IGFBP-1 levels were decreased in mice with polyoma virus middle T antigen (PyVmT)-induced breast cancer when compared with wild-type mice [47]. Additionally, IGFBP-1 inhibited breast cancer cell growth in a xenograft model using MCF-7 cells [48]. Studies addressing the role of IGFBPs in human cancer have had conflicting results; however, most evidence supports the role of a high IGF:IGFBP ratio resulting in an increased risk of developing cancer [49].

The Role of the Insulin Receptor in Cancer

Numerous animal and cell culture studies have been performed to investigate the role of the IR and insulin and cancer. Insulin is able to stimulate directional cell motility *in vitro* [50]. A study by Wang and colleagues demonstrated that a chimeric retrovirus with the IR intracellular cytoplasmic domain transformed chicken embryo fibroblasts and formed colonies in agar [51]. The first studies examining the role of insulin in mammary tumor growth in rats were conducted in the 1970s. Mammary tumors induced by the carcinogen 7,12 dimethylbenz(a)anthracene (DMBA) in the rat were inhibited when the rats were made diabetic by alloxan administration, which destroys pancreatic β-cells and causes insulin deficiency. Administration of insulin in these rats restored tumor growth [52]. In a nonobese mouse model of type II diabetes, hyperinsulinemia caused increased mammary tumor growth, while decreasing the circulating levels of insulin prevented this

enhanced growth [47]. Further, our lab showed that down-regulating the IR in breast cancer cells and xenografts caused reduced proliferation, metastasis, angiogenesis, and lymphangiogenesis [53]. These studies provide evidence that insulin may be involved in the growth of some malignancies. In addition to insulin, IR may be activated by IGF-II. This was demonstrated in MDA-MB-157 breast cancer cells, whereby autocrine IGF-II stimulated cell proliferation through IR activation [54].

Insulin receptor overexpression has been reported in both breast and prostate cancer cell lines and some cancer cell lines grown in hyperglycemic conditions demonstrate increased growth in response to insulin as well as activation of the IR signaling pathway [47, 55]. Additionally, elevated IR levels were detected on the endothelial cells of human breast, colon, kidney, lung, and pancreas cancer specimens. In breast cancer specifically, 80% of samples demonstrated elevated IR levels when compared to normal breast tissues [56]. Epidemiological studies suggest that obese individuals, especially those with type II diabetes are at a higher risk of dying from cancer than lean individuals that are not diabetic [57]. More recently, a study demonstrated that insulin therapy, specifically insulin glargine (a longer acting form of insulin) increased breast cancer risk [58]. This correlates with an earlier animal study that showed AspB10 (a high affinity insulin analog) caused mammary tumor formation in female rats [59].

In vitro, *in vivo*, and epidemiological evidence suggests that insulin and IR are involved in the formation and maintenance of the malignant phenotype. Future studies

examining the role of the IGF system in cancer should include the IR. Further, agents which target both receptors may be a better choice for cancer therapy.

Anti-IGF1R therapy

The role of the IGF system in cancer biology has been established, and it is clear that disrupting the IGF system may be beneficial in cancer therapy. Multiple strategies to disrupt the IGF system are currently being developed and tested in clinical trials. These strategies include suppression of IGF production through growth hormone inhibition, neutralization of IGF action using IGFBPs, monoclonal antibodies targeting IGF ligands and IGF1R, antisense oligonucleotides targeting IGF1R, dominant negative receptors, and tyrosine kinase inhibitors, which inhibit both IGF1R and the closely related IR. In addition, the value of combining IGF1R inhibitors with traditional therapies is also being examined.

Reduction and Neutralization of IGF Ligand

Increased circulating levels of IGF-I have been associated with increased risk of developing certain cancers; therefore, reducing circulating IGF-I levels could prove beneficial in treating cancers. IGF-I is produced by the liver in response to growth hormone (GH) secretion from the pituitary gland acting on GH receptors on hepatic cells. One way to decrease circulating IGF-I levels is to inhibit growth hormone action. Pegvisomant is a pegylated derivative of growth hormone and is used to treat patients with acromegaly by reducing circulating IGF-I levels [60]. Additionally, pegvisomant

has demonstrated efficacy in reducing tumor growth in colon and breast xenograft models in mice [61]. Clinical trials are underway examining the potential benefit of combining an IGF1R antibody (figitumumab) with pegvisomant in solid tumors (ClinicalTrials.gov identifier: NCT00976508).

In addition to decreasing the production of IGF-I, neutralizing circulating IGF-I could also prove beneficial in treating cancers. IGF binding-proteins (IGFBPs) are a family of proteins that bind to IGF-I and IGF-II and thereby regulate their bioavailability. In addition, some IGFBPs are thought to have additional functions such as the ability to induce apoptosis that may prove beneficial in cancer therapy. IGFBP-1 has been shown to inhibit the proliferation, anchorage-independent growth, and motility of breast cancer cells *in vitro* [48, 62]. Further, IGFBP-1 (administered using an infusion pump) inhibited the growth of MCF-7 xenografts in a mouse model [63]. Although effective *in vitro* and *in vivo*, the short half-life of IGFBP-1 would require modification to the protein to make it suitable for human use. A more recent strategy to neutralize IGF-I and IGF-II has been utilized through the development of monoclonal antibodies to the ligands. MEDI-573 is a dual specific IGF-I/IGF-II antibody that does not target insulin. A study performed by Gao, *et. al.* demonstrated that MEDI-573 can inhibit IGF-I and IGF-II stimulated signaling and proliferation *in vitro*. Further, the antibody can also inhibit the growth of IGF-I and IGF-II driven tumors in a xenograft model [64]. The overall advantage of ligand neutralizing strategies is the ability to target the mitogenic signaling of IGF-I and IGF-II without affecting the metabolic signaling mediated by insulin.

Inhibition of IGF1R Signaling

Blockade of growth factor receptors using monoclonal antibodies is a proven strategy to inhibit receptor-mediated effects and signaling. A prime example is the antibody-mediated blockade of EGFR and HER2 receptors in lung and breast cancers respectively. Several monoclonal antibodies against the IGF1R have been developed and are currently being evaluated in Phase I-III clinical trials. Monoclonal antibodies represent the anti-IGF1R strategy furthest along in clinical development. One of the earliest antibodies developed against IGF1R was αIR3, which inhibited the growth of multiple breast cancer cell lines *in vitro* [65]. The effects of αIR3 *in vivo* were less consistent; while the antibody inhibited the growth of MDA-MB-231 xenografts, it was unable to inhibit the growth of MCF-7 xenografts [66]. Interestingly, αIR3 was able to inhibit the *in vitro* growth of MCF-7 cells. A chimeric humanized single chain antibody (scFv-Fc) displaying partial agonist activity towards IGF1R significantly suppressed MCF-7 xenograft growth in a mouse model [67]. More recently, several purely antagonistic antibodies have been developed and proven efficacious *in vitro* and *in vivo*, which has led to their evaluation in clinical trials. EM164 and its humanized version AVE1642 demonstrated inhibitory activity against IGF-I and IGF-II stimulated proliferation, signaling, and survival in MCF-7 cells *in vitro* and in LCC6 xenografts in a mouse model [68, 69]. A fully humanized monoclonal antibody A12 also blocked IGF-I and IGF-II signaling and xenograft growth in MCF-7 cells [70]. Several other fully human monoclonal antibodies, including CP-751,871 (figitumumab) and MK-0646 (dalotuzumab) inhibited the *in vitro* and xenograft growth of several types of cancer,

including breast and lung [71, 72]. There are currently multiple clinical trials underway examining the efficacy of IGF1R antibodies in solid tumors.

Signaling through the IGF1R may also be inhibited by targeting the tyrosine kinase domain of the receptor. Early tyrosine kinase inhibitors were shown to inhibit IGF1R and IR autophosphorylation and kinase activity equally and they inhibited the growth of MDA-MB468 and MCF-7 breast cancer cells in monolayer and colony formation assays [73]. Newer tyrosine kinase inhibitors claim to more selectively target the IGF1R, although still inhibit the IR to some degree, due to the close homology in the tyrosine kinase domains. NVP-AEW541 and NVP-ADW742 demonstrated higher selectivity towards IGF1R using *in vitro* kinase assays in cells expressing only IGF1R or IR; however, in whole-cell systems which express both receptors, the inhibition appears to be similar for both receptors [74]. Both compounds inhibited the growth of multiple cancer cell lines *in vitro* and *in vivo*. Several more tyrosine kinase inhibitors have been developed, including BMS-536924 and BMS-754807 that have demonstrated efficacy in inhibiting cancer cell and xenograft growth in a variety of models [75]. Although dual targeting of IGF1R/IR was negatively viewed due to potential metabolic consequences, the demonstration of the role of the IR in cancer biology has advanced the development of dual IGF1R/IR tyrosine kinase inhibitors. Currently, clinical trials are underway examining the efficacy of IGF1R/IR tyrosine kinase inhibitors alone and in combination with conventional therapies.

Hormonal Therapy for Breast Cancer

The development of hormonal therapy began over 100 years ago when it was discovered that breast cancer growth could be controlled by a factor secreted from the ovary. Several decades later, it was determined that this factor (estrogen) had the ability to stimulate proliferation in several target tissues by acting on estrogen receptors. Further, patients whose tumors contained estrogen receptors (and responded to estrogen) benefited from hypophysectomy.

These findings suggested that inhibiting estrogen signaling could influence breast cancer cell growth. The development of tamoxifen, a selective estrogen receptor modulator (SERM) provided further support for this concept. Tamoxifen acted as an anti-estrogen and inhibited the growth of estrogen receptor positive breast cancer cells as well as the formation of mammary tumors in mice [76, 77]. Tamoxifen also showed promising activity in early breast cancer trials. Subsequently, tamoxifen has proven useful in all facets of breast cancer treatment, including prevention, adjuvant therapy, and metastatic disease.

The advent of tamoxifen led to the development of additional hormonal therapies. Aromatase inhibitors (AI's) do not interact with the estrogen receptor directly; rather they prevent the peripheral conversion of androgens into estrogens. Third generation AI's (non-steroidal competitive aromatase inhibitors letrozole and anastrozole, and the steroidal aromatase inactivator exemestane) are often used in post-menopausal breast cancer patients and are challenging tamoxifen as the initial therapy in advanced disease [78]. A more recent addition to endocrine therapies is the estrogen receptor antagonist

fulvestrant, which competitively binds to the estrogen receptor without eliciting agonistic effects. Fulvestrant has shown to be as effective as anastrozole in patients progressing on prior endocrine therapies. Further, it has demonstrated efficacy as a first line therapy in patients with hormone-receptor positive tumors [79].

Despite these developments, both acquired and *de novo* resistance to hormonal therapies is a major barrier in the management of advanced breast cancer. Initially, the only option following the development of endocrine resistance was to change the endocrine agent. Following the exhaustion of endocrine therapies, cytotoxic chemotherapy was administered. More recently, studies have been initiated to investigate the mechanisms behind endocrine resistance, which has led to the development of newer therapies targeting growth factor receptors. The efficacy of these new therapies, alone and in combination with endocrine therapies is actively being investigated.

Crosstalk between Insulin-Like Growth Factors and Estrogens

Synergy between estrogen and IGF-I has been observed in a number of breast cancer cell models. In these models, co-treatment with estrogen and IGF-I often leads to enhanced growth when compared to either ligand alone. In breast cancer cells, IGF-I can increase the expression of progesterone receptor, a transcriptional target of the ER [80]. Further, IGF-I can directly increase the transcriptional activity of the ER in the absence of estradiol in MCF-7 cells [81]. In addition, co-treatment with IGF-I and estradiol enhanced the transcriptional activation of the ER to a higher level than either ligand alone. It has also been shown that IGFBP-1, which inhibits IGF-I action, inhibits both

IGF-mediated activation of ER and estrogen-mediated activation of the ER [82]. These observations suggest that IGF activation of ER may be required for maximal receptor activation.

In addition to IGF influencing ER activation, ER also affects IGF1R signaling. Multiple studies have demonstrated that estrogen can affect all levels of IGF pathway, with the net result being a sensitization to IGF signaling. Estrogen can increase the expression of both IGF1R and IRS-1 in breast cancer cells, leading to enhanced IGF signaling and activation of downstream pathways [34]. Increased expression and phosphorylation of IRS-1 was also observed in MCF-7L xenografts grown in the presence of estrogen. These tumors also had elevated levels of phosphorylated MAPK, indicating an active signaling cascade through IRS-1. Removal of estrogen halted tumor growth and decreased both IRS-1 expression and MAPK activity [34]. In addition, estrogen has also been shown to increase expression of IGF-II, which can act as an autocrine stimulator of breast cancer cells. Estrogen can also down-regulate the negative signaling components of the IGF pathway. Estrogen decreases expression of IGFBP-3, a binding protein that can inhibit breast cancer cell growth by binding and sequestering IGF ligand [83]. Further, estrogen can down-regulate IGF-IIR expression, a receptor that binds and sequesters IGF-II ligand without transmitting an extracellular signal and leading to inhibition of breast cancer cell proliferation [84]. Thus, estrogen up-regulates the positive IGF growth factor signaling elements, as well as down-regulates the negative IGF signaling elements, leading to an overall enhancement of IGF signaling.

The long-term estrogen-mediated effects on the IGF pathway may be attributed to transcriptional regulation; however, more recent reports have demonstrated estrogen may also elicit rapid, non-nuclear effects on the IGF pathway. Work by Zhang, et al. has led to a model whereby estrogen stimulates cytosolic ER to interact with adapter proteins, such as Shc or the p85 subunit of PI3K. This ER/adapter protein complex translocates to the membrane where it associates with IGF1R and activates downstream signaling cascades [85]. Thus, estrogen and the ER interact with IGF signaling at multiple levels and in multiple manners, ultimately resulting in enhanced signaling through the IGF pathway.

Rationale and purpose of this study

IGF1R inhibitors are currently being evaluated in endocrine resistant populations and have demonstrated limited success. In vitro and in vivo evaluation of these agents has been performed using endocrine sensitive breast cancer cells and xenografts. Therefore, we sought was to examine the efficacy of anti-IGF1R therapies in an endocrine-resistant model, mimicking the clinical scenario.

Hypothesis

Estrogen receptor (ER) regulation of IGF1R expression in estrogen receptor positive breast cancer cells and xenografts determines responsiveness to anti-IGF1R therapy *in vitro* and *in vivo*.

Chapter 2

Generation and characterization of a tamoxifen resistant breast cancer cell line.

Introduction

The estrogen receptor (ER) is a nuclear ligand-activated transcription factor that activates estrogen's mitogenic and proliferative actions through gene transcription. In addition, the ER may act in a non-genomic manner at the membrane through interaction with growth factor receptors [86]. Nearly 70% of invasive breast cancers are positive for ER expression at diagnosis. Signaling through the ER may drive the growth of these tumors, thus inhibiting the function of the ER has been the mainstay of treatment for these patients. Estrogen receptor function may be inhibited directly by binding to the receptor and preventing transcription using selective estrogen receptor modulators (SERMS) or by binding and leading to receptor degradation using pure antiestrogens. Additionally, ER function may be inhibited directly using aromatase inhibitors, which inhibit the peripheral conversion of androgens into estrogens in post-menopausal women.

The first and arguably most effective targeted therapy for breast cancer involves inhibition of estrogen receptor (ER) function. Tamoxifen, a selective estrogen receptor modulator (SERM), has proven effective in both early and advanced stages of breast cancer [87]. Tamoxifen is a SERM with mixed agonist/antagonist activities, depending on the target tissue, that holds the ER in an inactive conformation thereby preventing gene transcription [88]. Although at least half of estrogen receptor positive breast cancer patients will respond to hormonal therapy, the disease ultimately progresses in most patients. Tamoxifen resistance remains an important clinical problem which influences the survival of a large group of women. Elucidating the mechanisms of tamoxifen

resistance is vital in not only understanding disease progression, but also in understanding how best to treat these patients going forward.

Two types of endocrine resistance exist; *de novo* resistance occurs when tumors fail to initially respond to endocrine therapy, whereas acquired resistance evolves after a tumor initially responds to treatment then relapses over time despite continued therapy. Resistance may occur through one of several mechanisms, including: loss of ER expression, loss of dependence on ER/enhanced growth factor signaling, or the switch of an endocrine agent from having antagonistic to agonistic properties [89]. Elucidating these mechanisms will help give us an understanding of both how to treat endocrine resistant cancers and potentially how to delay the process from occurring.

The majority of clinical trials examining new therapies in breast cancer are in patients with advanced disease (patients who have failed on prior therapies). Unfortunately, preclinical studies evaluating these agents are most often performed using non-advanced or non-resistant cell models. Although resistance, and cancer itself, is a heterogeneous disease, evaluating agents in a background similar to that which they will clinically be used in is more likely to translate to the patient population. The overall aim of our study was to generate and characterize a tamoxifen resistant breast cancer cell line in order to better understand endocrine resistance and its implications in evaluating emerging therapies. Herein, we reveal tamoxifen resistant (TamR) MCF-7L and T47D cells maintain estrogen receptor expression and proliferate in response to estrogen; furthermore, the pure antiestrogen fulvestrant can inhibit the growth of TamR cells. Interestingly, estrogen regulated gene transcription is diminished in TamR cells;

however, a unique set of genes is up-regulated in response to estrogen in TamR, but not parental cells. One of the estrogen-regulated genes that was down-regulated in our TamR cells was IGF1R, which is of particular interest, because there are currently clinical trials underway examining the efficacy of IGF1R antibodies in endocrine resistant patients. This finding underscores the importance of evaluating new therapies in a model system that matches the patient population the therapy will be used in.

Results

Tamoxifen resistant cells are refractory to tamoxifen treatment.

In order to learn more about endocrine resistance and its implications in breast cancer treatment, tamoxifen-resistant MCF-7L and T47D cells were generated. Cells were cultured in phenol-red-free IMEM containing 5% dextran-cleared-charcoal (DCC) serum and 100 nM 4-OH-tamoxifen for 6 months prior to characterizing cells. Initially, cells ceased to grow; however, after a period of approximately 3 months, cell growth resumed. Cells were passaged for an additional 3 months prior to characterization. After selection, TamR cells survived in the presence of increasing concentrations of tamoxifen; however, parental cells were inhibited with as little as 1 nM tamoxifen (figure 2.1). Further, TamR cells continued to survive over time (up to 14 days) in the presence of 100 nM tamoxifen; whereas parental cells did not grow (figure 2.2) Thus, TamR cells continued to survive and grow in the presence of tamoxifen, even up to concentrations of 1 μM, demonstrating resistance to the drug.

Tamoxifen resistant cells maintain estrogen receptor expression and respond to estrogen treatment.

We began our characterization of the TamR line by determining whether our TamR cells maintained responsiveness to estrogen. Clinically, the majority of tamoxifen resistant breast cancers maintain estrogen receptor expression [90]. Similar to some tamoxifen resistant cancers, TamR cells maintained expression of estrogen receptor (figure 2.3). Estrogen has previously been reported to stimulate proliferation in MCF-7 cells. Interestingly, TamR cells were able to proliferate in response to estrogen to a similar level to parental cells (figure 2.4). Further, the pure steroidal antiestrogen fulvestrant was able to inhibit the growth of both TamR and parental cells, indicating the estrogen receptor still plays a role in TamR cells (figure 2.5).

The estrogen receptor contains multiple phosphorylation sites, two prominent sites are serine-118, though to be phosphorylated by MAPK, and serine-167, thought to be phosphorylated by Akt. In our lab, we have found estrogen is able to phosphorylate the serine-118 site, whereas IGF-I and insulin are able to phosphorylate the serine-167 site [91]. MCF-7L TamR cells had basal phosphorylation of serine-118; however, the site was only phosphorylated in parental cells in response to estrogen treatment. Both insulin and IGF-I were able to phosphorylate serine-167 in parental cells; however, in TamR cells, only insulin was able to phosphorylate the site (figure 2.6).

Tamoxifen exerts its action by binding to the estrogen receptor and holding it in an inactive conformation, preventing gene transcription. Therefore, tamoxifen treatment should prevent the transcription of estrogen regulated genes. When we examined the

gene expression regulated by ER in TamR cells, we found basal levels of estrogen regulated genes, such as AREG, TFF1, PR, and KIAA0575 were down-regulated (figure 2.7 and data not shown); however, estrogen was able to stimulate transcription of these genes, although not to basal parental levels. Numerous publications list genes regulated by tamoxifen as well as genes up-regulated in tamoxifen resistant cells and tumors [92], [93]. Interestingly, we found genes (RAB30, KIAA0922) up-regulated in response to estrogen treatment in TamR, but not parental cells (figure 2.8). Although the transcriptional activity of the estrogen receptor was altered in TamR cells, the proliferative response to estrogen did not change. Similar to the clinical situation of tamoxifen resistance where some tumors remain dependent on estradiol, our cells maintained estrogen receptor expression and responded to estrogen treatment.

Tamoxifen resistant cells expressed low levels of IGF1R.

Previous reports have demonstrated a link between IGF1R and ER signaling [24, 34, 91, 94]. Prior to examining the effectiveness of anti-IGF therapy in TamR cells, we examined the IGF signaling pathway and its components. Interestingly, IGF1R protein levels were diminished as measured by Western blot (figure 2.9). Further, TamR cells failed to phosphorylate Akt and MAPK after IGF-I treatment. The cells retained expression of IR and insulin and IGF-II ligand treatment resulted in phosphorylation of Akt and MAPK. To examine whether this change in IGF1R expression was due to decreased transcription, we performed qRT-PCR to examine the message level of IGF1R. Indeed, IGF1R mRNA was decreased in TamR cells compared to parental cells (figure 2.10). Treating TamR cells with estrogen resulted in increased transcription of IGF1R,

but did not restore the receptor to parental levels. Insulin receptor mRNA levels were not significantly different between parental and resistant cells (figure 2.10). Further, estrogen treatment did not affect IR levels in either cell line. In agreement with the biochemical data, MCF-7L cells were able to proliferate in response to insulin, IGF-I, and IGF-II; however, TamR cells were only able to proliferate in response to insulin and IGF-II (figure 2.11). Similarly, insulin, IGF-I, and IGF-II were able to stimulate the anchorage independent growth of parental cells; however, TamR cells only grew in response to insulin and IGF-II (figure 2.12). These data demonstrate that tamoxifen resistant cells lack IGF1R expression, but maintain expression of IR and are able to signal, proliferate, and grow through IR.

Conclusion

Understanding the mechanisms of resistance to tamoxifen and other endocrine therapies will help to both identify new targets to overcome resistance and predict patient benefit from endocrine therapy. Although the selection of tamoxifen resistant cells in culture may not exactly mimic the selection pressures in women with breast cancer, cell culture systems can generate models of tamoxifen resistance that may be applicable *in vivo*. In fact, the data presented in this section are consistent with clinical findings indicating the estrogen receptor is present in tamoxifen resistant breast cancer [95, 96].

In this study we derived and characterized a TamR cell line using endocrine sensitive MCF-7L and T47D cells to study the mechanisms involved in acquired tamoxifen resistance in breast cancer cells. Both TamR cell lines expressed estrogen

receptors and proliferated in response to estrogen; furthermore, their growth could be inhibited using the pure antiestrogen fulvestrant. However, transcription of estrogen-regulated genes was significantly decreased in tamoxifen resistant cells. Interestingly, several genes were regulated by estrogen in TamR, but not parental cells. Other studies with tamoxifen resistant cell lines support our findings regarding a role for the ER in resistant cells [97]. In addition, clinical studies suggest the ER is still present and may have a function in tamoxifen resistant breast cancers. Specifically, clinical trials have demonstrated a benefit to using both aromatase inhibitors and SERDs such as fulvestrant in patients that have previously failed on tamoxifen therapy.

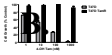
Our finding that classic ER function remains suppressed in tamoxifen-resistant tumors was shown both at the level of mRNA expression of selected classically ER-dependent genes and by protein expression for IGF1R. This suggests that tamoxifen resistance in our model is not the result of tamoxifen acting as an agonist at the level of gene transcription. Our results are in contrast to a previous observation *in vitro* involving HER2-overexpressing MCF-7 cells in which tamoxifen induced both classic genomic and nongenomic ER functions [98]. However, later findings by the same group suggest that tamoxifen resistance *in vivo* is predominantly mediated by nongenomic mechanisms and that classic ER genomic action does not play a major role. They go on to suggest that other" nonclassic" ER genomic mechanisms could also potentially play a role in this system [99]. The relative contribution of classic genomic versus nonclassic genomic versus nongenomic ER functions to tamoxifen resistance likely involves a spectrum

rather than a single mechanism, which may be dependent on multiple factors, including the tumor microenvironment and the duration of tamoxifen exposure.

Several models of tamoxifen resistance have demonstrated enhanced signaling through growth factor pathways as a mechanism of resistance [100, 101]. However, we found our tamoxifen-resistant cell lines lacked both protein and mRNA expression of IGF1R, but maintained expression of IR. Based on prior reports of ER transcriptional regulation of IGF1R, it is not surprising that IGF1R expression would be decreased following acute treatment with a selective estrogen receptor modulator such as tamoxifen [83, 102, 103]. Our finding of decreased IGF1R levels in resistant cells is in agreement with multiple *in vitro* models of tamoxifen resistance [104, 105]. However, clinical trials of IGF1R monoclonal antibodies are proceeding in endocrine resistant breast cancer patients. Although our *in vitro* model may not exactly match the development of resistance *in vivo*, at the very least it should warrant the stratification of patients based on IGF1R expression in clinical trials.

In summary, data from this *in vitro* model of tamoxifen resistance provide evidence that classic ER genomic functions remain suppressed; however, nonclassic and possibly nongenomic functions of ER remain important in resistant cells. These data add new understanding to the mechanisms of tamoxifen resistance and provide a strong rationale for evaluating new inhibitors in a preclinical model system that matches the clinical scenario they will be tested in.

A.



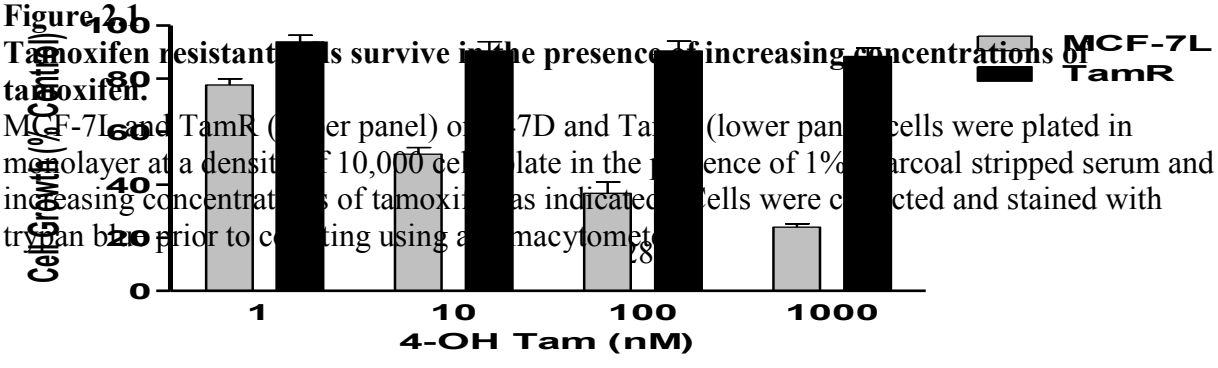
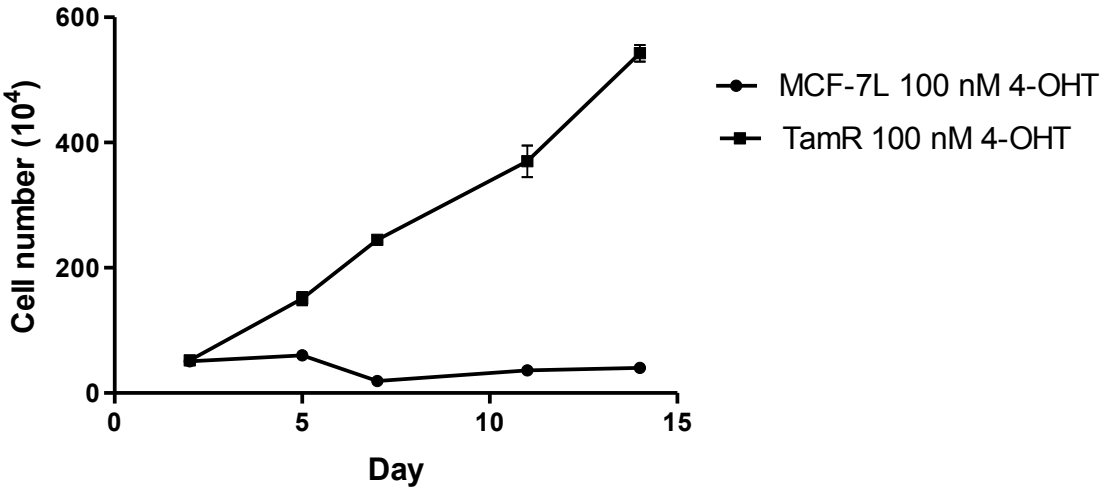


Figure 2.1
Tamoxifen resistant cells survive in the presence of increasing concentrations of tamoxifen.
MCF-7L and TamR (upper panel) or MCF-7D and TamR (lower panel) cells were plated in monolayer at a density of 10,000 cells/plate in the presence of 1% charcoal stripped serum and increasing concentrations of tamoxifen as indicated. Cells were collected and stained with trypan blue prior to counting using a hemacytometer.

A.



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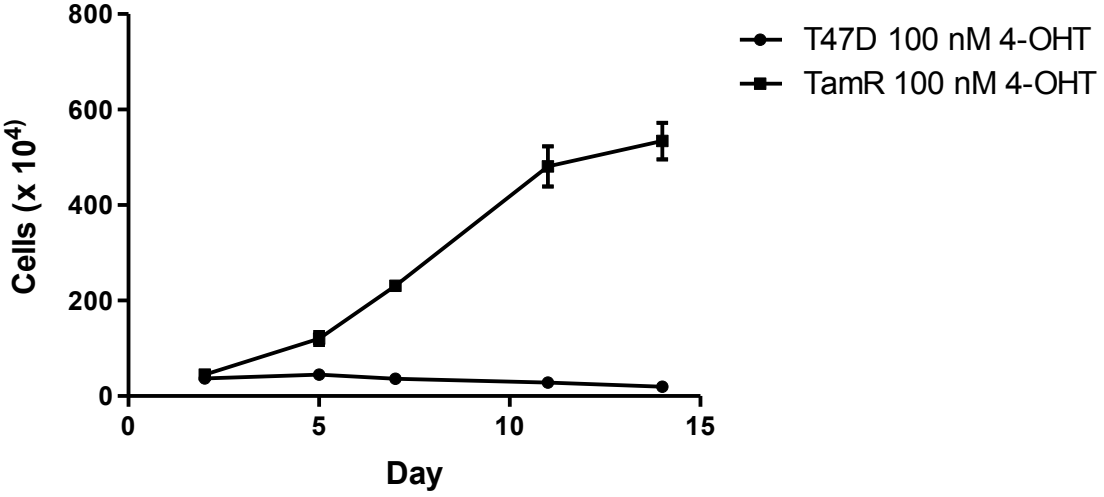


Figure 2.2
Tamoxifen resistant cells survive in the presence of tamoxifen over time.
MCF-7L and TamR (upper panel) or T47D and TamR (lower panel) cells were plated in monolayer at a density of 10,000 cells/plate in the presence of 1% charcoal stripped serum and 100 nM tamoxifen. Cells were collected and stained with trypan blue prior to counting using a hemacytometer at the days indicated.

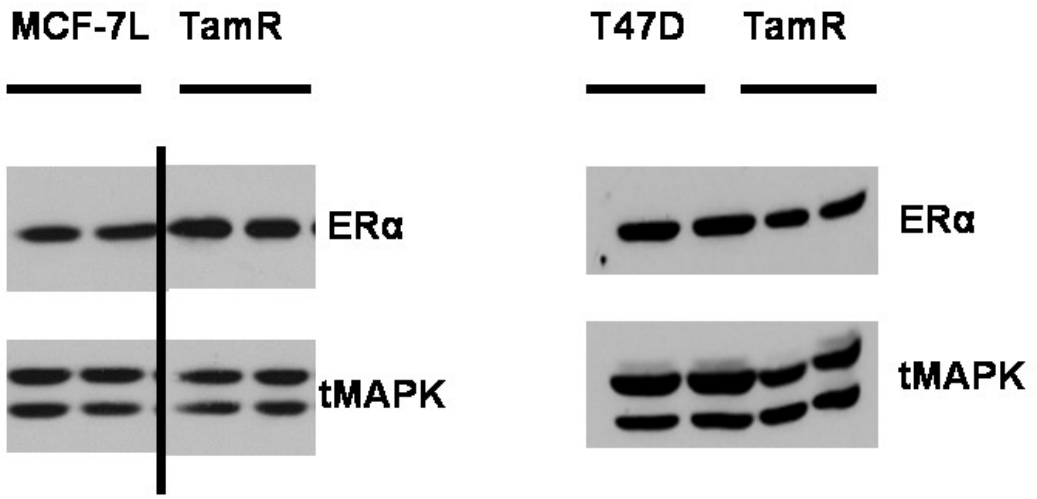
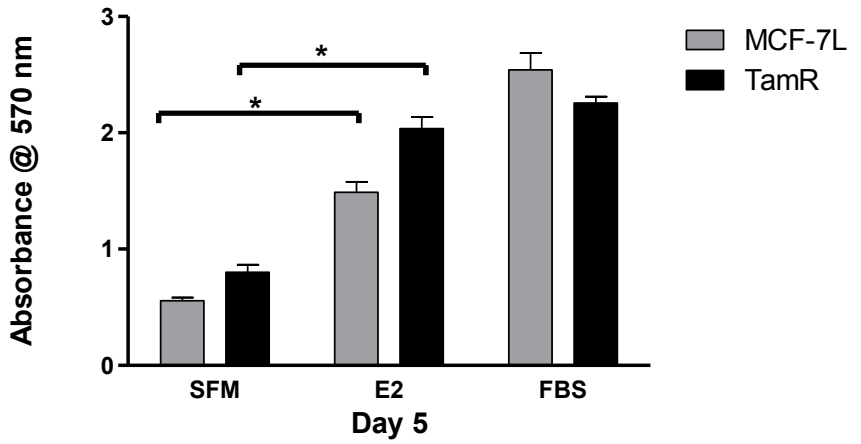


Figure 2.3
Tamoxifen resistant cells maintain estrogen receptor expression.
Cells were exposed to charcoal stripped serum for three days prior to harvesting lysates. Lysates were collected from MCF-7L and TamR (left panel) and T47D and TamR (right panel) cells and were separated by SDS-PAGE. Total protein levels of estrogen receptor (ER) and MAPK were assessed using specific antibodies by immunoblotting.

A.



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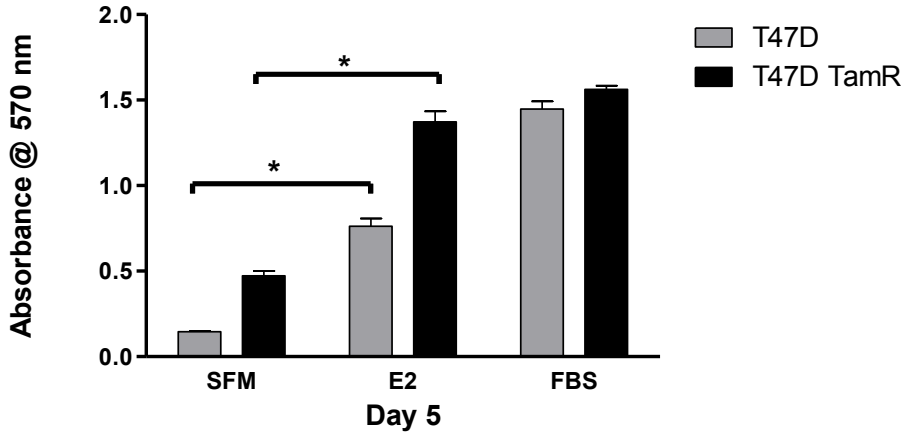


Figure 2.4
Tamoxifen resistant cells proliferate in response to estrogen.
MCF-7L and TamR (left panel) or T47D and TamR (right panel) cells were grown in charcoal stripped serum prior to serum starving cells overnight. Cells were treated with 1 nM E2 or 5% FBS and growth was assessed after 5 days using the MTT assay. An unpaired *t* test was used to compare the difference between untreated and treated samples. *$p<0.01$

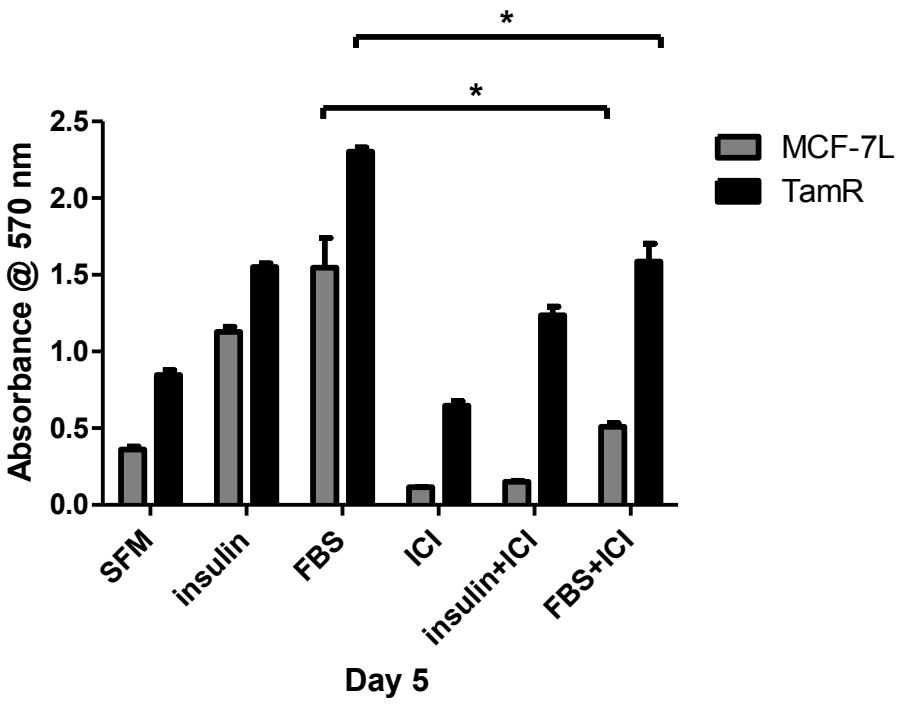


Figure 2.5
Fulvestrant inhibits the growth of tamoxifen resistant MCF-7L cells.
MCF-7L and TamR cells were grown in charcoal stripped serum prior to serum starving cells overnight. Cells were treated with 10 nM insulin or 5% FBS in the presence and absence of 100 nM fulvestrant (ICI) and growth was assessed after 5 days using the MTT assay. An unpaired *t* test was used to compare the difference between untreated and treated samples. *$p<0.005$

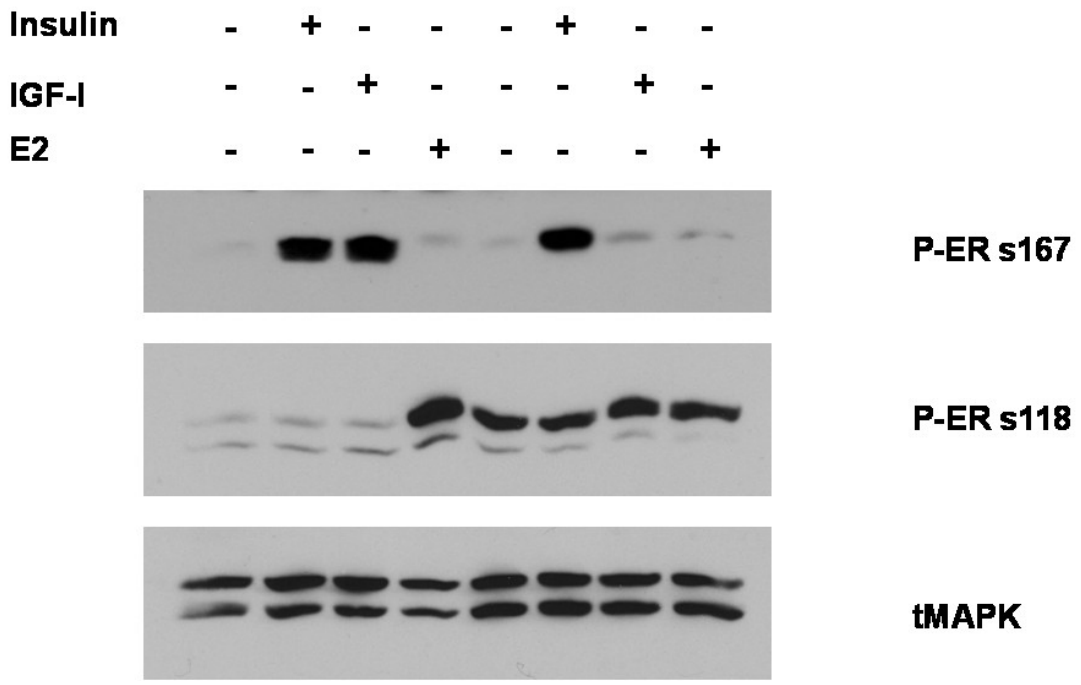
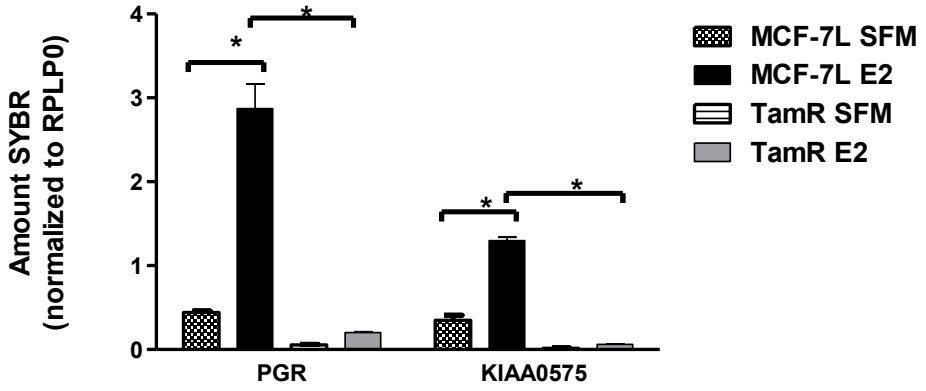


Figure 2.6
Estrogen receptor can be phosphorylated in both MCF-7L parental and TamR cells. Cells were exposed to charcoal stripped serum for three days and serum starved overnight prior to harvesting lysates. Cells were treated with indicated ligands for 30 minutes and lysates were collected from MCF-7L and TamR cells and were separated by SDS-PAGE. Total and phosphorylated protein levels of estrogen receptor (ER) and MAPK were assessed using specific antibodies by immunoblotting.

A.



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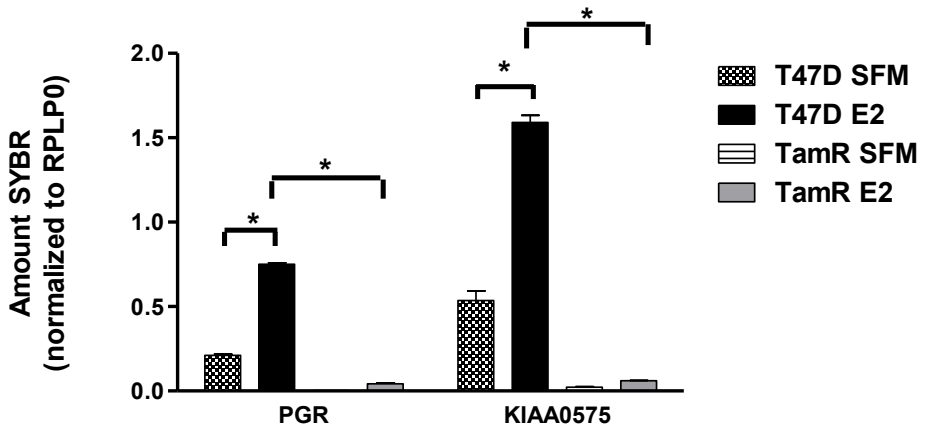


Figure 2.7
Expression of estrogen regulated genes is decreased in tamoxifen resistant cells. Cells were plated and exposed to charcoal stripped serum prior to serum starving and treating with estradiol for 4 hours. Total RNA was isolated from MCF-7L and TamR (left panel) or T47D and TamR (right panel) cells and was reverse transcribed. Expression of PGR and KIAA0575 was analyzed using qRT-PCR and was normalized to the RPLP0 housekeeper gene. One way ANOVA with a Tukey's post-test was used to analyze the data. * $p<0.01$

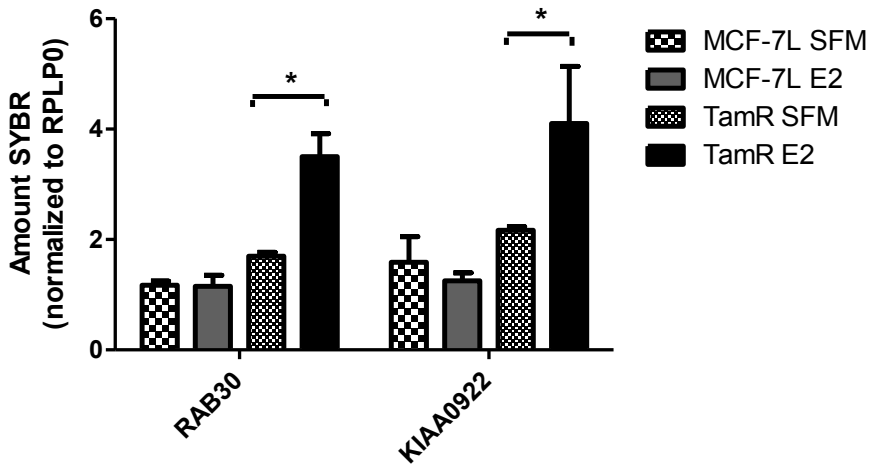


Figure 2.8
Estrogen enhances the expression of unique genes in TamR, but not parental cells. Cells were plated and exposed to charcoal stripped serum prior to serum starving and treating with estradiol for 4 hours. Total RNA was isolated from MCF-7L and TamR cells and was reverse transcribed. Expression of RAB30 and KIAA0922 was analyzed using qRT-PCR and was normalized to the RPLP0 housekeeper gene. An unpaired *t* test was used to compare the difference between untreated and treated samples. *$p<0.05$

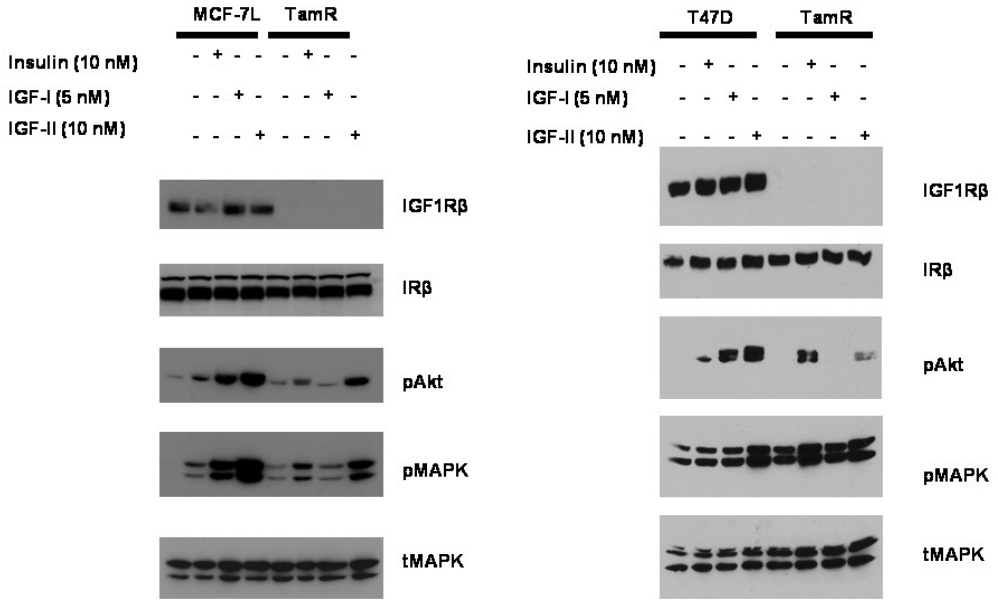
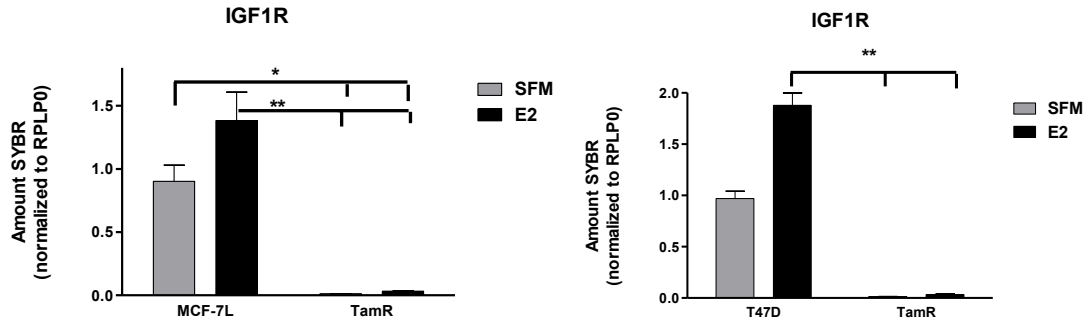


Figure 2.9
IGF1R levels and IGF-mediated signaling are reduced in TamR cells.
MCF-7L and TamR (upper panel) or T47D and TamR (lower panel) cells were serum starved overnight, then treated with 10 nM insulin, 5 nM IGF-I or 5 nM IGF-II for 10 minutes. Cellular lysates were separated by SDS-PAGE and levels of IGF1R, IR, phosphorylated Akt and MAPK, and total MAPK protein levels were assessed using specific antibodies by immunoblotting.

A.



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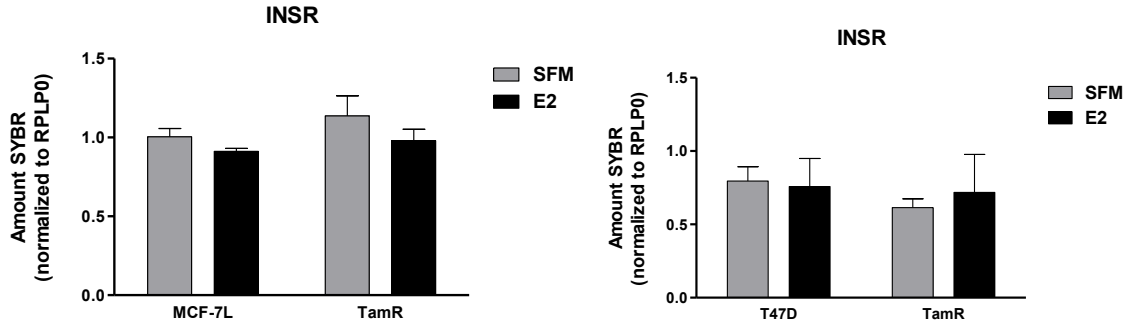
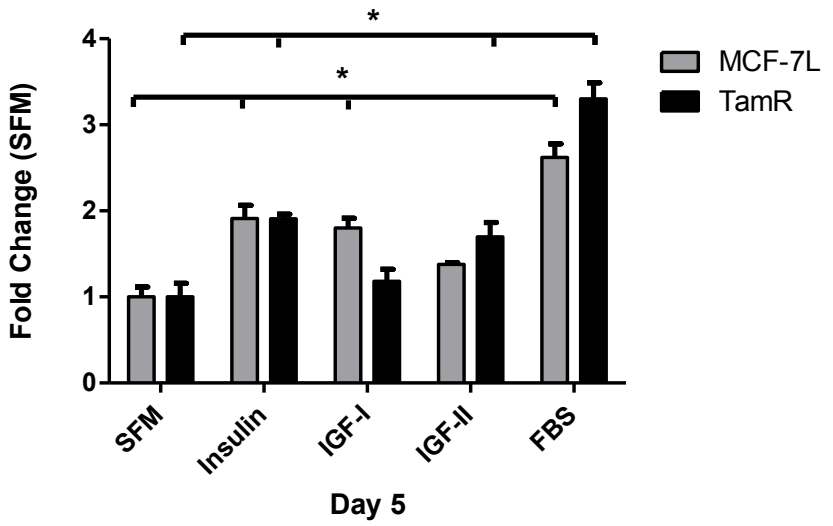


Figure 2.10
IGF1R mRNA levels are reduced in TamR cells, while IR levels remain unchanged. Cells were plated and exposed to charcoal stripped serum prior to an overnight starvation and a 4 hour estradiol treatment. Total RNA was isolated from MCF-7L and TamR (left panel) or T47D and TamR (right panel) cells and was reverse transcribed and IGF1R (A) and IR (B) levels were analyzed using qRT-PCR. Data was normalized to the RPLP0 housekeeper gene. One way ANOVA with Tukey's post-test was done to compare the statistical significance between the cell lines. *$p<0.05$, **$p<0.01$

A.



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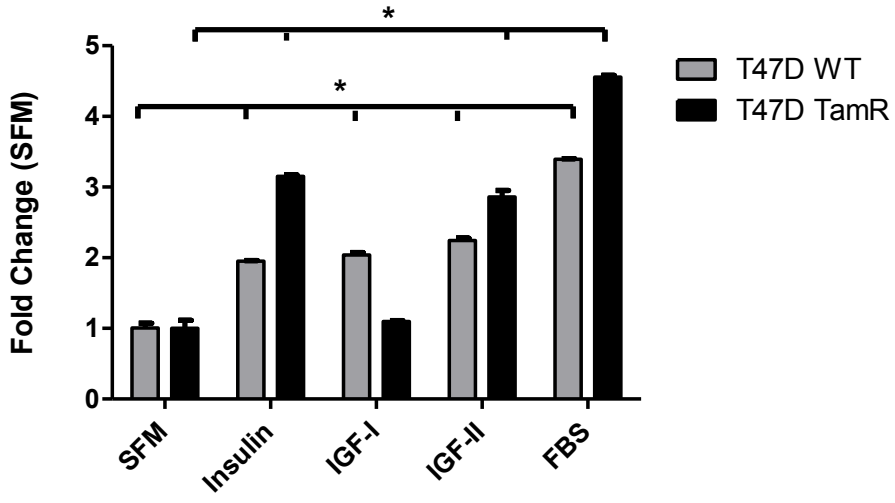
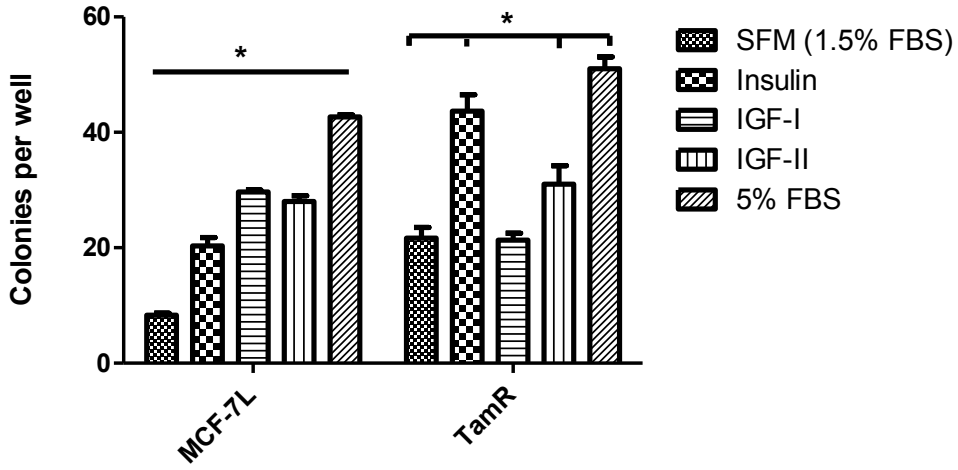


Figure 2.11
TamR cells have decreased proliferation in response to IGF-I.
MCF-7L and TamR (A) or T47D and TamR (B) cells were serum starved overnight prior to treating cells with indicated ligands. Proliferation was evaluated at day 5 using MTT assay, with results displayed as fold change (vs. SFM). One way ANOVA with Tukey's post-test was done to compare the statistical significance between the cell lines. *$p<0.05$

A.



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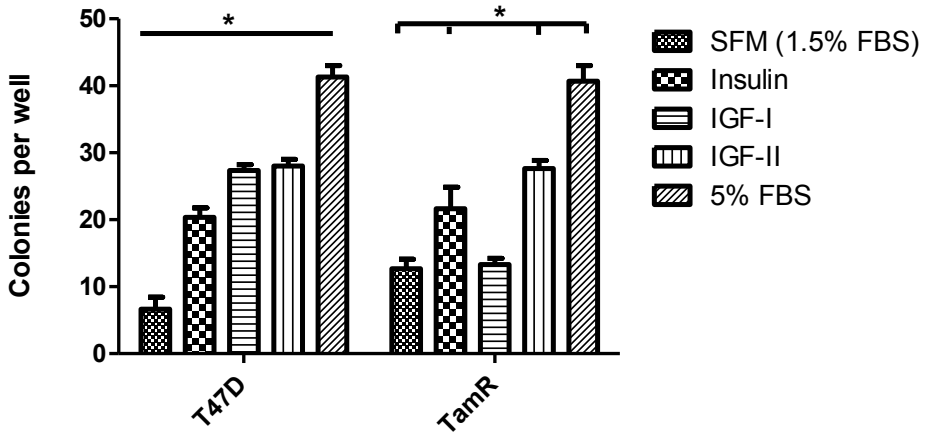

Figure 2.12
TamR cells do not grow in an anchorage independent manner in response to IGF-I, but do respond to IGF-II and insulin.
MCF-7L and TamR (A) or T47D and TamR (B) cells were serum starved and treated with anti-IGF antibody and ligand in 1.5% FBS in 0.45% agar and overlaid on 0.8% bottom agar. Colony growth in agarose was assessed after 14 days. Colonies formed were counted and averaged from 5 individual microscopic fields. Results displayed are the average number of colonies in 5 fields of 3 wells). One way ANOVA with Tukey's post-test was done to compare the statistical significance between the cell lines. *$p<0.05$

Chapter 3

Evaluation of the efficacy of IGF1R inhibitors in endocrine sensitive and resistant breast cancer cells.

Dedra H. Fagan, Ryan R. Uselman, Deepali Sachdev, and Douglas Yee.
Acquired resistance to tamoxifen is associated with loss of the type I insulin-like growth factor receptor (IGF1R): implications for breast cancer treatment.
Cancer Res. Published OnlineFirst May 9, 2012.

Introduction

The IGF1R is a receptor tyrosine kinase that exerts its biological effects through binding of the ligands IGF-I and IGF-II. Following, ligand binding and receptor activation, adaptor molecules are recruited, leading to activation of downstream pathways, including the MAPK and PI3K pathways, ultimately leading to proliferation, angiogenesis, resistance to apoptosis, and metastasis [106, 107]. The closely related insulin receptor behaves in a similar manner, through its ligands insulin and IGF-II.

Crosstalk between the IGF1R and estrogen receptor has been well-documented and has led to clinical trials investigating the combined use of IGF1R and ER-inhibitors. Multiple studies have shown that ERα can enhance IGF1R signaling through transcriptional upregulation of IGF1R, IRS-1, and IGF-II [34, 108-111]. Reciprocally, IGF1R has been shown phosphorylate and activate ER on serine-167 through an S6-kinase mechanism [91]. In addition to current IGF1R inhibitor clinical trials examining combined anti-IGF1R, anti-ER therapies, trials are also being performed in endocrine resistant populations.

The role of the IGF1R in cancer has been established and clinical trials evaluating inhibitors to this pathway are currently underway [112]. As noted, preclinical studies have documented crosstalk between the IGF1R and ER pathways [94], yet clinical trials conducted primarily in endocrine resistant patients have been disappointing [113]. *In vitro* and *in vivo* evaluation has been performed using endocrine sensitive cells, with

relatively little evidence demonstrating the effectiveness of anti-IGF1R therapy in endocrine resistant cells.

Two strategies of targeting the IGF1R are currently being evaluated in clinical trials. Monoclonal antibodies bind to the IGF1R, leading to receptor internalization and downregulation. Tyrosine kinase inhibitors bind to the ATP catalytic domain of the internal tyrosine kinase domain of the IGF1R and the closely related insulin receptor. While some view targeting of the IR dangerous due to metabolic consequences, recent data suggest a benefit to targeting the IR [114, 115]. Multiple reports have demonstrated a role for the insulin receptor in cancer biology [53, 116, 117]. Further, phase I clinical trials have shown limited metabolic consequences that can be treated using metformin [3]. Thus, the clinical benefit of using IGF1R/IR TKI's may outweigh their potential metabolic side effects.

The overall aim of our study was to investigate the effectiveness of anti-IGF therapies using an endocrine resistant model. Herein, we reveal tamoxifen-resistant cells lack expression of IGF1R, and hence, are unaffected by IGF1R monoclonal antibodies. Tamoxifen treated xenografts also have reduced levels of IGF1R and mice do not benefit from combined treatment with tamoxifen and dalotuzumab. Furthermore, complete and successful suppression of IGF1R signaling may require dual-inhibition of IGF1R and PI3K targets, as is currently under study in the clinic. Alternatively, endocrine resistant patients may require the use of tyrosine kinase inhibitors, which are effective through inhibition of IR signaling.

Results

IGF1R Antibodies Can Inhibit the Growth of Estrogen-Sensitive MCF-7L Cells.

Previous studies have demonstrated crosstalk between IGF1R and ER [34, 81, 91]. In general, ER-positive cells are able to grow and proliferate in response to IGF-I; whereas, ER-negative cells do not [53, 69]. We sought to determine whether various IGF1R antibodies could inhibit the growth of MCF-7L cells. Pretreating cells with two different IGF1R monoclonal antibodies resulted in IGF1R receptor downregulation. It also resulted in inhibition of IGF-I mediated signaling and slightly decreased IGF-II mediated signaling (figure 3.1). MCF-7L cells were treated with three different IGF1R monoclonal antibodies (R1507, AVE1642, or dalotuzumab [MK-0646]) in the presence of IGF-I or 5% FBS. All antibodies inhibited the IGF-I stimulated growth of MCF-7L cells (figure 3.2). To correlate these findings with a more relevant *in vivo* readout, we examined their ability to inhibit anchorage independent growth. All antibodies were also able to inhibit IGF-I stimulated anchorage-independent growth in an estrogen-containing environment (1% FBS) as shown in figure 3.3.

Dalotuzumab Can Inhibit the Growth of Estrogen-Sensitive MCF-7 Xenografts.

We next wanted to verify an IGF1R antibody (dalotuzumab) could inhibit the *in vivo* growth of MCF-7L cells. Initially, we used subcutaneous estrogen pellets to stimulate the growth of tumors. When tumors reached a volume of 100 mm^3, we began treating animals with dalotuzumab at a dose of 500 μg twice weekly. Dalotuzumab was effectively able to inhibit the growth of MCF-7L xenografts (figure 3.4).

Dalotuzumab inhibited signaling, proliferation, and anchorage-independent growth in parental, but not TamR cells.

Dalotuzumab (MK-0646) is a humanized monoclonal antibody that binds the IGF1R. It has been shown to down-regulate IGF1R *in vitro* and *in vivo* [72, 118]. In order to examine the ability of the antibody to inhibit IGF-induced signaling, we pretreated MCF-7L parental and TamR cells with 20 μg/ml dalotuzumab for 24 hours prior to stimulating cells with ligand. Dalotuzumab inhibited IGF-I signaling, as measured via Akt and MAPK phosphorylation, in MCF-7L (figure 3.5) and T47D parental cells and had a minimal effect on both insulin and IGF-II signaling. TamR cells did not respond to IGF-I, but pAkt was activated by IGF-II and insulin. Dalotuzumab did not affect response to any of the ligands in TamR cells, presumably due to lack of IGF1R expression. In order to examine if this difference was also biologically relevant, we examined the effect of dalotuzumab on proliferation and anchorage-independent growth using the MTT and soft agar assays, respectively. All IGF system ligands tested induced proliferation in MCF-7L (figure 3.6) and T47D parental cells; however, only proliferation in response to IGF-I was inhibited in the presence of dalotuzumab. In contrast, insulin and to a lesser extent IGF-II stimulated the proliferation of TamR cells and this proliferation was not inhibited by dalotuzumab. Similarly, all ligands induced the anchorage-independent growth of MCF-7L parental cells (figure 3.7) and dalotuzumab inhibited growth in response to IGF-I and IGF-II. In agreement with the signaling data, both insulin and IGF-II induced the anchorage-independent growth of TamR cells. This

growth was not inhibited by dalotuzumab. Thus, dalotuzumab inhibited IGF-induced signaling, proliferation, and anchorage-independent growth in MCF-7L parental cells, but had no effect in TamR cells, presumably due to their lack of IGF1R expression.

AEW541 inhibited signaling, proliferation, and anchorage-independent growth in parental and TamR cells.

AEW541 is a dual tyrosine kinase inhibitor (TKI) that targets both IGF1R and insulin receptor. In order to examine the effect of IGF1R TKI's in endocrine resistance, we pretreated MCF-7L parental and TamR cells for three hours with 0.5 μM AEW541 prior to stimulating cells with ligands. AEW541 inhibited insulin, IGF-I, and IGF-II signaling in MCF-7L cells (figure 3.8) and T47D cells. Further, AEW541 was also able to inhibit insulin and IGF-II stimulated phosphorylation of Akt and MAPK in TamR cells. To investigate whether this inhibition was also biologically important, we again examined proliferation and anchorage-independent growth. AEW541 was able to inhibit insulin, IGF-I, and IGF-II stimulated proliferation in MCF-7L (figure 3.9) and T47D cells and insulin and IGF-II stimulated proliferation in TamR cells. Additionally, AEW541 was also able to inhibit insulin, IGF-I, and IGF-II stimulated anchorage-independent growth in MCF-7L parental cells and insulin and IGF-II stimulated anchorage-independent growth in TamR cells (figure 3.10). Thus, AEW541 was able to inhibit signaling, proliferation, and anchorage-independent growth by suppressing both IGF1R and IR function in MCF-7L parental cells. Interestingly, AEW541 was also able to inhibit the growth of TamR cells presumably via suppression of IR signaling. These data

show that TKI's, which target both IGF1R and IR, are effective in parental and resistant cells, due to inhibition of IR signaling.

Dalotuzumab inhibited estrogen stimulated growth but did not add to tamoxifen-mediated growth inhibition *in vivo*.

We next examined the effect of dalotuzumab on the *in vivo* growth of MCF-7L cells. Ovariectomized athymic mice were injected in the second mammary fat pad with MCF-7L cells as previously described [67]. Mice were administered estrogen to stimulate tumor growth and tumors were allowed to establish (tumor volume of ~ 200 mm^3) prior to beginning treatment. Dalotuzumab (administered beginning at day 32) inhibited the growth of estrogen stimulated tumors (figure 3.11). To study the combination of tamoxifen and dalotuzumab, estradiol was withdrawn on day 32 and tamoxifen was started. Dalotuzumab treatment began simultaneously with tamoxifen (Tam+dalotuzumab) or when tumors began to grown on tamoxifen alone (Tam $\rightarrow$dalotuzumab) at approximately day 74. Tamoxifen by itself inhibited the growth of tumors; however, dalotuzumab co-administered with tamoxifen did not further suppress tumor growth. Further, dalotuzumab did not significantly inhibit the growth of tamoxifen-resistant tumors when administered after the tumors began to grow on tamoxifen.

We next sought to determine whether this lack of efficacy of dalotuzumab in tamoxifen treated xenografts was due to decreased IGF1R levels similar to the lack of IGF1R expression as observed *in vitro*. When tumors reached 1000 mm^3, mice were sacrificed and tumors were harvested for RNA isolation. Expression of IGF1R mRNA

was significantly reduced in tamoxifen treated xenografts when compared to estrogen treated xenografts regardless of dalotuzumab treatment (figure 3.12). Thus, tamoxifen treated xenografts do not benefit from dalotuzumab treatment, due to decreased IGF1R expression. However, estrogen treated xenografts express significantly more IGF1R and benefit from dalotuzumab treatment. These data suggest that the level of receptor expression is important in determining response to dalotuzumab treatment and that estrogen receptor plays an important role in regulating IGF1R expression.

Global gene expression profiling reveals significant changes between estrogen and tamoxifen treated xenografts.

In order to learn more about tamoxifen resistance *in vivo*, we performed global gene expression profiling on collected xenograft tumor samples. Specifically, we compared differences between tumors stimulated with estrogen versus tamoxifen. Tumors were harvested during the growth phase of tamoxifen treatment, indicating the tumors were resistant or no longer responding to tamoxifen treatment. We found ~1038 genes to be differentially regulated in estrogen treated compared to tamoxifen resistant tumors using a $p<0.05$ and fold change >1.5 (figure 3.13). Several network pathways, notably those involved in cellular development/proliferation and gene expression were modulated in tamoxifen resistant xenografts (figure 3.14, 3.15). Ingenuity® pathway analysis revealed significant alterations in ~180 pathways when comparing tamoxifen resistant xenografts to estrogen treated xenografts. Interestingly, two of the pathways found to be disregulated were the IGF-I signaling pathway and the estrogen-dependent breast cancer signaling pathway. Further, one of the ten most highly downregulated

genes in tamoxifen resistant xenografts (*IGF1R)* is a known estrogen regulated gene (figure 3.16). Multiple other estrogen-regulated genes were also found to be significantly decreased in resistant xenografts, including *PGR* and *GREB1*. These data support our qPCR findings of decreased IGF1R levels in tamoxifen treated xenografts. Further, these data support our findings from Chapter 2, which demonstrated classic genomic function of the estrogen receptor was suppressed in tamoxifen resistant cells.

Discussion

The recently published results of IGF1R antibodies in clinical trials demonstrating limited success in endocrine-resistant populations prompted us to investigate their efficacy using an endocrine resistant model. Previous investigations into the efficacy of anti-IGF therapies have been performed using endocrine-sensitive cell lines and xenograft models. Since IGF1R is an ER transcriptional target, understanding if IGF1R expression was affected by resistance to tamoxifen has clinical relevance. Interestingly, studies performed by Massarweh, et. al. using tamoxifen-resistant xenografts demonstrate decreased total levels of IGF1R, but basal phosphorylation of the receptor [99]. This discordance may be explained by a difference in dosage of tamoxifen in model systems. In our model, tamoxifen is continuously administered to cells, whereas, in the Massarweh study, animals are given tamoxifen five times weekly, leading to the possibility that ER function is not completely suppressed in this model. Furthermore, this study did not clearly distinguish between IGF1R or IR phosphorylation since the "phospho-specific" antibody detects both receptors.

The finding that tamoxifen resistant cells were refractory to IGF1R antibody treatment underscores the importance of using model systems similar to the patient populations the drug will be used in. Although several studies have demonstrated the efficacy of IGF1R monoclonal antibodies in breast cancer cells, these cells have been endocrine sensitive [67, 72]. The effect of combined anti-estrogen/anti-IGF1R treatment should also take into consideration whether the dose of anti-estrogen is sufficient, in and of itself, to suppress IGF1R function via receptor downregulation. Our *in vivo* results demonstrate that tamoxifen treatment results in decreased IGF1R mRNA levels. Initial results examining the effectiveness of IGF1R antibodies in endocrine resistant breast cancer populations have not demonstrated a definitive positive result [4, 119]. This may be due to the lack of IGF1R expression in these patient populations. A recent study examining mRNA expression in a cohort of tamoxifen-resistant breast cancer patients has demonstrated a decrease in IGF1R levels in the recurrent tumors, suggesting our findings *in vitro* may correlate with the clinical scenario [120].

The efficacy of TKI's in our tamoxifen resistant cells underscores the importance of co-targeting the IR, along with the IGF1R. Initially, development of IGF1R inhibitors aimed to avoid targeting the IR, due to potential metabolic consequences. However, numerous studies by us and others have demonstrated the IR does indeed play a role in cancer biology [3, 4, 115, 121]. Specifically, work by Hanahan and colleagues showed that IGF1R inhibition using antibodies is only successful in tumors/cells where the IGF1R/IR ratio is high. Additionally, they demonstrate the IR can actually serve as an escape mechanism, providing resistance to IGF1R antibodies [117]. Further, work by

Haluska and colleagues has shown that when figitumumab (an IGF1R monoclonal antibody) is administered to patients, there is an associated increase in plasma insulin [122]. This increase in insulin levels could potentially lead to increased IR signaling in tumor cells, providing yet another escape mechanism for the cancer cells to survive. These data are supported by a case report demonstrating increased copy number of IR in a woman with metastatic hormone refractory breast cancer [123]. The role of the IR in cancer biology has been clearly defined, and the metabolic consequences of its inhibition are actively being investigated. A recent study performed in mice demonstrated that an IGF1R/IR TKI alone or in combination with tamoxifen did not lead to a significant change in glucose homeostasis, suggesting the drugs are tolerable. This study also demonstrated the efficacy of the TKI in letrozole resistant xenografts, supporting our data that suggest TKI's are more effective than antibodies in tamoxifen resistant cells [124].

Although our data indicate IGF1R antibodies may not be effective in an endocrine resistant system, this does not mean they have little utility. Multiple trials are underway, examining the use of IGF1R antibodies in endocrine sensitive populations. One promising area of investigation is inhibiting both the upstream (IGF1R) and downstream (mTOR) components of the IGF1R pathway, leading to maximal inhibition of signaling. Inhibition of IGF1R has been shown to sensitize cells to mTOR inhibition [125]. Additionally, clinical trials are underway examining the efficacy of combining IGF1R antibodies with mTOR inhibitors [126, 127].

Overall, our data highlight the importance of using model systems that will match the patient population the drug will ultimately be used in. Additionally, when evaluating

IGF1R therapies, it will be important to carefully select the proper patient population, as well as to verify the target is present. Finally, our data suggest dual IGF1R/IR TKI's may be more effective than IGF1R antibodies, due to inhibition of IR. Combination therapy using IGF1R antibodies may require use of an mTOR inhibitor for complete suppression of the target, as is currently being evaluated in the clinic.

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Acknowledgements: We acknowledge Aaron Sarver from the Masonic Cancer Center Biomedical Informatics Core at the University of Minnesota for analysis of microarray data. We also acknowledge Merck for providing dalotuzumab and performing gene expression profiling.

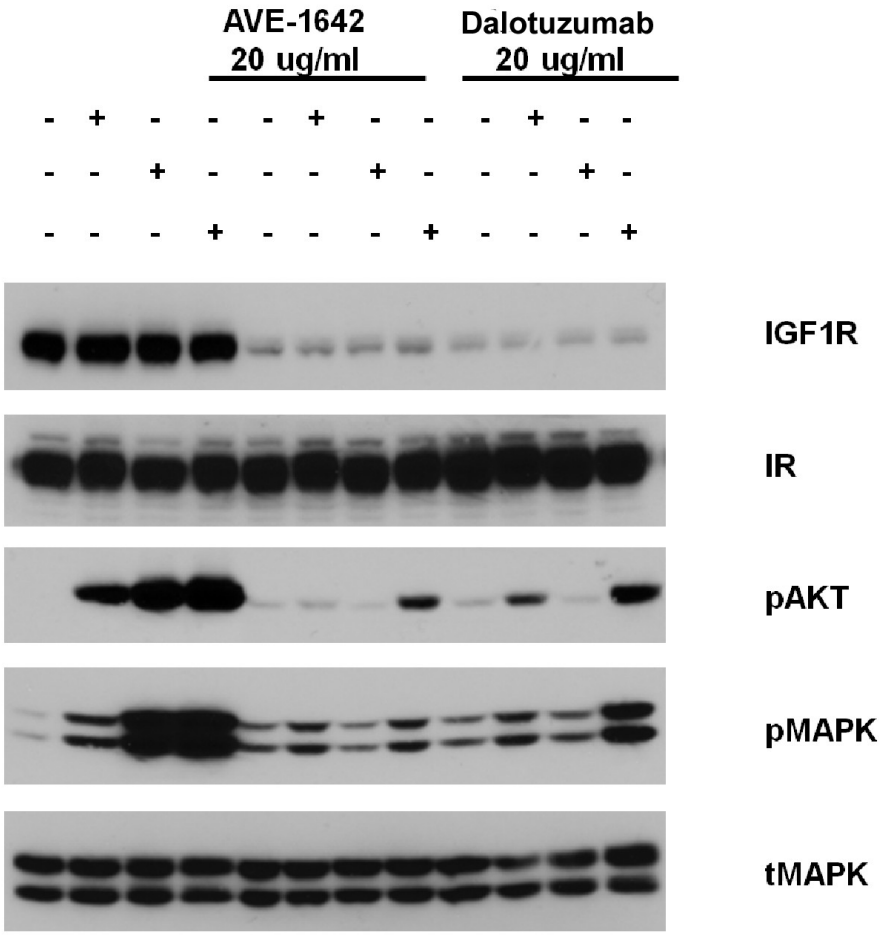

Figure 3.1
IGF1R antibodies are effective at inhibiting IGF-mediated signaling in estrogen receptor-positive breast cancer cells.
MCF-7L cells were pre-treated with indicated IGF1R antibodies for 24 hours in SFM prior to stimulating with ligands for 10 minutes. Lysates were collected and resolved using SDS-PAGE. Protein levels of IGF1R, pAkt, pMAPK, and total MAPK were assessed by using specific antibodies and immunblotting.

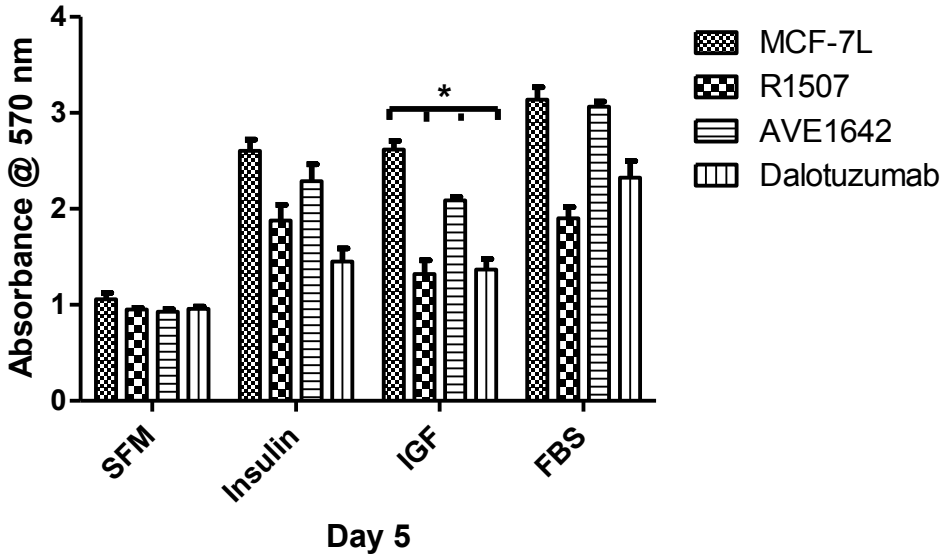

Figure 3.2
Monoclonal antibodies against IGF1R can inhibit the proliferation of MCF-7L cells. MCF-7L cells were serum starved and treated with indicated anti-IGF antibody along with indicated ligands. Proliferation was evaluated at day 5 using MTT assay, with results displayed as absorbance at 570 nm. One way ANOVA with Tukey's post-test was done to compare the statistical significance between the cell lines. *$p<0.05$

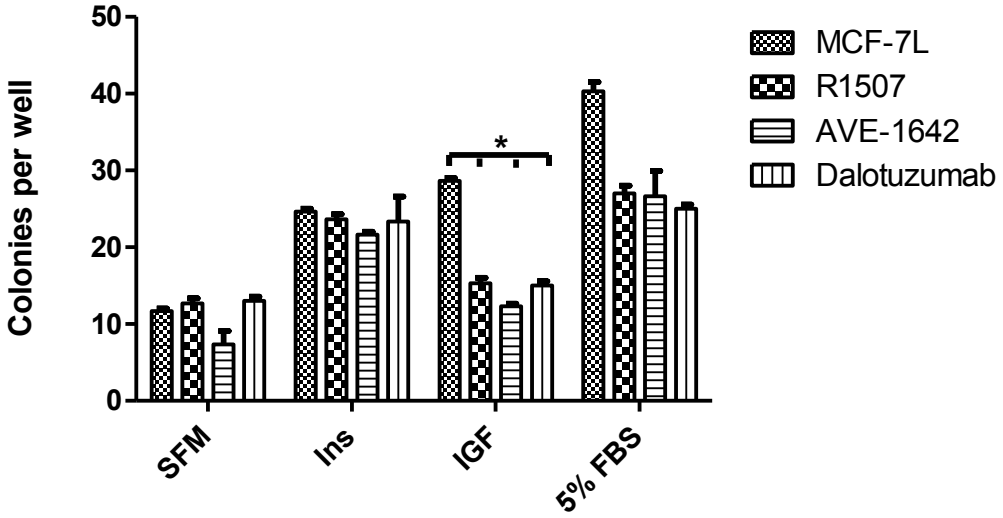

Figure 3.3
IGF1R monoclonal antibodies can inhibit the anchorage independent growth of MCF-7L cells.
MCF-7L cells were serum starved and treated with indicated anti-IGF1R antibody and ligand in 1.5% FBS in 0.45% agar and overlaid on 0.8% bottom agar. Colony growth in agarose was assessed after 14 days. Colonies formed were counted and averaged from 5 individual microscopic fields. Results displayed are the average number of colonies in 5 fields of 3 wells. One way ANOVA with Tukey's post-test was done to compare the statistical significance between the cell lines. *$p<0.05$

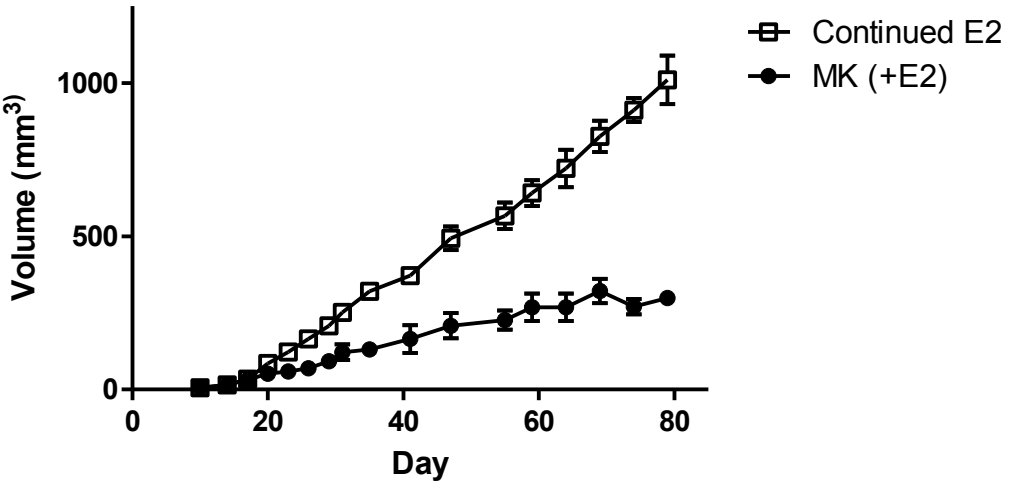

Figure 3.4
Dalozutumab can inhibit estrogen-stimulated growth in MCF-7L xenografts.
Ovariectomized athymic mice with MCF-7L xenograft tumors were treated with E2 +/- dalotuzumab. Tumor volumes were measured weekly and average volume was plotted.

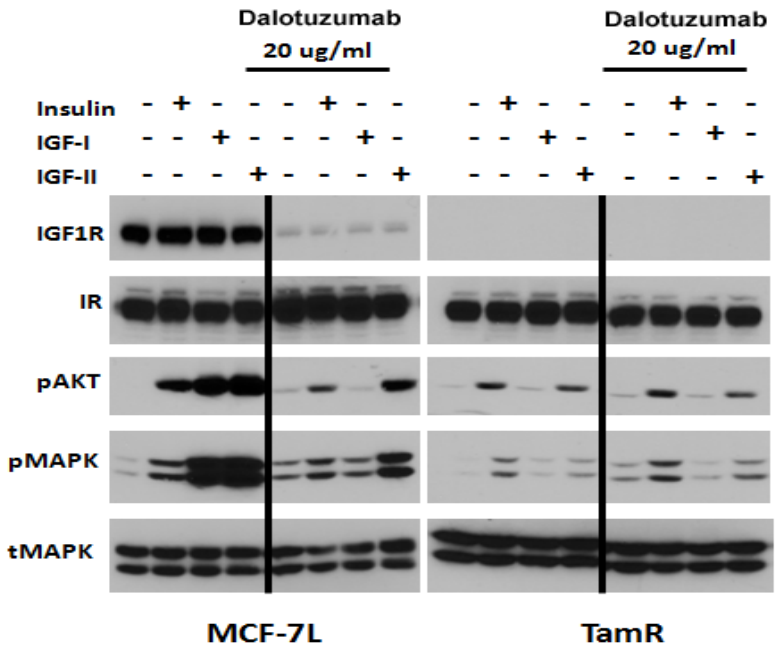

Figure 3.5
Treatment with an IGF1R antibody inhibits biochemical signaling in MCF-7L parental, but not TamR cells.
MCF-7L and TamR cells were serum starved overnight and pre-treated with 20 ug/ml antibody for 24 hours prior to treating the cells with 10 nM insulin, 5 nM IGF-I or 5 nM IGF-II for 10 minutes. Cellular lysates were separated by SDS-PAGE and levels of IGF1R, IR, phosphorylated Akt and MAPK, and total MAPK protein levels were assessed using specific antibodies by immunoblotting. (Adapted from figure 3.1).

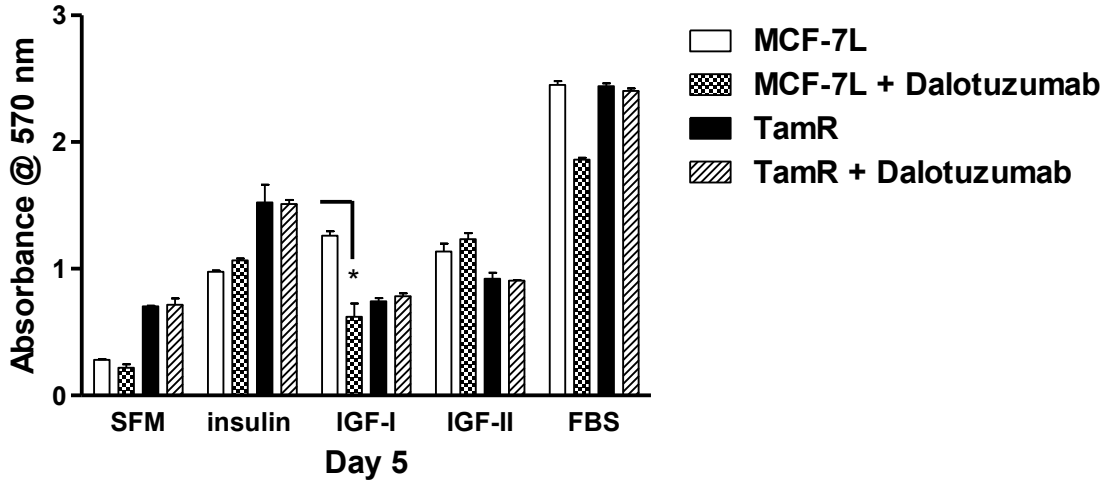

Figure 3.6
Tamoxifen resistant cells are refractory to IGF1R antibody treatment in a proliferation assay.
MCF-7L and TamR cells were serum starved and treated with anti-IGF antibody along with ligand. Proliferation was evaluated using MTT assay, with results displayed as absorbance at 570 nm. Two way ANOVA with Bonferroni comparison was used to compare the difference between antibody pre-treatment and un-treated samples. *$p<0.01$

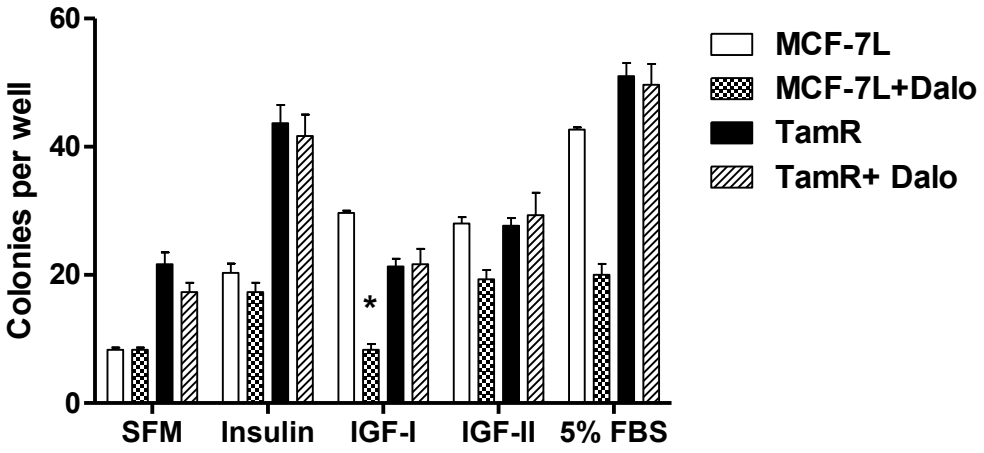

Figure 3.7
Treatment with an IGF1R antibody does not affect anchorage independent growth in tamoxifen resistant cells.
MCF-7L and TamR cells were serum starved and treated with anti-IGF antibody and ligand in 1% FBS in 0.45% agar and overlaid on 0.8% bottom agar. Colony growth in agarose was assessed after 14 days. Colonies formed were counted and averaged from 5 individual microscopic fields. Results displayed are the average number of colonies in 5 fields of 3 wells. Two way ANOVA with Bonferroni comparison was performed to compare the difference between antibody pre-treated and un-treated samples. *$p<0.01$

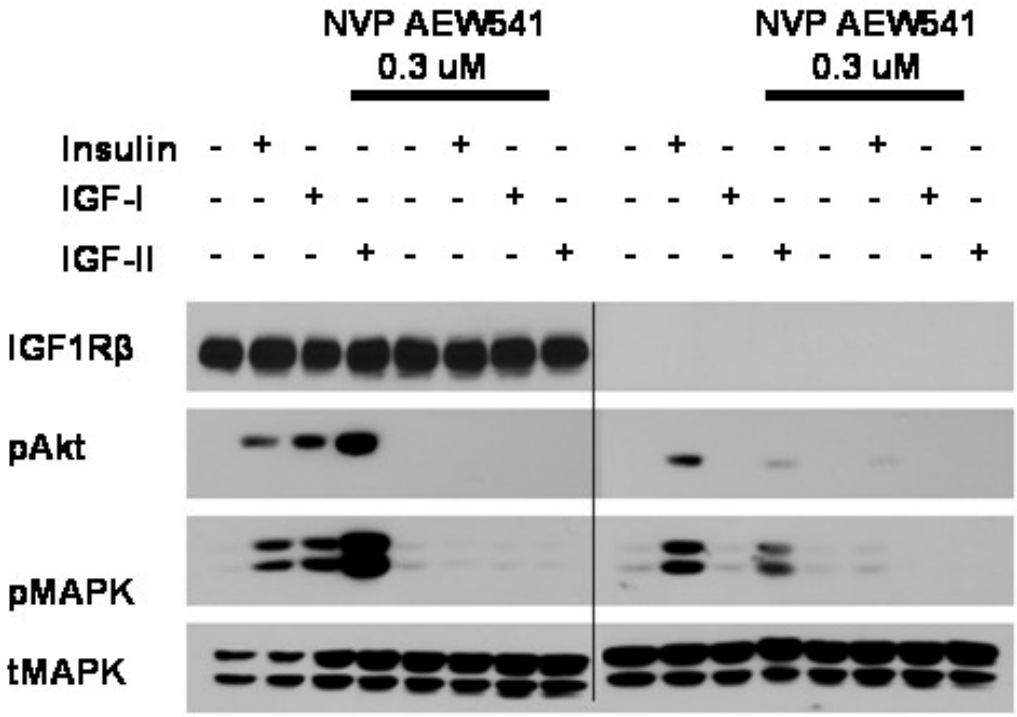

Figure 3.8
A dual IGF1R/IR tyrosine kinase inhibitor inhibits biochemical signaling in both MCF-7L parental and TamR cells.
MCF-7L and TamR cells were serum starved overnight and pre-treated with 0.3 uM TKI for 3 hours prior to treating the cells with 10 nM insulin, 5 nM IGF-I or 5 nM IGF-II for 10 minutes. Cellular lysates were separated by SDS-PAGE and levels of IGF1R, IR, phosphorylated Akt and MAPK, and total MAPK protein levels were assessed using specific antibodies by immunoblotting.

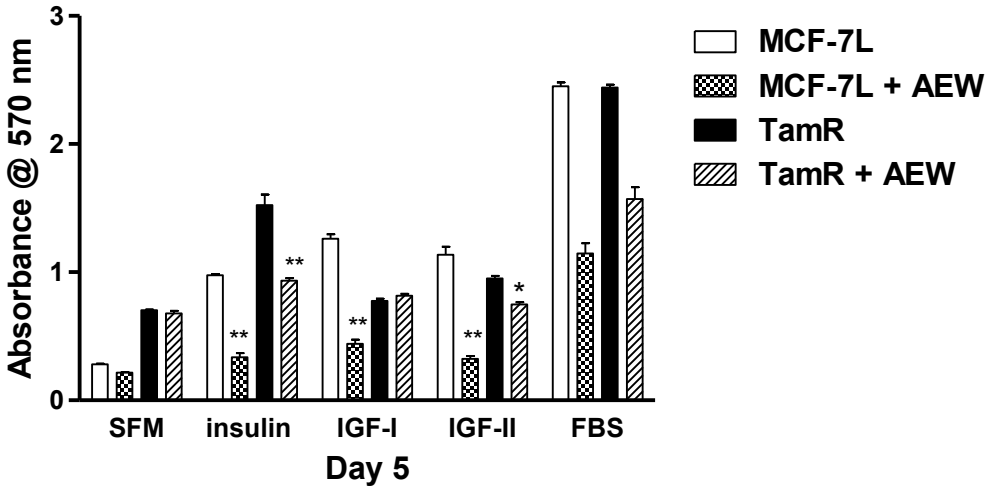

Figure 3.9
Proliferation in MCF-7L and TamR cells can be inhibited using an IGF1R/IR tyrosine kinase inhibitor.
MCF-7L and TamR cells were serum starved and treated with anti-IGF1R/IR TKI along with ligand. Proliferation was evaluated using MTT assay, with results displayed as absorbance at 570 nm. Two way ANOVA with Bonferroni comparison was used to compare the difference between TKI treatment and un-treated samples. *$p<0.05$, **$p<0.005$

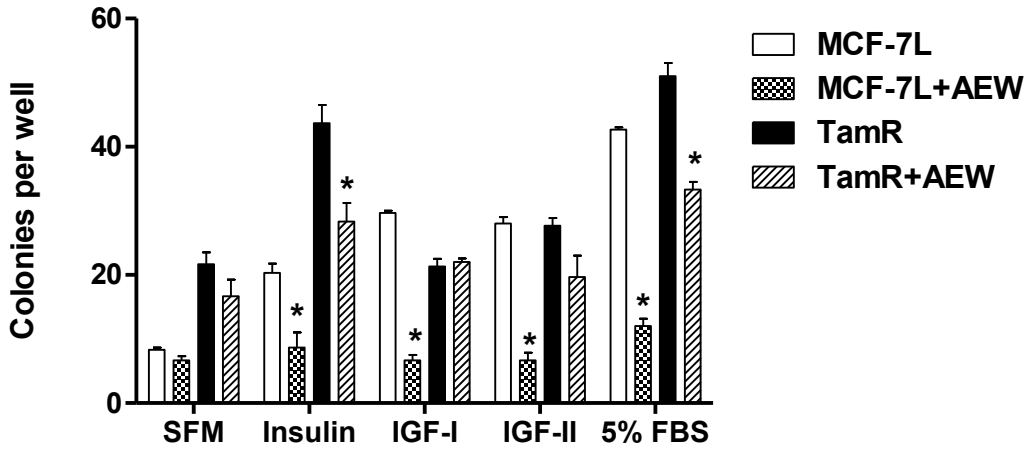

Figure 3.10
NVP-AEW541 (an IGF1R/IR tyrosine kinase inhibitor) can inhibit anchorage independent growth in both MCF-7L and TamR cells.
MCF-7L and TamR cells were serum starved and treated with anti-IGF1R/IR TKI and ligand in 1% FBS in 0.45% agar and overlaid on 0.8% bottom agar. Colony growth in agarose was assessed after 14 days. Colonies formed were counted and averaged from 5 individual microscopic fields. Results displayed are the average number of colonies in 5 fields of 3 wells. Two way ANOVA with Bonferroni comparison was performed to compare the difference between TKI treated and un-treated samples. *$p<0.01$

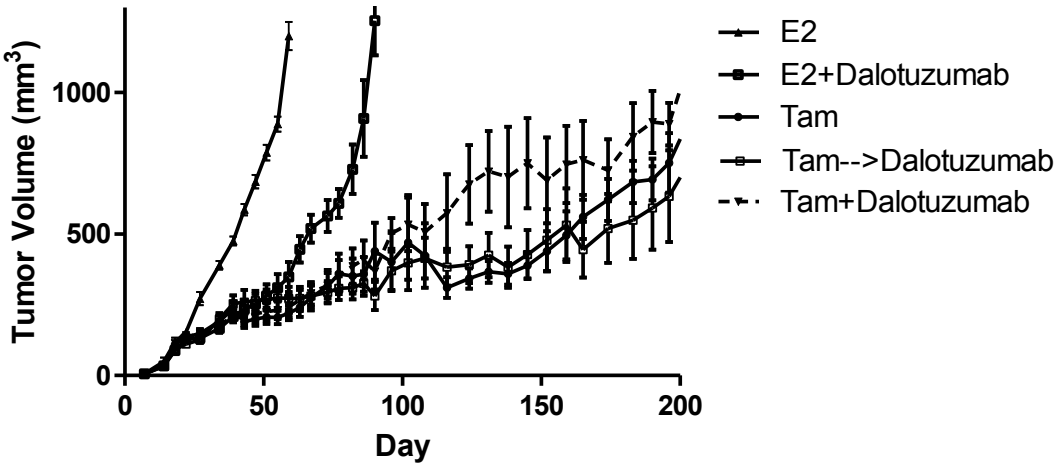

Figure 3.11
Dalotuzumab did not add to tamoxifen-mediated growth inhibition in MCF-7L xenografts.
Ovariectomized athymic mice with MCF-7L xenograft tumors were treated with E2 and tamoxifen, +/- dalotuzumab. Tumor volumes were measured weekly and average volume was plotted.

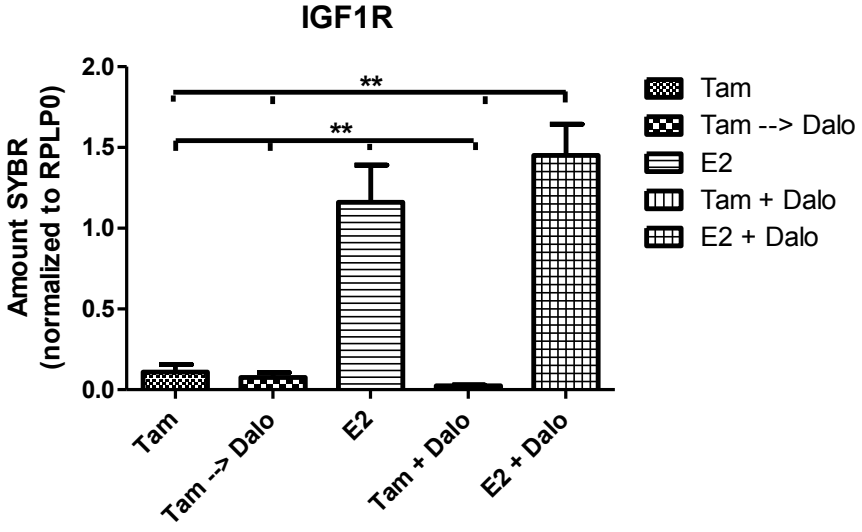

Figure 3.12
IGF1R mRNA levels were decreased in tamoxifen treated xenografts.
Xenografts were harvested from mice and total RNA was isolated using TriPure Reagent. RNA was reverse transcribed and analyzed using qRT-PCR. Results were normalized to the RPLP0 housekeeping gene. One way ANOVA with Tukey's comparison was used to compare the difference between treatment groups. *$p<0.05$, **$p<0.005$

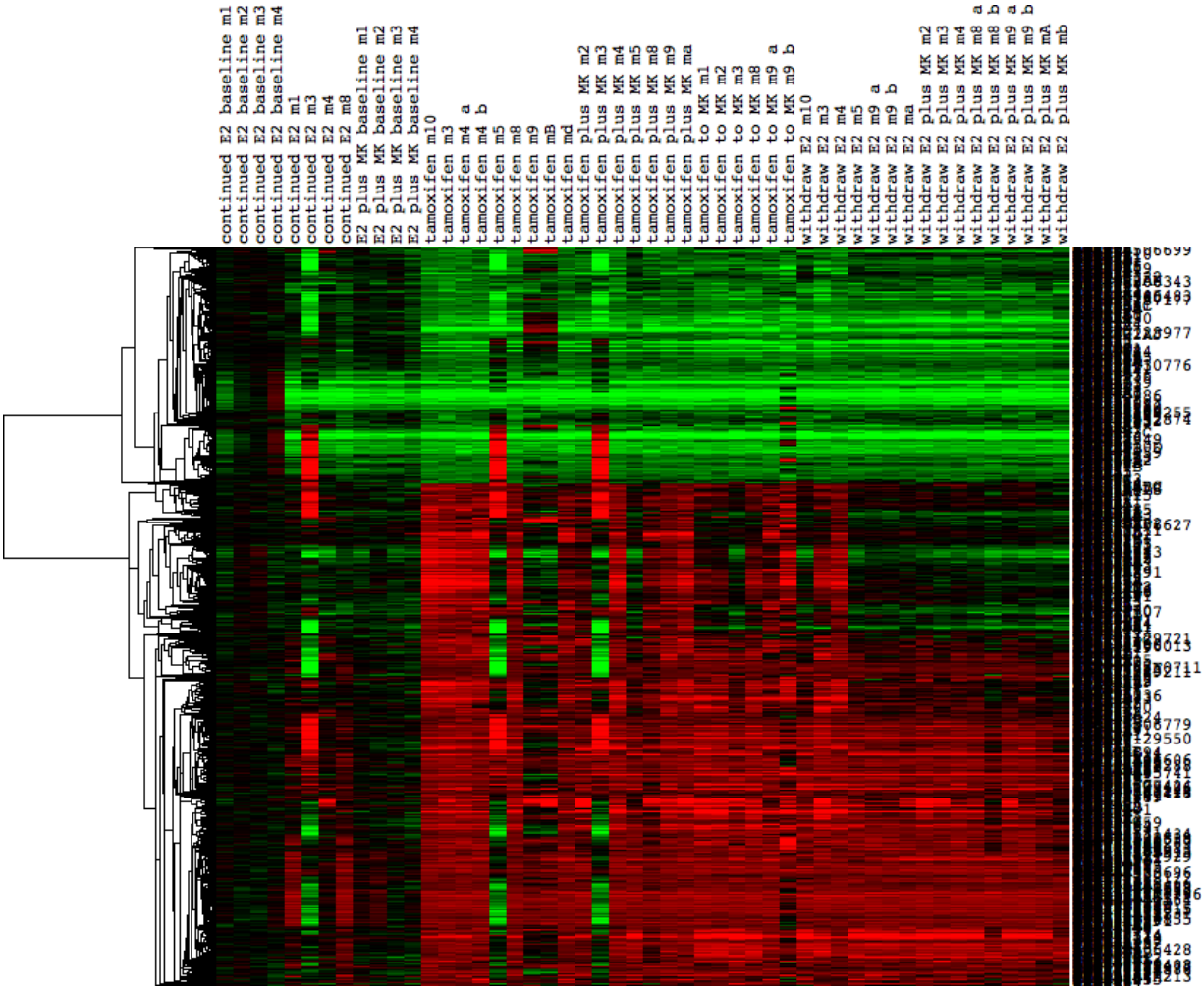

Figure 3.13
Global gene expression profiling reveals differences in estrogen and tamoxifen treated xenografts.
Comparative analysis of xenografts stimulated with estrogen (left most samples) and tamoxifen (center samples). The y-axis represents differentially expressed transcripts and the x-axis represents xenograft samples.

Figure 3.14
Multiple signaling networks are altered in tamoxifen treated xenografts.
Network analysis was carried out on the gene set found to be significantly changed between tamoxifen and estrogen treated xenografts. The top three networks from the analysis are depicted.

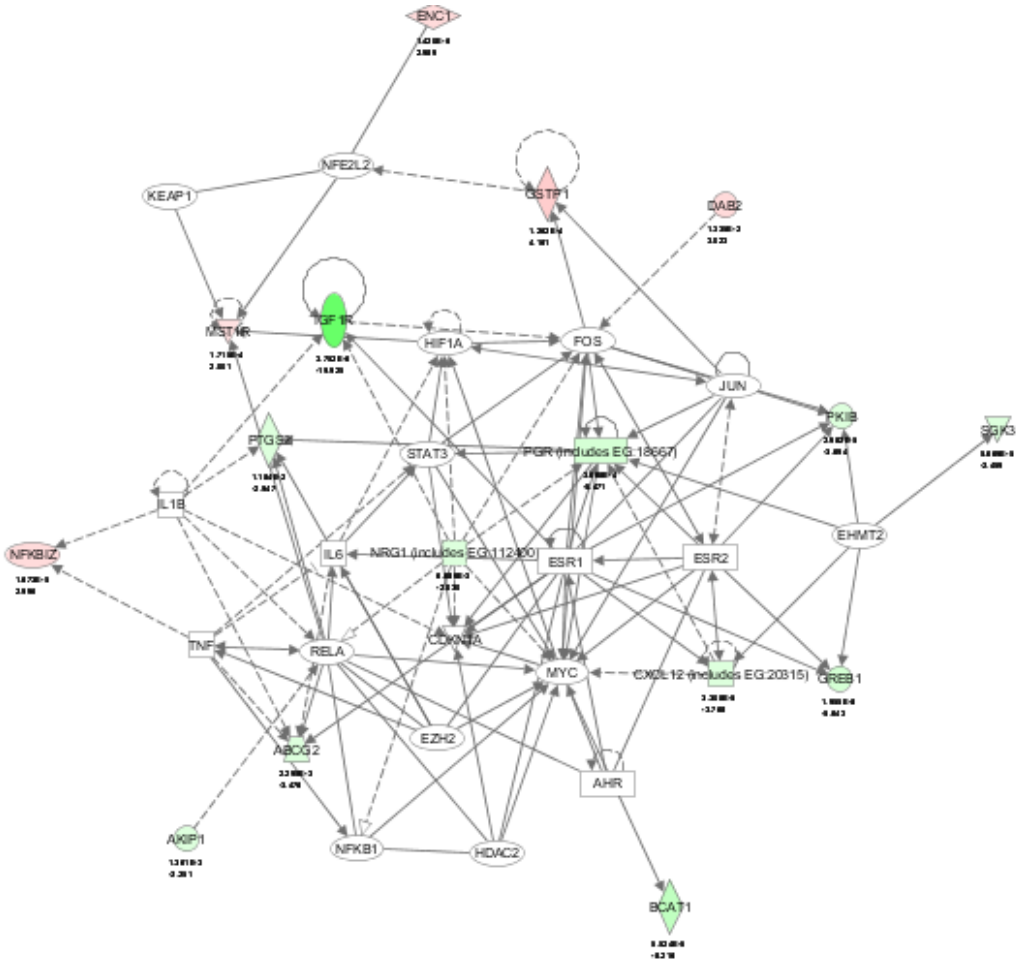

Figure 3.15
Pathway analysis reveals significant changes in key pathways in tamoxifen treated xenografts.
Transcripts significantly different (fold change >1.5, $p<0.05$) were subjected to Ingenuity® pathway analysis. The gene expression/estrogen regulation in breast cancer pathway is depicted as a representation of one of the ~170 pathways altered in tamoxifen treated xenografts.

Figure 3.16
Estrogen-regulated genes are downregulated in tamoxifen treated xenografts. Genes that were significantly different ($p<0.05$) in tamoxifen treated xenografts were sorted and ranked according to level of downregulation. The ten most highly downregulated genes are listed.

Chapter 4

Concluding Remarks

Studies of the ER have revealed a complexity in the biology of the receptor—this complexity may also be translated to the mechanisms responsible for endocrine resistance in breast cancer. Both genomic and non-genomic activities of ER, in addition to crosstalk with growth factor and kinase pathways, influence the cells (or tumors) sensitivity to endocrine therapy. Understanding this network will provide clear therapeutic advantages in treating and delaying endocrine resistance. Further, an understanding of endocrine resistance will provide insight into how best to use new therapeutic strategies in breast cancer. New breast cancer therapies are often tested in those patients who have failed on previous endocrine therapy; however, as demonstrated in this thesis it could be disadvantageous.

The data presented in this thesis provide evidence that IGF1R inhibitors, specifically monoclonal antibodies, are ineffective in a tamoxifen resistant model due to lack of IGF1R expression. Data from the first part of this work describe the generation and characterization of a tamoxifen resistant model system that has similar properties to the clinical scenario and may be useful in evaluating new therapies in an endocrine resistant setting. Using this model system, in the second part of this thesis, we examine the efficacy of IGF1R inhibitors in both endocrine sensitive and resistant breast cancer cells. Using multiple IGF1R monoclonal antibodies, we demonstrate that endocrine sensitive, ER positive breast cancer cells express high levels of IGF1R and are, therefore, sensitive to inhibition of this receptor. In contrast, TamR cells lack IGF1R expression and are not sensitive to IGF1R inhibition using monoclonal antibodies. TamR cells maintain expression of IR and their growth can be inhibited with a dual IGF1R/IR

tyrosine kinase inhibitor. This lack of IGF1R expression was further confirmed *in vivo* using tamoxifen treated xenografts.

The data presented is of important clinical significance as IGF1R monoclonal antibodies are currently being evaluated in clinical trials. These trials in breast cancer are primarily in endocrine resistant patients. Unfortunately, results from these trials have not been positive, potentially due to lack of IGF1R expression in these patients. Based on our findings, inhibition of IR may be necessary in tamoxifen resistant patients. At the very least, IGF1R expression in tumors should be evaluated prior to evaluating IGF1R monoclonal antibodies in clinical trials, especially in the endocrine resistant setting.

Materials and Methods

Reagents. All chemical reagents were purchased from Sigma unless otherwise indicated. IGF-I, IGF-II, and insulin were purchased from Novozymes Gro*Pep* Limited (Denmark) and Eli Lilly (USA) respectively.

Cell lines and culture. All cells were grown at 37° C in a humidified atmosphere containing 5% CO_2 and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin. MCF-7 cells were provided by C. Kent Osborne (Baylor College of Medicine, Houston, TX) and maintained in improved MEM Richter's modification medium (zinc option) supplemented with 5% FBS and 11.25 nM insulin. MCF-7 TamR cells were generated by culturing MCF-7 in phenol-red free IMEM (zinc option) supplemented with 11.25 nM insulin, 5% charcoal/dextran-treated FBS, and 100 nM 4-OH-tamoxifen. T47D cells were obtained from ATCC and maintained in MEM supplemented with 5% FBS and 6 ng/ml insulin. T47D TamR cells were generated by culturing T47D cells in phenol-red free IMEM supplemented with 5% charcoal/dextran-treated FBS, and 100 nM 4-OH-tamoxifen. TamR cells were grown in the presence of 4-OH-tamoxifen for 6 months to allow resistance to develop prior to characterizing cells. As a control, parental cells were cultured for the same amount of time in regular media. Following the establishment of resistance, cells were passed for no more than 3 months.

Antibodies. Horseradish peroxidase-conjugated antiphosphotyrosine (PY-20) was purchased from BD Biosciences (Sparks, MD). The ERα antibody used for Western blot

analysis was purchased from Neomarkers Lab Vision (Fremont, CA). The IRβ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phosphorylated Akt, IGF1Rβ, and total and phospho-p44/42 (MAPK/ERK) were purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce (Rockford, IL).

Growth Curve Analysis. Cells were plated at a density of 1 x 10^4 in 6-well plates and allowed to equilibrate overnight. Full medium was replaced with phenol-red free IMEM supplemented with 1% dextran-coated-charcoal (DCC)-FBS. 4-OH-tamoxifen was added to cells at concentration and time as indicated in the figures. Cells were stained with trypan blue and counted using a hemacytometer.

Immunoblot. Cells were plated at a density of 3×10^5 in 60-mm-diameter dishes and allowed to equilibrate overnight. Full medium was replaced with dextran-coated-charcoal (DCC)-treated fetal calf serum for the next 3–5 d, after which cells were switched to serum-free medium (SFM) for 24 h. Upon reaching 70% confluency, cells were treated, placed on ice, washed twice with ice-cold PBS, and lysed with lysis buffer of 50 mM Tris-Cl (pH 7.4), 1% Nonidet P-40, 2 mM EDTA (pH 8.0), 100 mMNaCl, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin). Lysates were clarified by centrifugation at 12,000 × rpm for 15 min at 4 C. Protein concentrations were determined using the bicinchoninic acid protein assay reagent kit (Pierce). Cellular protein (50 μg) was separated by SDS-PAGE,

transferred to a nitrocellulose membrane, and immunoblotted according to manufacturer guidelines.

Monolayer growth assay. Cells were plated in 24-well plates at a density of 30,000 cells per well, allowed to equilibrate overnight and starved in SFM media for 24 hours. After 5 days of treatment, growth was assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously [128]. 60 uL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in SFM was added to each well. After incubation for 3 h at 37°C, wells were aspirated and formazan crystals were lysed with 500 µL of solubilization solution (95% DMSO + 5% IMEM). Absorbance was measured with a plate reader at 570 nm using a 650 nm differential filter to assess growth.

Anchorage-independent growth. A 1-ml layer of 0.8% SeaPlaque-agarose (BioWhittaker, Rockland, ME) in 1% FBS-containing growth media was solidified into each well of a six-well plate. The bottom layer was overlaid with 0.8 ml of a 0.45% top agar mixture for 10,000 cells per well with appropriate treatment. All plates were incubated at 37 C. After 12 d colony number was assessed on a light microscope with an ocular grid. Five random fields were counted per well and only colonies exceeding two thirds of a grid square were scored.

RNA Isolation and qRT-PCR. Cells were plated at a density of 1×10^6 in 100-mm-diameter dishes, allowed to equilibrate overnight, DCC starved for 3 d, and incubated overnight in SFM. Cellular RNA was isolated using TriPure Reagent according to the manufacturer (Roche). For quality control and to determine concentration, a 260:280

assay was performed on a spectrophotometer. Forward and reverse primers were designed to target the following transcripts: RPLP0 and IGF1R. A total of 2 μg of RNA was reverse transcribed using the Transcriptor reverse transcriptase kit, and quantitative PCR was performed using the Universal SYBR Green kit according to the manufacturer's recommended protocol (Roche) on an Eppendorf Mastercycler Realplex^{4}machine (Hamburg, Germany). The relative concentration of mRNA was calculated using cycle threshold values that were derived from a standard curve and normalized to ribosomal protein, large, P0 as an internal control.

Xenograft growth. All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. MCF-7L cells (5 x 10^6) were injected into the mammary fat pad of 5-week-old female ovariectomized athymic mice. One day prior to injection, mice were administered estrogen via drinking water at a concentration of 1 μM as described previously [129]. Tumors were allowed to achieve an average volume of 200 mm^3 prior to beginning treatment. Tamoxifen citrate (Sigma) was subcutaneously administered at a dose of 500 μg in a peanut oil emulsion daily for 5 of 7 days per week. Dalotuzumab was administered twice weekly via intraperitonealy injection at a dose of 500 μg. Control animals were injected with histidine-based buffer and peanut oil alone. Tumor growth was measured bidirectionally and tumor volumes were calculated using the formula length x $breadth^2/2$.

Microarray - RNA isolation and analysis. Tumors were harvested after reaching a volume of 1000 mm^3 and placed in RNALater solution (Qiagen). Tumors were homogenized and RNA was isolated using TriPure reagent (Qiagen). Prior to gene array

analysis, RNA was purified using RNeasy mini kit (Qiagen). RNA samples were submitted to Rosetta/Merck for analysis of purity and subsequent microarray analysis using a Merck custom chip created by Affymetrix.

Microarray - statistical analysis. Statistical analysis of arrays was performed by the Biomedical Informatics Core Facility at the Masonic Cancer Center-University of Minnesota. All arrays were normalized using GC-RMA process embedded in GeneData refiner. T-tests were performed between groups using GeneData expressionist with P-values < 0.05 and a minimum average fold change of 1.5 was employed. Hierarchical clustering was carried out on log base 2 transformed data generated using Gene Cluster 3.0. Data was visualized and images generated using Java TreeView.

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