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Type-I Insulin-Like Growth Factor Receptor (IGF1R)-Estrogen Receptor (ER) Crosstalk Contributes to  
Antiestrogen Therapy Resistance in Breast Cancer Cells

PRINCIPAL INVESTIGATOR:  
Dedra Fagan

CONTRACTING ORGANIZATION:  
University of Minnesota  
Minneapolis, MN 55455-2070

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| 6. AUTHOR(S)<br><br>Dedra Fagan<br><br><br>E-Mail: ober0165@umn.edu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                  |                                  |                                      | 5d. PROJECT NUMBER                                   |                                            |
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| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                  |                                  |                                      |                                                      |                                            |
| 14. ABSTRACT<br>Tamoxifen, the first targeted therapy, has shown great success in treating estrogen receptor (ER) positive breast cancer. However, both acquired and <i>de novo</i> resistance to this therapy prevents it from being effective in all situations. Multiple lines of evidence indicate that increased signaling through growth factor pathways, such as the IGF pathway, mediates resistance to tamoxifen. The link between ER and ICGF1R leads us to hypothesize that IGF system crosstalk with the ER contributes to tamoxifen resistance. Tamoxifen resistance has thus provided researchers with a reason to investigate other growth factor pathways involved in breast cancer. As new targeted therapies are being developed, it will be important to examine their benefit with existing therapies. In order to examine the effectiveness of anti-IGF1R inhibitors <i>in vitro</i> , tamoxifen resistant (TamR) cells were generated by culturing MCF-7L and T47D cells in the presence of 4-OH-tamoxifen for >6 months. TamR cells had diminished levels of IGF1R, with unchanged levels of insulin receptor (IR). Further, TamR cells failed to respond to IGF-I induced p-AKT activation, while retaining responsiveness to both insulin and IGF-II. Additionally, IGF-I failed to enhance the proliferation and anchorage-independent growth of TamR cells; however, both insulin and IGF-II were able to enhance proliferation in MCF anchorage-independent growth. An IGF1R antibody was effective in inhibiting signaling, anchorage-independent growth, and proliferation in MCF-7L cells, but had no effect in TamR cells. In contract, an IGF1R/TR tyrosine kinase inhibitor was effective in both MCF-7L and TamR cells. In a xenograft model, an IGF1R antibody was able to inhibit estrogen stimulated tumor growth, but had no additive effect when combined with tamoxifen treatment. Further, tamoxifen-treated xenografts had diminished IGF1R levels. |                  |                                  |                                      |                                                      |                                            |
| 15. SUBJECT TERMS<br>Endocrine resistance, IGF1R, breast cancer, tamoxifen                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                  |                                  |                                      |                                                      |                                            |
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## **Introduction:**

Tamoxifen, the first targeted breast cancer therapy, has shown great success in treating estrogen receptor (ER) positive breast tumors. However, both acquired and *de novo* resistance to this therapy prevents it from being effective in all situations. While newer therapies, such as aromatase inhibitors, targeting the ER have been developed, some women (such as pre-menopausal women) do not benefit. Therefore, tamoxifen remains an important and clinically useful drug in a subset of ER positive breast cancer patients. Multiple lines of evidence indicate that increased signaling through growth factor pathways, such as the IGF pathway, mediates resistance to tamoxifen. The link between ER and IGF1R leads us to hypothesize that IGF system crosstalk with the ER contributes to tamoxifen resistance. Tamoxifen resistance has thus provided researchers with a reason to investigate other growth factor pathways involved in breast cancer development and progression. As new targeted therapies are being developed, it will be important to examine their benefit with existing therapies. Completion of this project will help address the rationale for combining IGF1R inhibitors with tamoxifen. Further, while clinical trials investigating the combined use of aromatase inhibitors and IGF1R inhibitors have been examined, combined tamoxifen/IGF1R inhibition has not been examined.

## **Body:**

**Specific Aim 1: Determine the role of the estrogen receptor in tamoxifen resistant cells.**

***1.1 Determine if tamoxifen resistant cells are stimulated by tamoxifen treatment.***

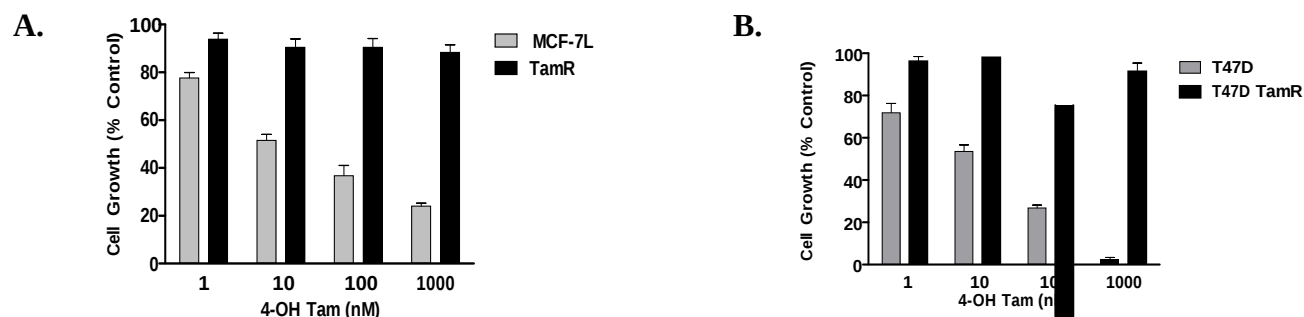
***1.2 Determine if the estrogen receptor is expressed and remains functional in tamoxifen resistant cells.***

***1.3 Determine if tamoxifen resistant cells are responsive to alternate anti-estrogen therapies.***

## **Results**

**Tamoxifen resistant cells are refractory to tamoxifen treatment.**

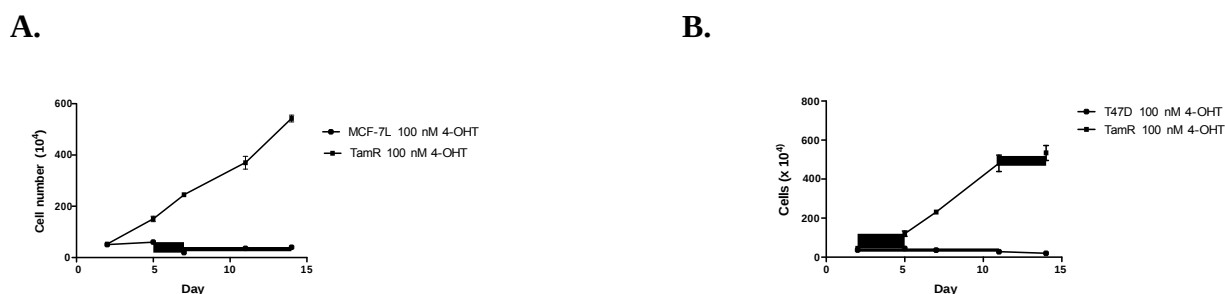
In order to learn more about endocrine resistance and its implications in breast cancer treatment, tamoxifen-resistant MCF-7L and T47D cells were generated. Cells were cultured in phenol-red-free IMEM containing 5% dextran-cleared-charcoal (DCC) serum and 100 nM 4-OH-tamoxifen for 6 months prior to characterizing 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 1.1). 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 1.2) Thus, TamR cells continued to survive and grow in the presence of tamoxifen, even up to concentrations of 1  $\mu$ M, demonstrating resistance to the drug.



**Figure 1.1**

**Tamoxifen resistant cells survive in the presence of increasing concentrations of tamoxifen.**

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 increasing concentrations of tamoxifen as indicated. Cells were collected and stained with trypan blue prior to counting using a hemacytometer.



**Figure 1.2**

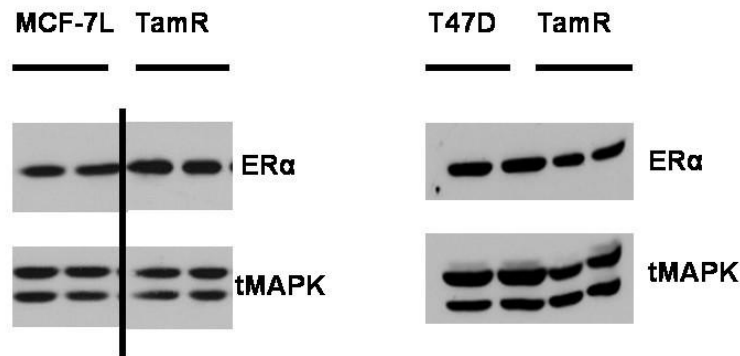
**Tamoxifen resistant cells survive in the presence of tamoxifen over time.**

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.

## 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 [1]. Similar to some tamoxifen resistant cancers, TamR cells maintained expression of estrogen receptor (figure 1.3). 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 1.4). 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 1.5).

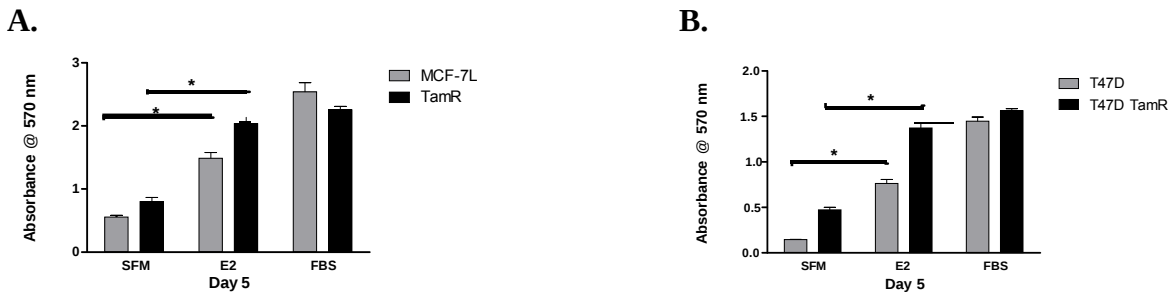
The estrogen receptor contains multiple phosphorylation sites, two prominent sites are serine-118, thought to be phosphorylated by MAPK, and serine-167, thought to be phosphorylated by Akt. In our lab, we have found estrogen is able to phosphorylate the serine-118 site, whereas IGF-I and insulin are able to phosphorylate the serine-167 site. 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 1.6).



**Figure 1.3**

**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.

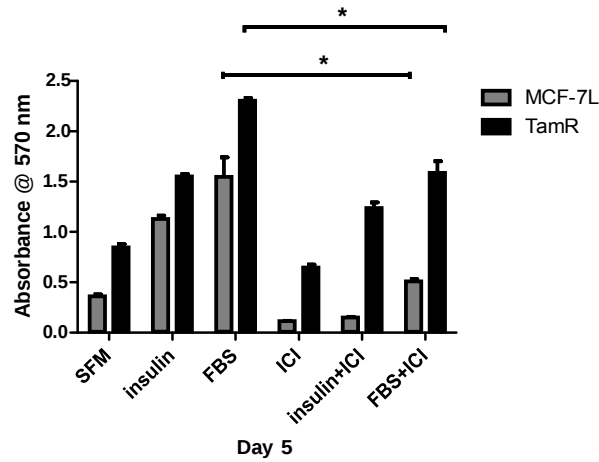


**Figure 1.4**

**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

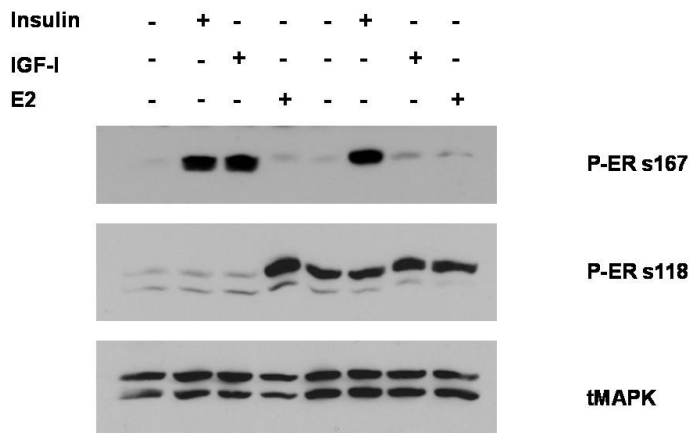
Tamoxifen exerts its action by binding to the estrogen receptor and holding it in an inactive conformation, preventing gene transcription. Therefore, tamoxifen treatment should prevent the transcription of estrogen regulated genes. When we examined the gene expression regulated by ER in TamR cells, we found basal levels of estrogen regulated genes, such as AREG, TFF1, PR, and KIAA0575 were down-regulated (figure 1.7 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 [2]. Interestingly, we found genes (RAB30, KIAA0922) up-regulated in response to estrogen treatment in TamR, but not parental cells (figure 1.8). 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.



**Figure 1.5**

**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

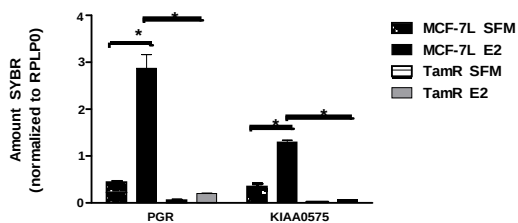


**Figure 1.6**

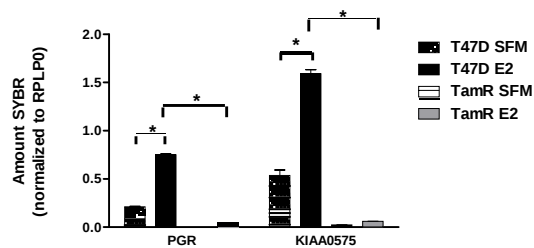
**Estrogen receptor can be phosphorylated in both MCF-7L parental and TamR cells.**

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.**



**B.**



**Figure 1.7**

**Expression of estrogen regulated genes is decreased in tamoxifen resistant cells.**

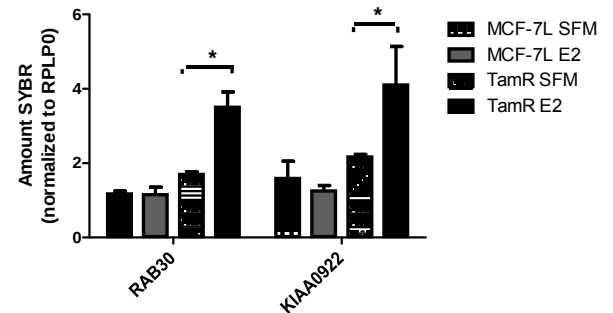
Cells were plated and exposed to charcoal stripped serum prior to serum starving and treating with estradiol for 4 hours. Total RNA was isolated from MCF-7L and TamR (left panel) or T47D and TamR (right panel) cells and was reverse

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 1.8**

**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. \* $p < 0.05$



**Determine the role of the IGF system in tamoxifen resistant breast cancer cells and the responsiveness of tamoxifen resistant cells to anti-IGF therapy.**

**2.1 Determine if IGF signaling is altered in tamoxifen resistant cells.**

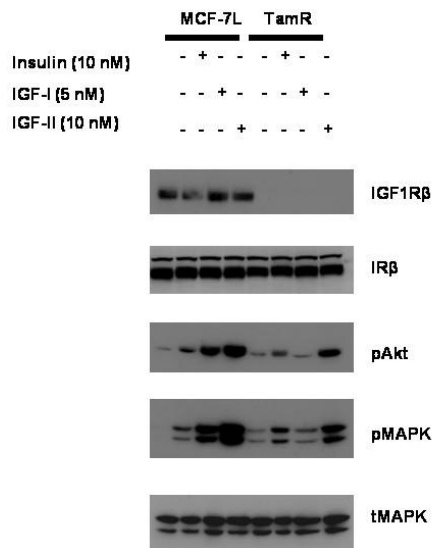
**2.2 Determine if tamoxifen resistant cells displayed altered sensitivity towards IGF1R inhibition and if tamoxifen resistant cells have a biological and biochemical response toward IGF1R inhibition in vitro.**

**2.3 Determine if tamoxifen resistant tumors are responsive to IGF1R inhibition and if this inhibition is enhanced when compared to tamoxifen sensitive xenograft tumors.**

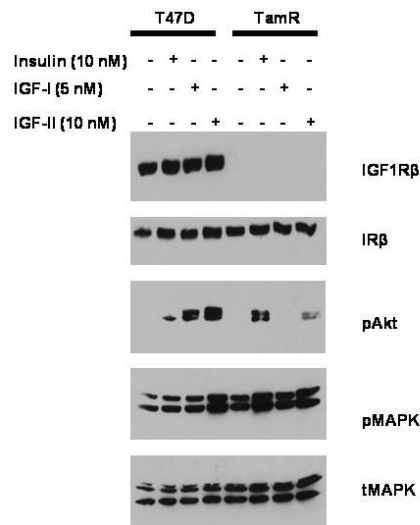
**Tamoxifen resistant cells expressed low levels of IGF1R.**

Previous reports have demonstrated a link between IGF1R and ER signaling [3-6]. 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 2.1). 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. Our original hypothesis and specific aims, which postulated IGF1R would be activated in tamoxifen resistance, thusly had to be modified. Our revised statement of work was re-submitted in 2012 (appendix A). 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 2.2). 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 2.2). Further, estrogen treatment did not affect IR levels in either cell line.

A.



B.

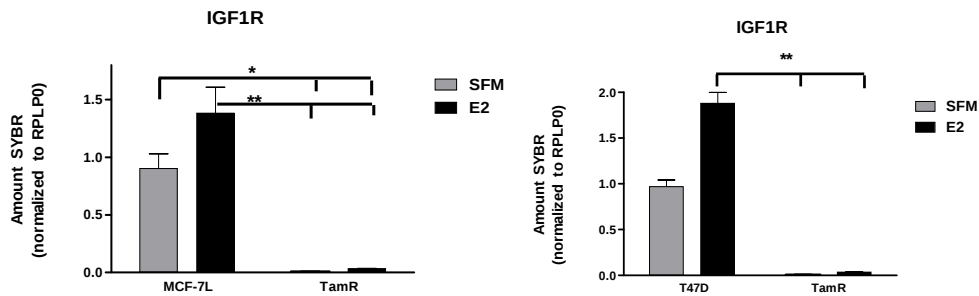


**Figure 2.1**  
**IGF1R levels and IGF-mediated signaling are reduced in TamR cells.**

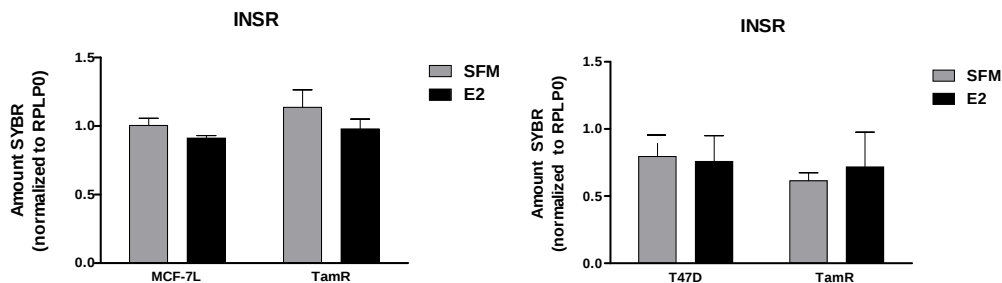
MCF-7L and TamR (upper panel) or T47D and TamR (lower panel) cells were serum starved overnight, then treated with 10 nM insulin, 5 nM IGF-I or 5 nM IGF-II for 10 minutes. Cellular lysates were separated by SDS-PAGE and levels of IGF1R, IR, phosphorylated Akt and MAPK, and total MAPK protein levels were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

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 2.3). 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 2.4). 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.

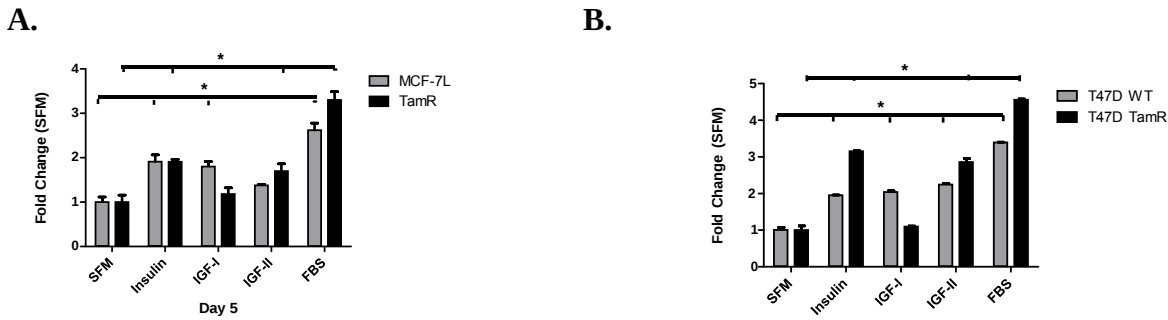
A.



B.



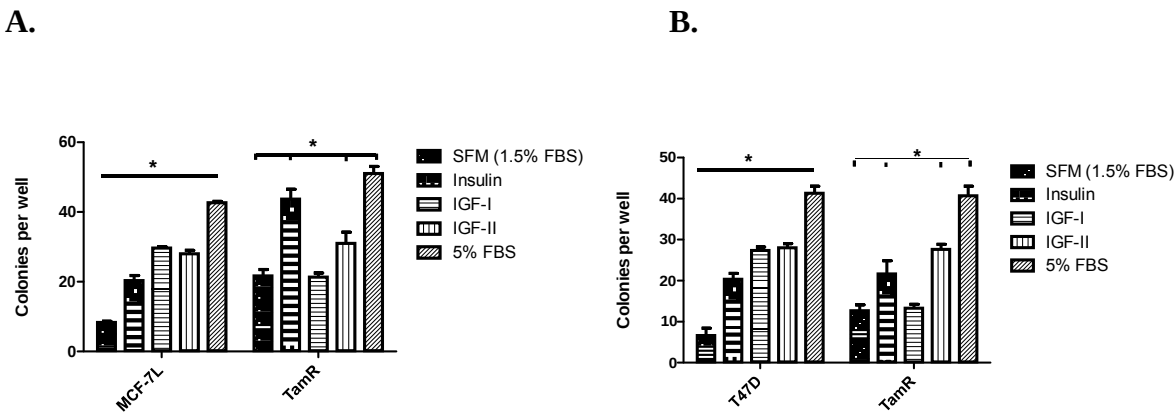
**Figure 2.2**  
**IGF1R mRNA levels are reduced in TamR cells, while IR levels remain unchanged.**  
Cells were plated and exposed to charcoal stripped serum prior to an overnight starvation and a 4 hour estradiol treatment. Total RNA was isolated from MCF-7L and TamR (left panel) or T47D and TamR (right 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. \* $p < 0.05$ , \*\* $p < 0.01$



**Figure 2.3**

**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. \* $p < 0.05$



**Figure 2.4**

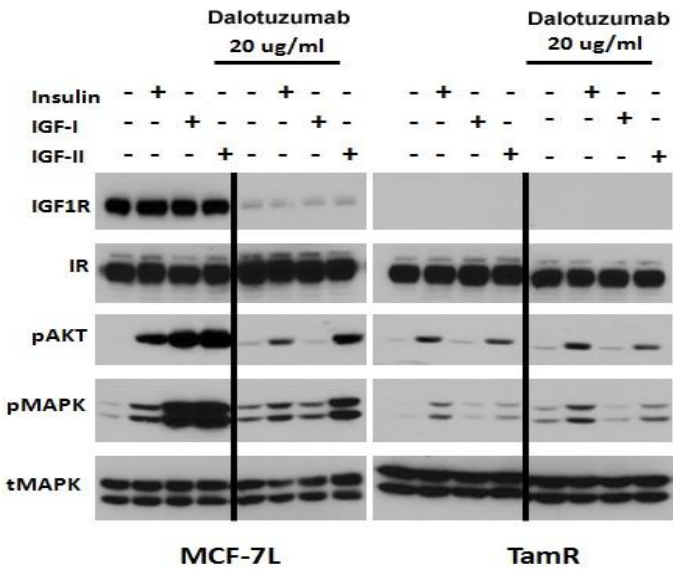
**TamR cells do not grow in an anchorage independent manner in response to IGF-I, but do respond to IGF-II and insulin.**

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. \* $p < 0.05$

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

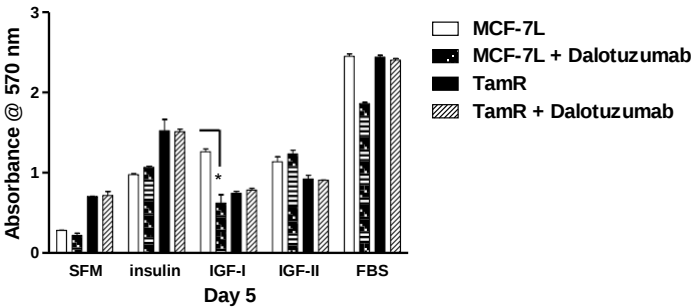
Dalotuzumab (MK-0646) is a humanized monoclonal antibody that binds the IGF1R. It has been shown to down-regulate IGF1R *in vitro* and *in vivo* [7, 8]. In order to examine the ability of the antibody to inhibit IGF-induced signaling, we pretreated MCF-7L parental and TamR cells with 20  $\mu\text{g/ml}$  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 2.5) 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 2.6) 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 2.7) 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.



**Figure 2.5**  
**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.



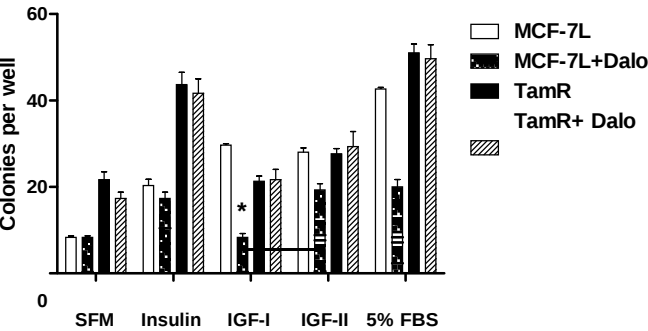
**Figure 2.7**  
**Treatment with an IGF1R antibody does not affect anchorage independent growth in tamoxifen resistant cells.**

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

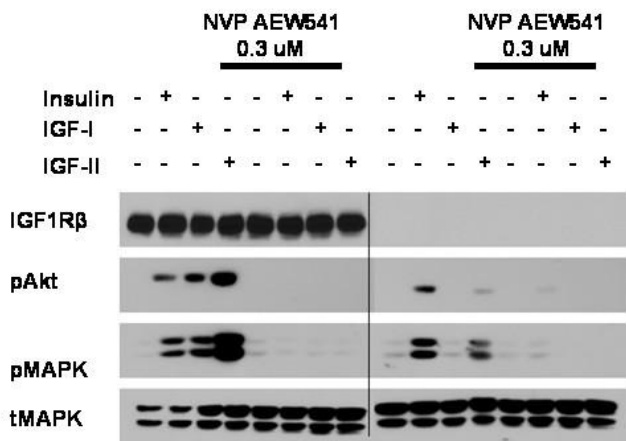
**Figure 2.6**  
**Tamoxifen resistant cells are refractory to IGF1R antibody treatment in a proliferation assay.**

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

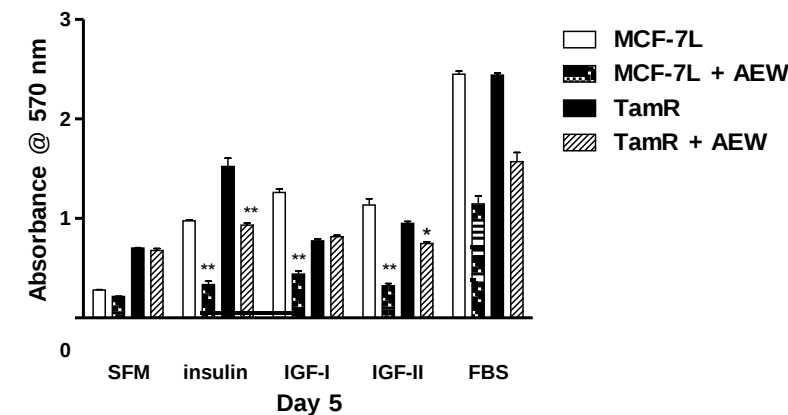


# **AEW541 inhibited signaling, proliferation, and anchorage-independent growth in parental and TamR cells.**

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  $\mu$ M AEW541 prior to stimulating cells with ligands. AEW541 inhibited insulin, IGF-I, and IGF-II signaling in MCF-7L cells (figure 2.8) 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 2.9) 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 2.10). 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.



**Figure 2.8**  
**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  $\mu$ M 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.

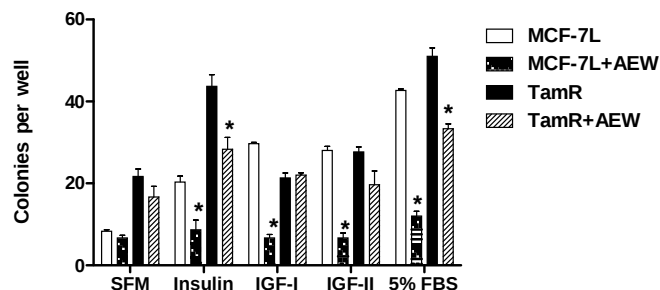


**Figure 3.9**  
**Proliferation in MCF-7L and TamR cells can be inhibited using an IGF1R/IR tyrosine kinase inhibitor.**  
MCF-7L and TamR cells were serum starved and treated with anti-IGF1R/IR TKI along with ligand. Proliferation was evaluated using MTT assay, with results displayed as absorbance 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.**

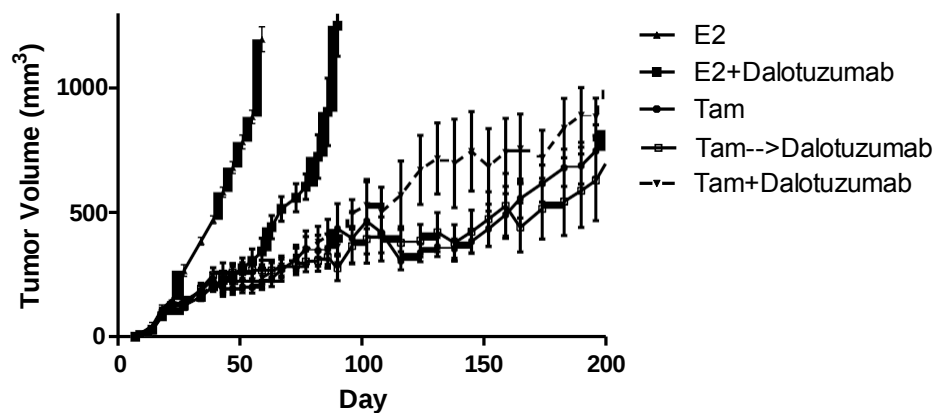
MCF-7L and TamR cells were serum starved and treated with anti-IGF1R/IR TKI and ligand in 1% FBS in 0.45% agar and overlaid on 0.8% bottom agar. Colony growth in agarose was assessed after 14 days. Colonies formed were counted and averaged from 5 individual microscopic fields. Results displayed are the 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. \* $p < 0.01$



### **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 [9]. Mice were administered estrogen to stimulate tumor growth and tumors were allowed to establish (tumor volume of  $\sim 200 \text{ mm}^3$ ) prior to beginning treatment. Dalotuzumab (administered beginning at day 32) inhibited the growth of estrogen stimulated tumors (figure 2.11). 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  $1000 \text{ mm}^3$ , 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 2.12). 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.



**Figure 2.11**

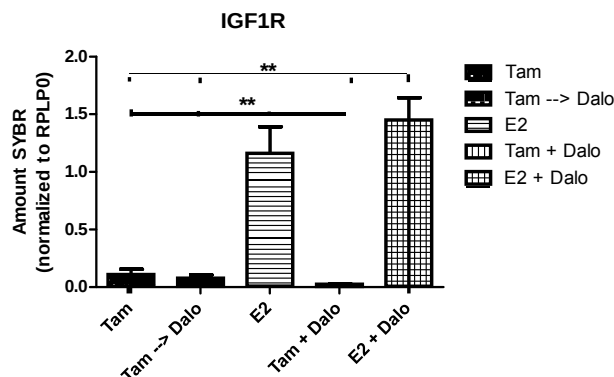
**Dalotuzumab did not add to tamoxifen-mediated growth inhibition in MCF-7L xenografts.**

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 2.12**

**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. \* $p < 0.05$ , \*\* $p < 0.005$



## 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 2.13). Several network pathways, notably those involved in cellular development/proliferation and gene expression were modulated in tamoxifen resistant xenografts (figure 2.14, 2.15). 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 dysregulated 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 2.16). 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 aim 1, which demonstrated classic genomic function of the estrogen receptor was suppressed in tamoxifen resistant cells.



Cellular Development, Cellular Growth and Proliferation,  
Cellular Movement

↑ACSL1, ↓AKR1C3, ↑ALDH1A3, ↓ANXA1, ↓BCL2, ↓CD44 (includes EG:100330801), ↓CDC42EP1,  
↓CXCL12 (includes EG:20315), CXCR4, ↑CXCR7, ↑CYP19A1, ↓EGR3, ERBB4, F3, ↓GAL, GATA4, ↑GSTM3, ↓HAS2,  
↑HOXB4, HYAL2, ↓IGF1R, ↑IL8, IL1B, ↑IL1R1, ↑MYLK, ↓NR5A2, ↓NRG1 (includes EG:112400),  
↓PGR (includes EG:18667), ↓PLAU, ↓PTGS2, RAC1, SHC1 (includes EG:20416), SNCG, ↓SPP1 (includes EG:20750),  
↓VIM

Cellular Development, Cellular Growth and Proliferation,  
Tumor Morphology

↑ADAMTS1, AKT1, ↑ANGPT1, ↓BCL2, BIRC5, CASP2, ↑CD74, ↑CDKN2B, ↓COL3A1, ↑COL5A2, ↓EMP3, ↓FHIT,  
↓FSCN1, ↓GFRA1, GRN, ↓HKDC1, ↑HLA-DRA, HOXB7, IGFBP5, IL6, ITGA5, ITGA6, JUN, KRT18, LGALS3,  
↑mir-181, MYC, ↑NRP1 (includes EG:18186), PTEN, RB1, ↑SALL4, SMARCA4, STAT3, TGFB1 (includes EG:21803),  
VEGFA

Gene Expression, Cell Death, Cellular Development

↓ABCG2, AHR, ↓AKIP1, ↓BCAT1, CDKN1A, ↓CXCL12 (includes EG:20315), ↑DAB2, EHMT2, ↑ENC1, ESR1, ESR2,  
EZH2, FOS, ↓GREB1, ↑GSTP1, HDAC2, HIF1A, ↓IGF1R, IL6, IL1B, JUN, KEAP1, ↑MST1R, MYC, NFE2L2, NFKB1,  
↑NFKBIZ, ↓NRG1 (includes EG:112400), ↓PGR (includes EG:18667), ↓PKIB, ↓PTGS2, RELA, ↓SGK3, STAT3, TNF

**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.



## Key Research Accomplishments

- Generation and characterization of tamoxifen resistant cells in two different breast cancer cell lines (MCF-7L and T47D).
- Tamoxifen resistant cells maintain estrogen receptor expression and are able to proliferate in response to estrogen.
- Tamoxifen resistant cells have diminished levels of IGF1R and fail to respond to IGF-I treatment.
- Tamoxifen resistant cells maintain IR expression and respond to both insulin and IGF-II.
- IGF1R antibodies are effective in inhibiting the proliferation and anchorage-independent growth of MCF-7L cells, but are not effective in TamR cells.
- Tyrosine kinase inhibitors, targeting both the IR and IGF1R are effective in biochemical and biological inhibition of both MCF-7L cells and TamR cells.
- Tamoxifen treated xenografts have reduced levels of IGF1R and do not respond to IGF1R antibody treatment.
- Careful consideration should be taken in the design of clinical trials using anti-IGF1R therapy in order to select the proper patient population which will achieve the most benefit from therapy.

## Reportable Outcomes

- **Manuscripts**
  - Fagan DH, Uselman RR, Sachdev D, Yee D. Acquired resistance to tamoxifen is associated with loss of the IGF1 receptor: implications for breast cancer treatment. *Cancer Res.* 2012 Jul 1;72(13):3372-80. (Appendix B)
- **Abstracts/Posters Presented**
  - Fagan, DH and Yee, D. Effectiveness of anti-IGF therapy in tamoxifen-resistant breast cancer cells. DOD Era of Hope Meeting. Orlando, FL. August 2011. *Poster Presentation*
  - Fagan, DH and Yee, D. Effectiveness of anti-IGF therapy in tamoxifen-resistant breast cancer cells. Robert Hebbel Medicine Research Day. Minneapolis, MN. November 2011. *Poster Presentation*
  - Fagan, DH and Yee, D. Effectiveness of anti-IGF therapy in tamoxifen-resistant breast cancer cells. AACR San Antonio Breast Cancer Symposium. San Antonio, TX. November 2011. *Poster Presentation*
  - Fagan DH, Uselman RR, Sachdev D, Yee D. Acquired resistance to tamoxifen is associated with loss of the IGF1 receptor: implications for breast cancer treatment. Masonic Cancer Center Research Symposium. Minneapolis, MN. May, 2012. *Poster Presentation*
  - Fagan DH, Uselman RR, Sachdev D, Yee D. Acquired resistance to tamoxifen is associated with loss of the IGF1 receptor: implications for breast cancer treatment. Robert Hebbel Medicine Research Day. Minneapolis, MN. November, 2012. *Poster Presentation*
- **Degree's Obtained**
  - **Ph.D., Pharmacology:** June 2012  
University of Minnesota School of Medicine, Minneapolis, MN

## Conclusion

Tamoxifen resistant breast cancer cells lack IGF1R expression and do not respond to IGF-I treatment. However, TamR cells maintain expression of IR and respond to both insulin and IGF-II treatment. Monoclonal antibodies, which target only the IGF1R, are in-effective in blocking either proliferation or anchorage-

independent growth in TamR cells. In contrast, TKI's, which target both the IR and IGF1R inhibit both the proliferation and anchorage, independent growth of TamR cells. Further, in a xenograft model, tamoxifen treated animals have decreased IGF1R expression in xenografts and do not respond to IGF1R antibody treatment.

### **Significance**

The majority of anti-IGF1R clinical trials are in estrogen receptor-positive patients who have progressed on prior endocrine therapy. Although these agents have been extensively evaluated using *in vitro* and *in vivo* modeling systems, their effect in endocrine-resistant models, mimicking the clinical trial scenario, has not been adequately investigated. Our data suggest that IGF1R tyrosine kinase inhibitors may be more effective than IGF1R antibodies in patients resistant to endocrine therapy due to inhibition of IR signaling.

## References

1. Arpino, G., et al., *Molecular mechanism and clinical implications of endocrine therapy resistance in breast cancer*. Oncology, 2009. **77 Suppl 1**: p. 23-37.
2. Masri, S., et al., *Genome-wide analysis of aromatase inhibitor-resistant, tamoxifen-resistant, and long-term estrogen-deprived cells reveals a role for estrogen receptor*. Cancer Res, 2008. **68**(12): p. 4910-8.
3. Becker, M.A., et al., *The IGF pathway regulates ERalpha through a S6K1-dependent mechanism in breast cancer cells*. Mol Endocrinol, 2011. **25**(3): p. 516-28.
4. Fagan, D.H. and D. Yee, *Crosstalk between IGF1R and estrogen receptor signaling in breast cancer*. J Mammary Gland Biol Neoplasia, 2008. **13**(4): p. 423-9.
5. Lee, A.V., et al., *Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo*. Mol Endocrinol, 1999. **13**(5): p. 787-96.
6. Mulligan, C., et al., *Microarray analysis of insulin and insulin-like growth factor-1 (IGF-1) receptor signaling reveals the selective up-regulation of the mitogen heparin-binding EGF-like growth factor by IGF-1*. J Biol Chem, 2002. **277**(45): p. 42480-7.
7. Goetsch, L., et al., *A recombinant humanized anti-insulin-like growth factor receptor type I antibody (h7C10) enhances the antitumor activity of vinorelbine and anti-epidermal growth factor receptor therapy against human cancer xenografts*. Int J Cancer, 2005. **113**(2): p. 316-28.
8. Pandini, G., et al., *Functional responses and in vivo anti-tumour activity of h7C10: a humanised monoclonal antibody with neutralising activity against the insulin-like growth factor-1 (IGF-1) receptor and insulin/IGF-1 hybrid receptors*. Eur J Cancer, 2007. **43**(8): p. 1318-27.
9. Sachdev, D., et al., *A chimeric humanized single-chain antibody against the type I insulin-like growth factor (IGF) receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I*. Cancer Res, 2003. **63**(3): p. 627-35.

## Appendix A

### Statement of Work

#### Specific Aim 1

##### **Determine the role of the estrogen receptor (ER) in tamoxifen resistant breast cancer cells. (revised aim)**

- |                     |                                                                                                                                                     |
|---------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| Task 1: Month 1-6   | Generate MCF-7L and T47D tamoxifen resistant cells. Verify resistance to tamoxifen using dose response curves and cell counting or MTT assay.       |
| Task 2: Month 6-11  | Determine the functional status of the estrogen receptor using MTT assay, Western blotting, and qRT-PCR with estrogen treatment in resistant cells. |
| Task 3: Month 11-12 | Determine if tamoxifen resistant cells retain responsiveness to alternative endocrine therapies (i.e. SERDs) as is seen in the clinic.              |

#### Specific Aim 2

##### **Determine the role of the IGF system in tamoxifen resistant breast cancer cells and the responsiveness of tamoxifen resistant cells to anti-IGF therapy. (revised aim)**

- |                     |                                                                                                                                                                                                                                                                                                                                                                                                               |
|---------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Task 1: Month 12-16 | Determine if IGF signaling is altered in tamoxifen resistant cells using Western blotting, MTT assay, soft agar, and qRT PCR techniques.                                                                                                                                                                                                                                                                      |
| Task 2: Month 16-21 | Determine if tamoxifen resistant cells displayed altered sensitivity towards IGF1R inhibition and if tamoxifen resistant cells have a biological and biochemical response toward IGF1R inhibition in vitro. Following our discovery that tamoxifen resistant cells lack IGF1R expression, we opted to also perform this aim using a TKI, which targets both IGF1R/IR since TamR cells maintain IR expression. |
| Task 3: Month 12-27 | Generate tamoxifen resistant xenograft tumors in athymic, nude mouse model using MCF-7L cells. Determine if tamoxifen resistant tumors are responsive to IGF1R inhibition and if this inhibition is enhanced when compared to tamoxifen sensitive xenograft tumors.                                                                                                                                           |
| Task 4: Month 24-29 | Analyze data and submit for publication in peer-reviewed journal.                                                                                                                                                                                                                                                                                                                                             |
| Task 5: Month 27-35 | Perform microarray gene expression analysis on tumors harvested from Task 3. Characterize differences between treatment groups and validate using qRT-PCR. Analyze data and prepare for submission for publication.                                                                                                                                                                                           |

## Appendix B

## Acquired Resistance to Tamoxifen Is Associated with Loss of the Type I Insulin-like Growth Factor Receptor: Implications for Breast Cancer Treatment

Dedra H. Fagan<sup>2</sup>, Ryan R. Usefman\ Deepali Sachdev<sup>1,3</sup>, and Douglas Yee<sup>1,2,3</sup>

### Abstract

The role of the insulin-like growth factor (IGF) system in breast cancer is well defined, and inhibitors of this pathway are currently in clinical trials. The majority of anti-IGF1R clinical trials are in estrogen receptor-positive patients who have progressed on prior endocrine therapy; early reports show no benefit for addition of IGF1R inhibitors to endocrine therapy in this setting. In this study, we examined the effectiveness of IGF1R inhibitors *in vitro* by generating tamoxifen-resistant (TamR) cells. We found that TamR cells had diminished levels of IGF1R with unchanged levels of insulin receptor (IR), and failed to respond to IGF-I-induced Akt activation, proliferation, and anchorage-independent growth while retaining responsiveness to both insulin and IGF-II. The IGF1R antibody dalotuzumab inhibited IGF-I-mediated Akt phosphorylation, proliferation, and anchorage-independent growth in parental cells, but had no effect on TamR cells. An IGF1R tyrosine kinase inhibitor, AEW541, with equal potency for the IGF1R and IR, inhibited IGF-I-, IGF-II-, and insulin-stimulated Akt phosphorylation, proliferation, and anchorage-independent growth in parental cells. Interestingly, AEW541 also inhibited insulin- and IGF-II-stimulated effects in TamR cells. Tamoxifen-treated xenografts also had reduced levels of IGF1R, and dalotuzumab did not enhance the effect of tamoxifen. We conclude that cells selected for tamoxifen resistance *in vitro* have downregulated IGF1R making antibodies directed against this receptor ineffective. Inhibition of IGF1R may be necessary to manage tamoxifen-resistant breast cancer. *Cancer Res*; 72(13); 3372-80. ©2012 AACR.

### Introduction

The first and arguably most effective targeted therapy for breast cancer involves inhibition of estrogen receptor (ER) function. Tamoxifen, a selective estrogen receptor modulator, has proven effective in both early and advanced stages of breast cancer (1). In addition, depriving receptors of ligand using aromatase inhibitors and degrading receptors through pure nonsteroidal anti-estrogens have also proven effective. Unfortunately, after initial success, a large portion of these tumors will develop resistance. This has led to the exploration and identification of additional targeted therapies, namely against growth factor receptors, such as EGFR, HER2, and IGF1R.

The IGF1R is a receptor tyrosine kinase that exerts its biologic 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 mitogen-activated pro-

tein kinase (MAPK) and PI3K pathways, ultimately leading to proliferation, angiogenesis, resistance to apoptosis, and metastasis (2, 3). The closely related insulin receptor behaves in a similar manner, through its ligands insulin and IGF-II.

Cross-talk 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 ERa can enhance IGF1R signaling through transcriptional upregulation of *IGF1R*, *IRS-1*, and *IGF-1* (4-8). Reciprocally, IGF1R has been shown phosphorylate and activate ER on serine-167 through an S6-kinase mechanism (9). In addition to current IGF1R inhibitor clinical trials examining combined anti-IGF1R, anti-ER therapies, trials are also being conducted 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 (10). As noted, preclinical studies have documented cross-talk between IGF1R and ER pathways (11), yet clinical trials conducted primarily in endocrine-resistant patients have been disappointing (12). *in vitro* and *in vivo* evaluation has been conducted using endocrine sensitive cells, with relatively little evidence showing 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

**Authors' Affiliations:** <sup>1</sup>Masonic Cancer Center; and Departments of <sup>2</sup>Pharmacology and <sup>3</sup>Medicine, University of Minnesota, Minneapolis, Minnesota

**Corresponding Author:** Douglas Yee, Masonic Cancer Center, University of Minnesota, MMC 806, 420 Delaware St SE, Minneapolis, MN 55455. Phone: 612-626-8487; Fax: 612-626-3069; E-mail: yee006@umn.edu  
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domain of the internal tyrosine kinase domain of the IGF1R and the closely related insulin receptor. Although some view targeting of the IR dangerous because of metabolic consequences, recent data suggest a benefit to targeting the IR (13, 14). Multiple reports have showed a role for the insulin receptor in cancer biology (15–17). Furthermore, phase I clinical trials have shown limited metabolic consequences that can be treated using metformin (18). Thus, the clinical benefit of using IGF1R/IR tyrosine kinase inhibitors (TKI) 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.

## Materials and Methods

### Reagents

All chemical reagents were purchased from Sigma-Aldrich unless otherwise indicated. IGF-I, IGF-II, and insulin were purchased from Novozymes GroPep Limited and Eli Lilly, respectively.

### Cell lines and culture

All cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin. MCF-7 cells were provided by C. Kent Osborne (Baylor College of Medicine) and maintained in improved MEM Richter's modification medium (zinc option) supplemented with 5% FBS and 11.25 nmol/L insulin. MCF-7 TamR cells were generated by culturing MCF-7 in phenol-red free IMEM (zinc option) supplemented with 11.25 nmol/L insulin, 5% charcoal/dextran-treated FBS, and 100 nmol/L 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 nmol/L 4-OH tamoxifen. TamR cells were grown in the presence of 4-OH tamoxifen for 6 months to allow resistance to develop before 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 anti-phosphotyrosine (PY-20) was purchased from BD Biosciences. The ERα antibody used for Western blot analysis was purchased from

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

### Growth curve analysis

Cells were plated at a density of  $1 \times 10^4$  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  $3 \times 10^5$  in 60-mm-diameter dishes and allowed to equilibrate overnight. Full medium was replaced with DCC-treated fetal calf serum for the next 3 to 5 days, after which cells were switched to serum-free medium (SFM) for 24 hours. Upon reaching 70% confluency, cells were treated, placed on ice, washed twice with ice-cold PBS, and lysed with lysis buffer of 50 mmol/L Tris-Cl (pH 7.4), 1% Nonidet P-40, 2 mmol/L EDTA (pH 8.0), 100 mmol/L NaCl, 10 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 µg/mL leupeptin, and 20 µg/mL aprotinin. Lysates were clarified by centrifugation at 12,000 rpm for 15 minutes 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 (19). Sixty microliters 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 hours 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) in 1% FBS-containing growth media was solidified into each well of a 6-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 days 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 quantitative real-time PCR

Cells were plated at a density of  $1 \times 10^6$  in 100-mm-diameter dishes, allowed to equilibrate overnight, DCC starved for 3 days, and incubated overnight in SFM. Cells were treated with SFM or 1 nmol/L estradiol for 4 hours. Cellular RNA was isolated using TriPure Reagent according to the manufacturer (Roche). For quality control and to determine concentration, a 260:280 assay was conducted on a spectrophotometer. Forward and reverse primers were designed to target the following transcripts: *PGR*, *KIAA0575*, *INSR*, *RPLPO* and *IGF1R*. A total of 2  $\mu$ g of RNA was reverse transcribed using the Transcriptor Reverse Transcriptase Kit, and quantitative PCR was conducted using the Universal SYBR Green Kit according to the manufacturer's recommended protocol (Roche) on an Eppendorf Mastercycler Realplex<sup>4</sup> machine. The relative concentration of mRNA was calculated using cycle threshold values that were derived from a standard curve and normalized to ribosomal protein, large, P<sub>0</sub> as an internal control.

#### Xenograft growth

AJ1 animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. MCF-7L cells ( $5 \times 10^6$ ) were injected into the mammary fat pad of 5-week-old female ovariectomized athymic mice. One day before injection, mice were administered estrogen via drinking water at a concentration of 1  $\mu$ mol/L as described previously (20). Tumors were allowed to achieve an average volume of 200 mm<sup>3</sup> before beginning treatment. Tamoxifen citrate (Sigma-Aldrich) was subcutaneously administered at a dose of 500  $\mu$ g in a peanut oil emulsion daily for 5 of 7 days per week. Dalotuzumab was administered twice weekly via intraperitoneal injection at a dose of 500  $\mu$ g. 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  $\times$  breadth<sup>2</sup>/2.

#### Results

##### Tamoxifen-resistant cells are refractory to tamoxifen treatment but respond to estrogen treatment

To examine the effect of anti-IGF therapy in endocrine resistance, tamoxifen-resistant MCF-7L and T47D cells were generated. After selection, TamR cells survived in the presence of increasing concentrations of tamoxifen; however, parental cells were inhibited with as little as 1 nmol/L tamoxifen (Fig. 1A). Thus, TamR cells continued to survive and grow in the presence of tamoxifen, even up to concentrations of 1  $\mu$ mol/L, showing resistance to the drug. Similar to some tamoxifen-resistant cancers, TamR cells maintained expression of estrogen receptor (Fig. 1B). Furthermore, TamR cells were able to proliferate in response to estrogen (Fig. 1C). When we examined gene expression regulated by ER in TamR cells, we found basal levels of estrogen regulated genes such as *KIAA0575* (GREB1), *PGR* (Fig. 1D), *TFF1*, *AREG*, *CTSD*, and *IGF1R* (data not shown) were downregulated; however, estrogen was still able to stimulate transcription of these genes. Similar to the clinical situation of tamoxifen resistance where

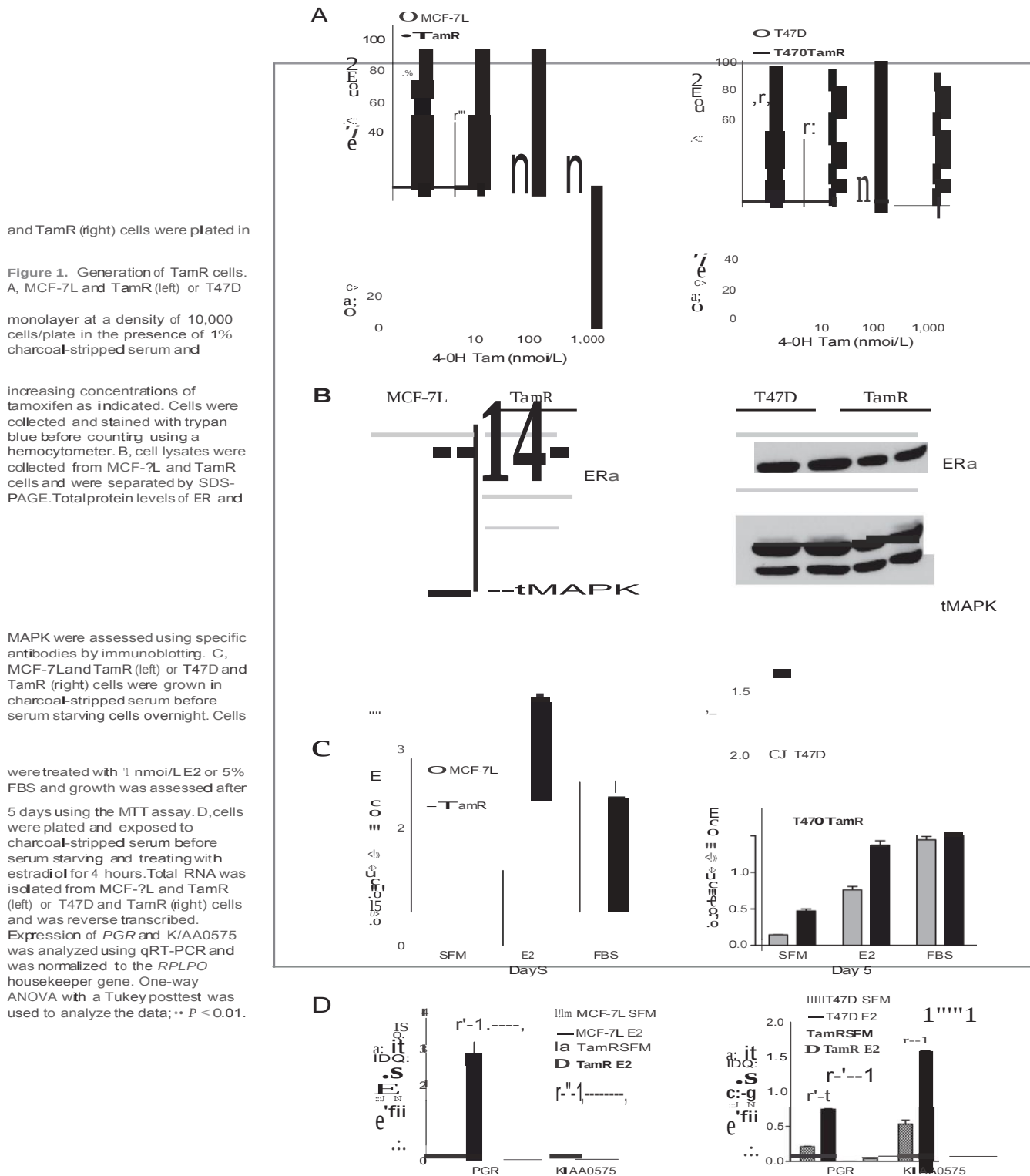
some tumors remain dependent on estradiol, our cells maintained estrogen receptor expression and responded to estrogen treatment.

##### Tamoxifen-resistant cells expressed low levels of IGF1R

Before 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 (Fig. 2A). Furthermore, TamR cells failed to phosphorylate Akt and MAPK after IGF-1 treatment. The cells retained expression of IR and insulin and IGF-11 ligand treatment resulted in phosphorylation of Akt and MAPK. To examine whether this change in IGF1R expression was because of decreased transcription, we conducted quantitative real-time PCR (qRT-PCR) to examine the message level of IGF1R. Indeed, IGF1R mRNA was decreased in TamR cells compared with parental cells (Fig. 2B). Treating TamR cells with estrogen resulted in a small increase in IGF1R mRNA, but did not restore the receptor to parental levels (Fig. 2B). Insulin receptor mRNA levels were not significantly different between parental and resistant cells (Fig. 2C). Furthermore, estrogen treatment did not affect IR levels in either cell line. These data show that tamoxifen-resistant cells lack IGF1R expression, but maintain expression of TR and are able to signal through IR.

##### 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 downregulate IGF1R *in vitro* and *in vivo* (21, 22). To examine the ability of the antibody to inhibit IGF-induced signaling, we pretreated MCF-7L parental and TamR cells with 20  $\mu$ g/mL dalotuzumab for 24 hours before stimulating cells with ligand. Dalotuzumab inhibited IGF-1 signaling, as measured via Akt and MAPK phosphorylation, in MCF-7L (Fig. 3A) and T47D (data not shown) parental cells and had a minimal effect on both insulin and IGF-11 signaling. TamR cells did not respond to IGF-1, but Akt was activated by IGF-11 and insulin. Dalotuzumab did not affect response to any of the ligands in TamR cells, presumably because of lack of IGF1R expression. 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 and T47D (data not shown) parental cells; however, only proliferation in response to IGF-1 was inhibited in the presence of dalotuzumab (Fig. 3B). In contrast, insulin and to a lesser extent IGF-11 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 (Fig. 3C) and dalotuzumab inhibited growth in response to IGF-1 and IGF-11. In agreement with the signaling data, both insulin and IGF-11 induced the anchorage-independent growth of TamR cells. This growth was not inhibited by dalotuzumab. Thus, dalotuzumab inhibited IGF-1 induced signaling, proliferation, and anchorage-independent growth in MCF-7L parental cells, but had no



effect in TamR cells, presumably because of their lack of IGF1R expression.

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

cells

AEW541 is a dual TKI that targets both IGF1R and insulin receptor. To examine the effect of IGF1R TKIs in endocrine

resistance, we pretreated MCF-7L parental and TamR cells for 3 hours with 0.3  $\mu$ M AEW541 before stimulating cells with ligands. AEW541 inhibited insulin, IGF-1, and IGF-11 signaling in MCF-7L cells (Fig. 4A) and T47D cells (data not

shown). Furthermore, AEW541 was also able to inhibit insulin and JGF-11-stimulated phosphorylation of Akt and MAPK in TamR cells. To investigate whether this inhibition was also biologically important, we again examined

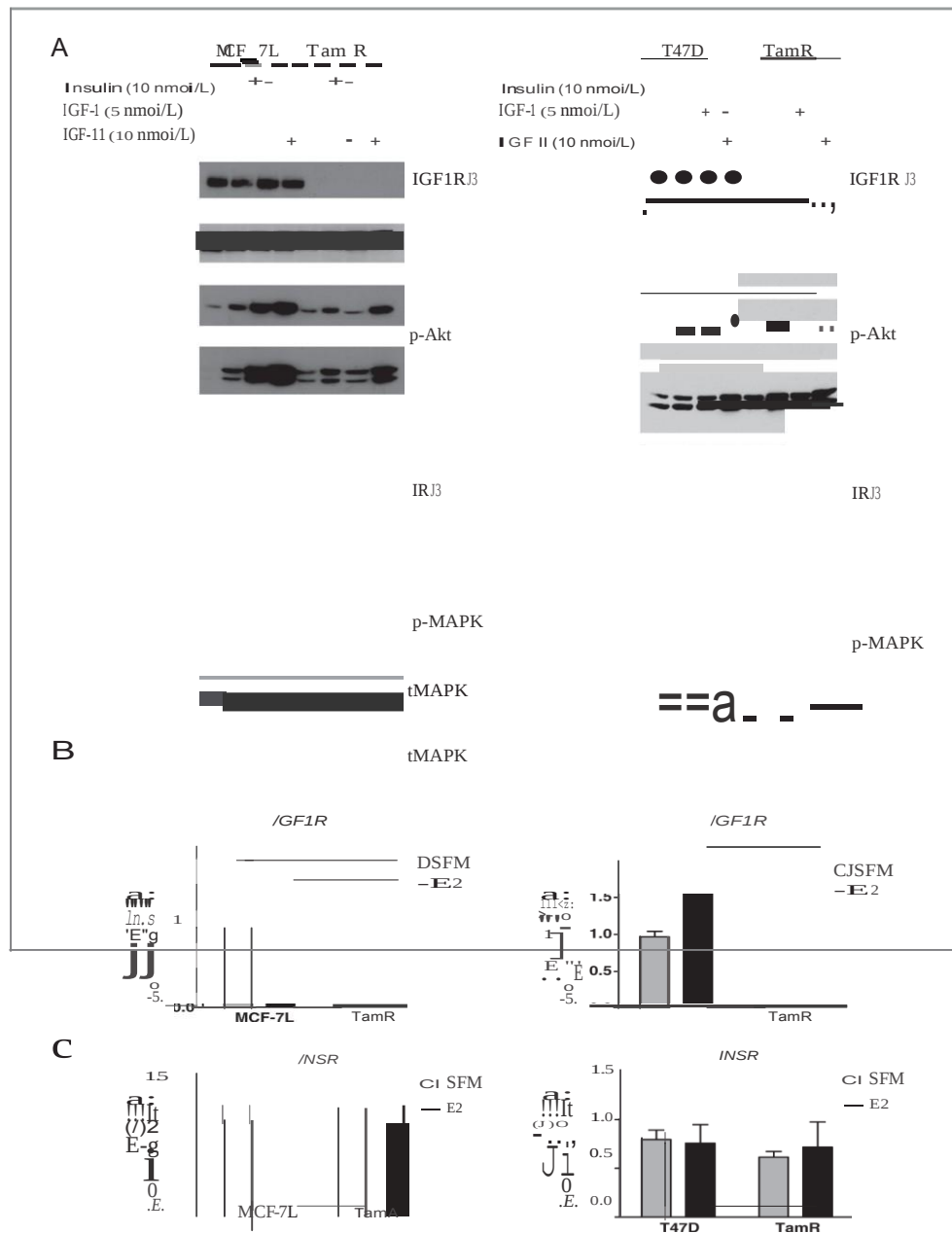


Figure 2. TamR cells have decreased IGF1R levels and fail to respond to IGF-I treatment. A, MCF-7L and TamR (top) or T47D and TamR (bottom) cells were serum starved overnight, then treated with 10 nmol/L insulin, 5

nmol/L IGF-I, or 10 nmol/L 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. B, cells were plated and exposed to charcoal-stripped serum before an overnight starvation and a 4-hour estradiol treatment. Total RNA was isolated from MCF-7L and TamR (left) or T47D and TamR (right) cells and was reverse transcribed and IGF1R levels were analyzed using qRT-PCR. Data were normalized to the *RPLPO* housekeeper gene. One-way ANOVA with Tukey posttest was done to compare the statistical significance between the cell lines; \*\*  $P < 0.05$ ; \*  $P < 0.01$ . C, cells were plated and exposed to charcoal-stripped serum, serum starved overnight, and treated for 4 hours with estradiol. Total RNA was isolated from MCF-7L and TamR cells and was reverse transcribed and IR levels were analyzed using qRT-PCR. Data were normalized to the *RPLPO* housekeeper gene.

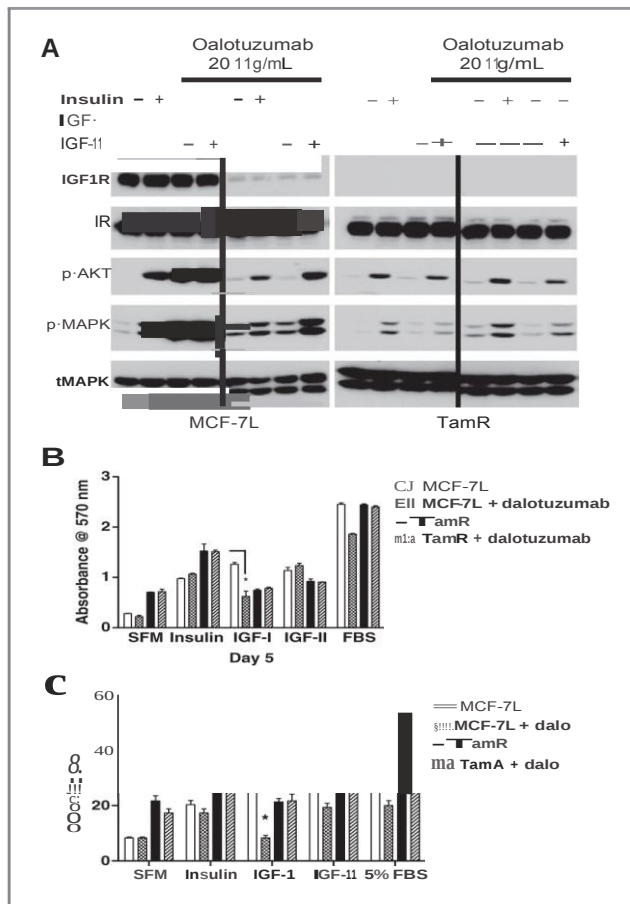
proliferation and anchorage-independent growth. AEW541 was able to inhibit insulin, IGF-I, and IGF-II-stimulated proliferation in MCF-7L and T47D (data not shown) cells and insulin and IGF-II-stimulated proliferation in TamR cells (Fig. 4B). In addition, AEW541 was also able to inhibit insulin, IGF-I, and IGF-II-stimulated anchorage-independent growth in MCF-7L parental cells and insulin and IGF-II-stimulated anchorage-independent growth in TamR cells (Fig. 4C). 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 TKIs, which target both IGF1R and IR, are effective in parental and resistant cells, because of 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 (23). Mice were administered estrogen to stimulate tumor growth and tumors were allowed to establish (tumor volume of 200 mm<sup>3</sup>) before beginning treatment. Dalotuzumab (administered beginning at day 32) inhibited the growth of estrogen-stimulated tumors (Fig. 5A). 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 grow on tamoxifen alone (Tam + Dalotuzumab) at approximately day 74.

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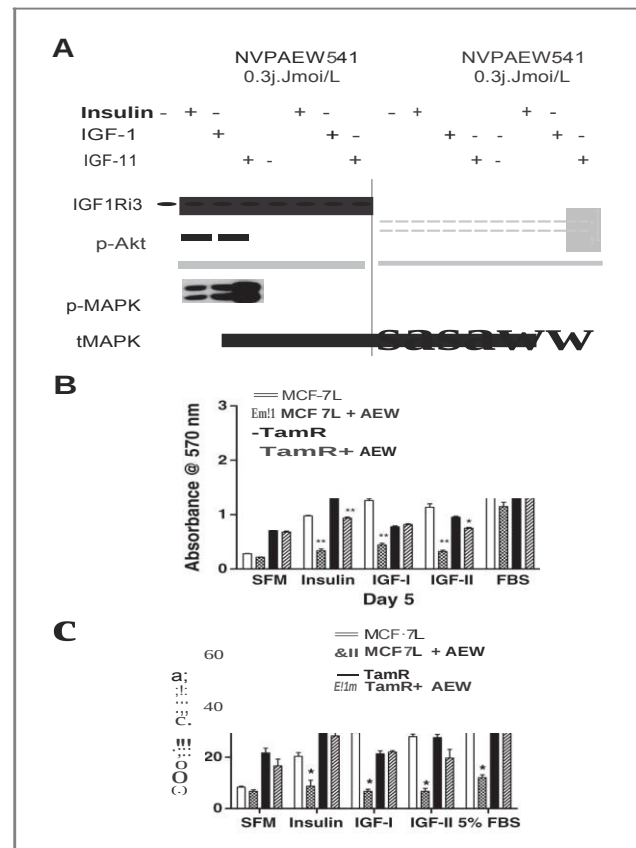


**Figure 3.** Dalotuzumab can inhibit the growth of MCF-7L parental but not TamR cells. **A**, MCF-7L and TamR cells were serum starved overnight and pretreated with 20  $\mu$ g/mL antibody for 24 hours before treating the cells with 10 nmol/L insulin, 5 nmol/L IGF-1, or 10 nmol/L IGF-II for 10 minutes. 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. **B**, MCF-7L and TamA 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 pretreatment and untreated samples; \*,  $P < 0.01$ . **C**, MCF-7L and TamA 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 conducted to compare the difference between antibody pretreated and untreated samples; \*\*,  $P < 0.01$ .

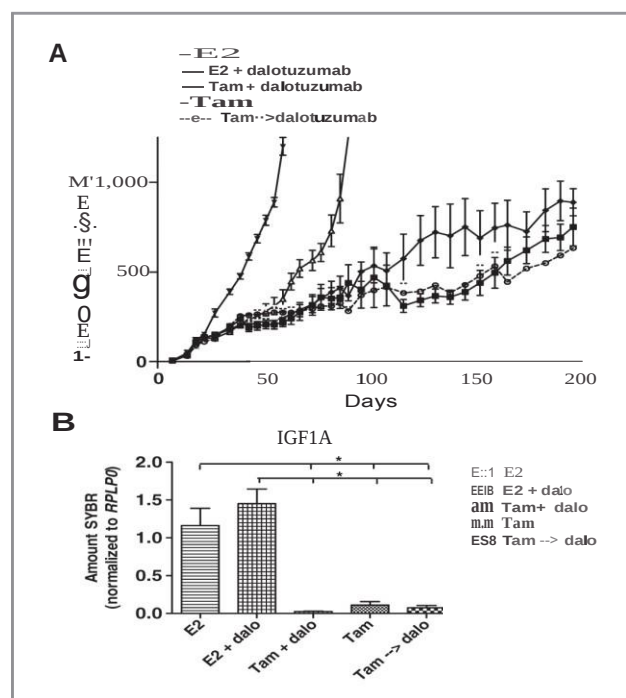
Tamoxifen by itself inhibited the growth of tumors; however, dalotuzumab coadministered with tamoxifen did not further suppress tumor growth. Furthermore, dalotuzumab did not

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 treatment was similar to the lack of IGF1R expression as observed *in vitro*. When tumors reached



**Figure 4.** AEW541 can inhibit the growth of MCF-7L and TamR cells. **A**, MCF-7L and TamR cells were serum starved overnight and pretreated with 0.3  $\mu$ M TKI for 3 hours before treating the cells with 10 nmol/L insulin, 5 nmol/L IGF-1, or 10 nmol/L 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. **B**, MCF-7L and TamR cells were serum starved and treated with anti-IGF1R 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 untreated samples; \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . **C**, MCF-7L and TamA cells were serum starved and treated with anti-IGF1R 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 conducted to compare the difference between TKI-treated and untreated samples; \*,  $P < 0.01$ .



**Figure 5.** Tamoxifen-treated MCF-7L xenografts have reduced IGF1R levels and do not respond to dalotuzumab treatment. **A**, ovariectomized athymic mice were given estrogen to stimulate MCF-7L xenograft tumor growth. At day 32, estrogen was withdrawn (unless indicated) and treatments began. For the Tam dalotuzumab group, tamoxifen was started at day 32, and dalotuzumab was started when tumors began to grow despite tamoxifen treatment at approximately day 74. Tumor volumes were measured weekly and average volume was plotted. **B**, 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. An unpaired *t* test was used to compare the difference between treatment groups; \*,  $P < 0.005$ .

1,000 mm<sup>3</sup>, mice were sacrificed and tumors were harvested for RNA isolation. Expression of IGF1H mRNA was significantly reduced in tamoxifen-treated xenografts when compared with estrogen-treated xenografts regardless of dalotuzumab treatment (Fig. 5B). Thus, tamoxifen-treated xenografts do not benefit from dalotuzumab treatment, because of decreased IGF1R expression. However, estrogen-treated xenografts express significantly more IGF1H 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 IGF1H expression.

## Discussion

The recently published results of IGF1R antibodies in clinical trials showing 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 conducted using endocrine-sensitive cell lines and xenograft models. Because IGF1H is an ER transcriptional target, understanding if IGF1H expression was affected by resistance to tamoxifen has clinical relevance. We found our tamoxifen-resistant cell lines lacked both protein and mRNA expression of IGF1H, but maintained expression of ER. This is in contrast to a report by Westley and colleagues showing that tamoxifen resistance in MCF-7 cells was associated with a dependence on IGF-1 (24). This contrary finding may be a result of a difference in the way the tamoxifen-resistant cells were generated; these investigators used low serum conditions during generation of tamoxifen resistance while we used complete media. When cells were selected in this manner, tamoxifen became an ER agonist. Although seemingly contradictory, these findings are consistent with our own. IGF1H expression requires agonism of ER. In Westley and colleagues, their "tamoxifen-resistant" cells tamoxifen-stimulated ER function. In our tamoxifen-resistant cells, we saw no evidence of agonistic activity stimulated by tamoxifen (Fig. 10).

On the basis of prior reports of ER transcriptional regulation of IGF1H, it is not surprising that IGF1H expression would be decreased after acute treatment with a selective estrogen receptor modulator such as tamoxifen (25–27). Interestingly, studies conducted by Massarweh and colleagues using tamoxifen-resistant xenografts show decreased total levels of IGF1H, but basal phosphorylation of the receptor (28). 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 5 times weekly, leading to the possibility that ER function is not completely suppressed in this model. Furthermore, this study did not clearly distinguish between IGF1H or IGF1R phosphorylation because 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 shown the efficacy of IGF1R monoclonal antibodies in breast cancer cells, these cells have been endocrine sensitive (21, 23). The effect of combined anti-estrogen/anti-IGF1H 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 show that tamoxifen treatment results in decreased IGF1R mRNA levels. Initial results examining the effectiveness of IGF1H antibodies in endocrine-resistant breast cancer populations have not showed a definitive positive result (29, 30). This may be because of the lack of IGF1H expression in these patient populations. A recent study examining mRNA expression in a cohort of tamoxifen-resistant patients with breast cancer has showed a decrease in IGF1R levels in the recurrent tumors, suggesting our findings *in vitro* may correlate with the clinical scenario (31).

The efficacy of TKJ's in our tamoxifen-resistant cells underscores the importance of cotargeting the IR, along with the IGF1R. Initially, development of IGF1R inhibitors aimed to avoid targeting the IR, because of potential metabolic consequences. However, numerous studies by us and others have showed that the IR does indeed play a role in cancer biology (14, 18, 29, 32). Specifically, work by Hanahan and colleagues showed that IGF1R inhibition using antibodies is only successful in tumors/cells where the IGF1R/TR ratio is high. In addition, they show the IR can actually serve as an escape mechanism, providing resistance to IGF1R antibodies (17). Furthermore, 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 (33). 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 showing increased copy number of IR in a woman with metastatic hormone-refractory breast cancer (34). 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 conducted in mice showed 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 showed the efficacy of the TKI in letrozole resistant xenografts, supporting our data that suggest TKJ's are more effective than antibodies in tamoxifen-resistant cells (35).

Although our data indicate IGF1R antibodies may not be effective in an endocrine-resistant system, this does not mean they have little use. 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 (36). In addition, clinical trials are underway examining the efficacy of combining IGF1R antibodies with mTOR inhibitors (37, 38).

Overall, our data highlight the importance of using model systems that will match the patient population the drug will ultimately be used in. In addition, 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/TR TKI's may be more effective than IGF1R antibodies, because of 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.

#### Disclosure of Potential Conflicts of Interest

D. Yee has received a commercial research grant from Merck. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

Conception and design: D.J.I. Fagan, D. Sachdev, D. Yee  
Development of methodology: D.J.I. Fagan, D. Sachdev  
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.J.I. Fagan, H.R. Uselman, D. Sachdev  
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.J.I. Fagan, D. Yee  
Writing, review, and/or revision of the manuscript: D.J.I. Fagan, H.R. Uselman, D. Sachdev, D. Yee  
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Yee  
Study supervision: D. Yee

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#### References

- MacGregor JI, Jordan VC. Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev* 1998;50:151-96.
- Tan no S, Mitsuuchi Y, Altomare DA, Xiao GH, Testa JR. AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells. *Cancer Res* 2001;61:589-93.
- Ciampolillo A, De Tullio C, Giordano F. The IGF-1/IGF-1 receptor pathway: implications in the pathophysiology of thyroid cancer. *Curr Med Chem* 2005;12:2881-91.
- Lee AV, Darbre P, King RJ. Processing of insulin-like growth factor-II (IGF-II) by human breast cancer cells. *Mol Cell Endocrinol* 1994;99:211-20.
- Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, et al. Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression *in vitro* and *in vivo*. *Mol Endocrinol* 1999;13:787-96.
- Molloy CA, May FE, Westley BR. Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line. *J Biol Chem* 2000;275:12565-71.
- Murphy W, Murphy LC, Friesen HG. Estrogen induces insulin-like growth factor-I expression in the rat uterus. *Mol Endocrinol* 1987;1:445-50.
- Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, et al. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. *J Biol Chem* 1994;269:16433-42.
- Becker MA, Ibrahim YH, Cui X, Lee AV, Yee D. The IGF pathway regulates ERalpha through a S6K1-dependent mechanism in breast cancer cells. *Mol Endocrinol* 2011;25:516-28.
- Osborne R. Commercial interest waxes for IGF-1 blockers. *Nat Biotechnol* 2008;26:719-20.
- Fagan DH, Yee D. Crosstalk between IGF1R and estrogen receptor signaling in breast cancer. *J Mammary Gland Biol Neoplasia* 2008;13:423-9.
- Kaufman PA, Ferrero JM, Bourgeois H, Kennecke H, De Boer R, Jacot W, et al. A randomized, double-blind, placebo-controlled, phase 2 study of AMG 479 with Exemestane (E) or Fulvestrant (F) in postmenopausal women with hormone-receptor positive (HR+) metastatic (M) or locally advanced (LA) breast cancer (BC). *Cancer Res* 2010;70:76s.

13. Avnet S, Sciacca L, Salerno M, Gancitano G, Cassarino MF, Longhi A, et al. **Insulin receptor isoform A and insulin-like growth factor II as additional treatment targets in human osteosarcoma.** *Cancer Res* 2009;69:2443-52.
14. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, et al. **The role of insulin receptors and IGF-1 receptors in cancer and other diseases.** *Arch Physiol Biochem* 2008;114:23-37.
15. Zhang H, Fagan DH, Zeng X, Freeman KT, Sachdev D, Yee D. **Inhibition of cancer cell proliferation and metastasis by insulin receptor down-regulation.** *Oncogene* 2010;29:2517-27.
16. Zhang H, Pelzer AM, Kiang DT, Yee D. **Down-regulation of type I insulin-like growth factor receptor increases sensitivity of breast cancer cells to insulin.** *Cancer Res* 2007;67:391-7.
17. Ulanet DB, Ludwig DL, Kahn CR, Hanahan D. **Insulin receptor functionally enhances multistage tumor progression and conveys intrinsic resistance to IGF-1R targeted therapy.** *Proc Natl Acad Sci U S A* 2010;107:10791-8.
18. Pollak M. **Targeting insulin and insulin-like growth factor signalling in oncology.** *Curr Opin Pharmacol* 2008;8:384-92.
19. Twentyman PR, Luscombe M. **A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity.** *Br J Cancer* 1987;56:279-85.
20. Levin-Allemand JA, Sokol K, Smith JD. **Safe and effective method for chronic 17beta-estradiol administration to mice.** *Contemp Top Lab Anim Sci* 2003;42:33-5.
21. Goetsch L, Gonzalez A, Leger O, Beck A, Pauwels PJ, Haeuw JF, et al. **A recombinant humanized anti-insulin-like growth factor receptor type I antibody (h7C10) enhances the antitumor activity of vinorelbine and anti-epidermal growth factor receptor therapy against human cancer xenografts.** *Int J Cancer* 2005;113:316-28.
22. Pandini G, Wurch T, Akla B, Corvaia N, Belfiore A, Goetsch L. **Functional responses and *in vivo* anti-tumour activity of h7C10: a humanised monoclonal antibody with neutralising activity against the insulin-like growth factor-1 (IGF-1) receptor and insulin/IGF-1 hybrid receptors.** *Eur J Cancer* 2007;43:1318-27.
23. Sachdev D, Li SL, Hartell JS, Fujita-Yamaguchi Y, Miller JS, Yee D. **A chimeric humanized single-chain antibody against the type I insulin-like growth factor (IGF) receptor renders breast cancer cells refractory to the mitogenic effects of IGF-1.** *Cancer Res* 2003;63:627-35.
24. Wiseman LR, Johnson MD, Wakeling AE, Lykkesfeldt AE, May FE, Westley BR. **Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells.** *Eur J Cancer* 1993;29A:2256-B4.
25. Freiss G, Rochefort H, Vignon F. **Mechanisms of 4-hydroxytamoxifen anti-growth factor activity in breast cancer cells: alterations of growth factor receptor binding sites and tyrosine kinase activity.** *Biochem Biophys Res Commun* 1990;173:919-26.
26. Huynh H, Yang X, Pollak M. **Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 auto-crine loop in human breast cancer cells.** *J Biol Chem* 1996;271:1016-21.
27. Pratt SE, Pollak MN. **Estrogen and antiestrogen modulation of MCF7 human breast cancer cell proliferation is associated with specific alterations in accumulation of insulin-like growth factor-binding proteins in conditioned media.** *Cancer Res* 1993;53:5193-8.
28. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, et al. **Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function.** *Cancer Res* 2008;68:826-33.
29. Pollak M. **Insulin and insulin-like growth factor signalling in neoplasia.** *Nat Rev Cancer* 2008;8:915-28.
30. Teicher AW, Sarantopoulos J, Patnaik A, Papadopoulos K, Lin CC, Roden J, et al. **Phase I, pharmacokinetic, and pharmacodynamic study of AMG 479, a fully human monoclonal antibody to insulin-like growth factor receptor 1.** *J Clin Oncol* 2009;27:580Q-7.
31. Drury S, Detre S, Leary A, Salter J, Reis-Filho J, Barbashina V, et al. **Changes in breast cancer biomarkers in the IGF1R/P13K pathway in recurrent breast cancer after tamoxifen treatment.** *Endocr Relat Cancer* 2011;18:565-77.
32. Gallagher EJ, LeRoith D. **The proliferating role of insulin and insulin-like growth factors in cancer.** *Trends Endocrinol Metab* 2010;21:610-8.
33. Haluska P, Shaw HM, Batzel GN, Yin D, Molina JR, Molife LR, et al. **Phase I dose escalation study of the anti insulin-like growth factor-1 receptor monoclonal antibody CP-751,871 in patients with refractory solid tumors.** *Clin Cancer Res* 2007;13:5834-40.
34. Shah SP, Morin RD, Khattri J, Prentice L, Pugh T, Burleigh A, et al. **Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution.** *Nature* 2009;461:809-13.
35. Haluska P, Hou X, Huang F, Harrington S, Greer A, Macedo L, et al. **Complete IGF signaling blockade by the dual-kinase inhibitor, BMS-754807, is sufficient to overcome tamoxifen and letrozole resistance *in vitro* and *in vivo*.** *Cancer Res* 2009;69:402.
36. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, et al. **mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt.** *Cancer Res* 2006;66:150Q-8.
37. DiCosimo S, Bendell JC, Cervantes-Ruiperez A, Roda D, Prudkin L, Stein MN, et al. **A phase I study of the oral mTOR inhibitor ridaforolimus (AIDA) in combination with the IGF-1R antibody dalotuzumab (DALO) in patients (pts) with advanced solid tumors.** *J Clin Oncol* 28:15s, 2010 (suppl; abstr 3008).
38. Nang A, LoRusso P, Gupta S, Benjamin S, Rohren E, Chen HK, et al. **Dual inhibition of IGF1R and mTOR pathways.** *J Clin Oncol* 28:15s, 2010 (suppl; abstr 3007).