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14. ABSTRACT

Obesity is a major risk factor for breast cancer, and is associated with reduced treatment response and reduced overall survival. The obesity-associated hormones IGF-I and leptin and their receptors, IGF-IR and leptin receptor (Ob-R), are elevated in breast cancer. Co-immunoprecipitation and immunoblotting demonstrated that IGF-IR and Ob-R interact in the breast cancer cell lines MDA-MB-231, MCF7, BT474, and SKBR3. Stimulation of cells with IGF-I promoted Ob-R phosphorylation, which was blocked by IGF-IR kinase inhibition. In addition, IGF-I activated downstream signaling molecules in the leptin receptor and IGF-IR pathways. In contrast to IGF-I, leptin did not induce phosphorylation of IGF-IR, indicating that receptor cross signaling is unidirectional, occurring from IGF-IR to Ob-R. Our results demonstrate for the first time a novel interaction and cross talk between the IGF-I and leptin receptors in human breast cancer cells. Our data also showed that inhibition of JAK2, which is immediately downstream of the leptin receptor, reduced proliferation of MCF7 breast cancer cells. Leptin specifically reduced sensitivity to docetaxel, and not to targeted therapies trastuzumab or lapatinib. Further, an unrelated obesity-associated cytokine did not reduce docetaxel sensitivity, suggesting that chemoresistance may be specifically induced by leptin and not by all adipocytokines.

15. SUBJECT TERMS

Breast cancer, leptin, insulin-like growth factor-I, growth factor receptor signaling

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INTRODUCTION

Obesity is an important risk factor associated with the development and progression of breast cancer (1-7), reduced therapeutic efficacy, and higher mortality rates among breast cancer patients (8-11). The obesity-associated hormones insulin-like growth factor-I (IGF-I) and leptin are found at high levels in breast cancer patients (12-15), and their receptors, IGF-IR and Ob-R (leptin receptor is also known as obesity receptor), are overexpressed in a majority of breast cancers (15-17). Increased expression of leptin and Ob-R correlate with increased risk for distant metastasis and reduced overall survival in breast cancer patients (15). Leptin induces proliferation of breast cancer cells via activation of STAT3 (18,19), a transcriptional activator of the anti-apoptotic protein Bcl-2 (20,21). STAT3-dependent overexpression of Bcl-2 was associated with resistance to the chemotherapeutic agent paclitaxel in breast cancer cells (21). In our initial grant proposal, we proposed the following model (**Figure 1**): IGF-IR and leptin receptor interact, inducing phosphorylation of Ob-R, which then activates downstream STAT3 and Bcl-2, resulting in taxane resistance. Our long-term goal was to establish markers of leptin receptor signaling as predictors of taxane response. The ultimate impact would be that these markers of leptin signaling, including serum levels of leptin and IGF-I, and tissue levels of phosphorylated Ob-R, STAT3 and Bcl-2, could be used (1) to identify patients most likely to respond to taxanes, and (2) as therapeutic targets to improve response rates to taxanes in the treatment of breast cancer.

BODY

<u>Task 1.</u> Apply nanotechnology-based methods for visualization of IGF-IR and leptin receptor (Ob-R) in real time. In the first year of this grant, we collaborated with Dr. Konstantin Sokolov at MD Anderson to label nanoparticles with antibodies against IGF-IR or Ob-R. However, nanotechnology was not initially available for this aim when the PI changed institutions from MD Anderson to Emory University. Thus, this aim has used non-nanotechnology based approaches to assess IGF-IR levels in breast cancer cell lines. We visualized IGF-IR expression in a panel of breast cancer lines by Western blotting, and found a wide variation in levels of phosphorylated and total IGF-IR (**Figure 2**). We used the MCF7 line for most of our work below, as it expressed the highest level of IGF-IR. Additional lines (MDA231 and SKBR3) were used to determine if our data extended to more than one cell line. During the last year of this award, we began collaborating with Dr. Khalid Salaita in the Department of Chemistry at Emory University. Dr. Salaita is an expert on nanoparticle-based approaches to study receptor interactions and signaling. As an initial experiment, Dr. Salaita used our BT474 cell line, which expresses high

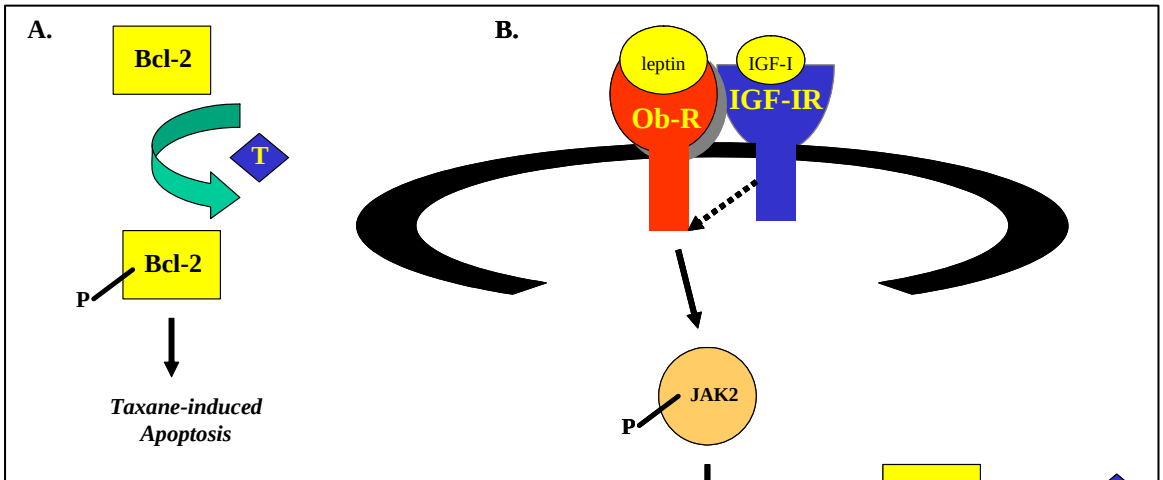

Figure 1. Model for leptin receptor-mediated taxane resistance. A) Taxanes (T) phosphorylate and inactivate Bcl-2, inducing apoptosis (22). B) Leptin activates JAK2 and STAT3 (18, 19), and STAT3 induces Bcl-2 expression (20,21). We demonstrate that IGF-IR and Ob-R interact (Figure 2). Here, we propose that IGF-IR, which is activated by IGF-I, cross talks to Ob-R, such that leptin and IGF-I will both activate JAK2/STAT3 phosphorylation downstream of the leptin receptor. STAT3 will then increase transcription of Bcl-2, resulting in decreased response of breast cancer cells to taxanes.

levels of IGF-IR and the receptor HER2. He initially used an anti-HER2 antibody to test binding of fluorophore-conjugated antibodies to the membrane of these cells. Streptavidin-biotin labeling of the antibody was performed; cells showed efficient binding of the antibody to the cell surface by fluorescent microscopy. Thus, labeling was successfully achieved in this initial experiment. We are continuing to collaborate with Dr. Salaita's group to label anti-IGF-IR antibody and anti-Ob-R antibody with streptavidin-biotin and expose MCF-7 and BT474 cells to these antibodies in the absence or presence of IGF-I or leptin. This aim was initially delayed due to difficulty in establishing a new collaboration at the PI's institution since the PI changed institutions in 2007. Now that the collaboration has been established, we are able to continue studying receptor-receptor interactions in real time using antibody-labeled nanoparticles.

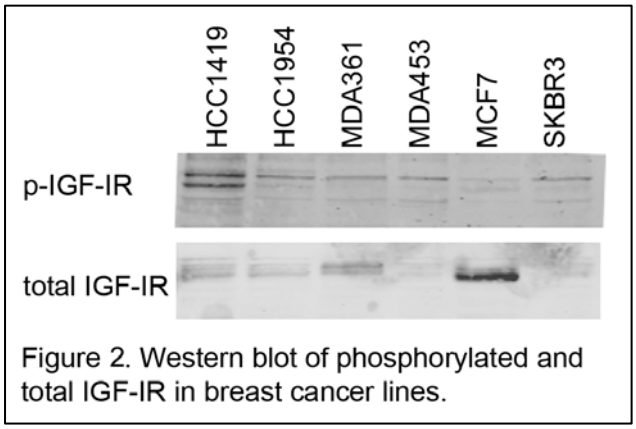

Figure 2. Western blot of phosphorylated and total IGF-IR in breast cancer lines.

<u>Task 2.</u> Demonstrate that IGF-I activates the leptin receptor via IGF-IR crosstalk.

During the course of this award, aim 2 was completed and published (reference 22, which is attached in the Appendix). The breast cancer cell lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were examined for IGF-IR and leptin receptor expression and interaction. Cells were lysed for protein and leptin receptor was immunoprecipitated from cells. Immunoblotting demonstrated that IGF-IR was pulled down with the leptin receptor, indicating that they form a complex (**Figure 3A**). Conversely, immunoprecipitation of IGF-IR showed pull down of leptin receptor in all cell lines (**Figure 3B**). Total receptor levels were also examined in the cell lines (**Figure 4**). The leptin receptor has two isoforms. The long isoform is approximately 120 kDa, and possesses kinase activity. The short isoform is 90 kDa and lacks signaling capability, and its function is unclear. All cells showed expression of the long isoform of the leptin receptor, with BT474 cells showing very low levels of the short isoform. IGF-IR expression varied among lines, with MCF7 and BT474 cells showing highest levels. We next wanted to determine the functional consequence of this interaction. MCF7 cells were serum starved overnight to remove hormonal stimulation. Cells were then stimulated with 100ng/mL IGF-I for the indicated time course (**Figure 5A**). Cells were lysed for protein, and immunoblots were performed for total and phosphorylated leptin receptor (using phospho-tyrosine 1141- specific antibody for leptin receptor, Santa Cruz Biotechnology), and for total and phosphorylated IGF-IR (using anti-phospho-tyrosine 1162/1163 insulin receptor/IGF-IR antibody, Biosource). IGF-IR phosphorylation was stimulated within 5 minutes (min).

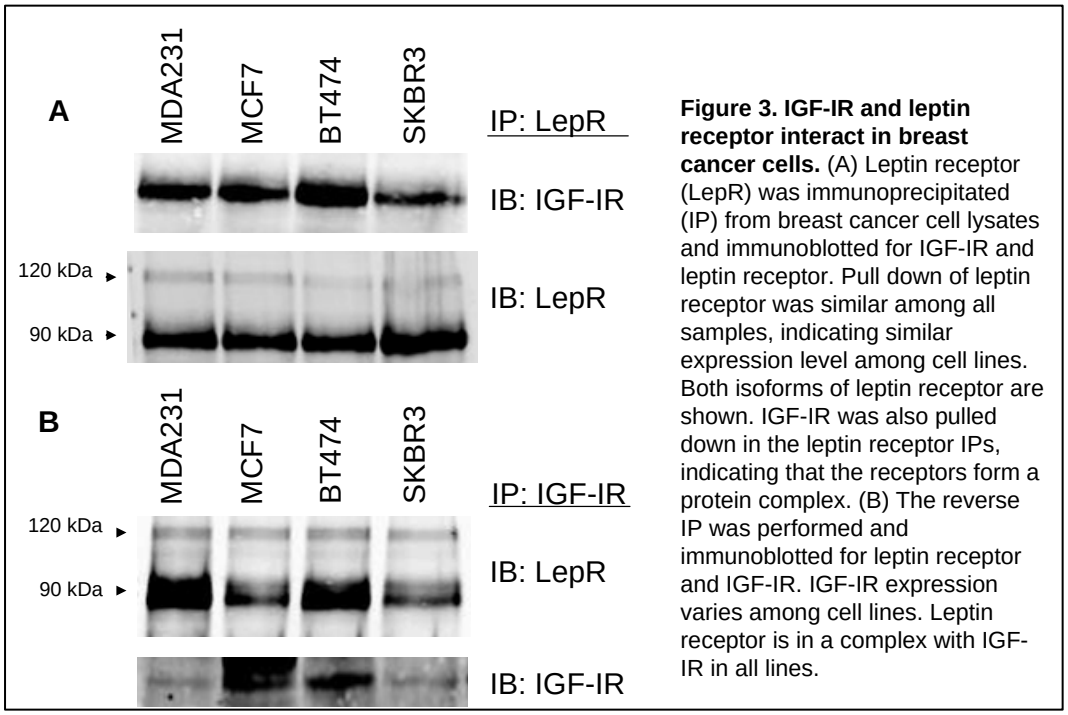

Figure 3. IGF-IR and leptin receptor interact in breast cancer cells. (A) Leptin receptor (LepR) was immunoprecipitated (IP) from breast cancer cell lysates and immunoblotted for IGF-IR and leptin receptor. Pull down of leptin receptor was similar among all samples, indicating similar expression level among cell lines. Both isoforms of leptin receptor are shown. IGF-IR was also pulled down in the leptin receptor IPs, indicating that the receptors form a protein complex. (B) The reverse IP was performed and immunoblotted for leptin receptor and IGF-IR. IGF-IR expression varies among cell lines. Leptin receptor is in a complex with IGF-IR in all lines.

Importantly, leptin receptor phosphorylation was also stimulated within 5 min, indicating that IGF-IR activation corresponds with activation of leptin receptor, suggesting potential cross talk from IGF-IR to leptin receptor. Total levels of IGF-IR and leptin receptor isoforms did not change. Downstream of the receptors, the signaling pathways including JAK2, STAT3, ERK1/2, and Akt were examined (**Figure 5B**). The major molecules activated were JAK2 and ERK1/2, and to a lesser extent STAT3 and Akt. We have also confirmed these results in another cell line, MDA231, in which we also observed phosphorylation of leptin receptor in response to IGF-I stimulation (**Figure 6A**) and activation of JAK2 and ERK1/2 as the major changes in downstream signaling (**Figure 6B**). Hence, the potential biological consequences of IGF-IR cross talk to leptin receptor may be mediated by the JAK2 and MAPK signaling pathways. Cross talk in the opposite direction, from leptin receptor to IGF-IR, was also examined. For this experiment, MCF7 cells were serum starved overnight, and stimulated with 100ng/mL leptin for the indicated time points (**Figure 7**). Immunoblots of total lysates were probed for phospho-leptin receptor and phospho-IGF-IR. Our preliminary results indicated that leptin receptor is phosphorylated as expected, but we did not detect phosphorylated IGF-IR. Next, MCF7 cells were serum-starved overnight, and then treated with 10μM Src kinase inhibitor I (EMD Chemicals, Gibbstown, NJ) overnight, followed by IGF-I (100ng/mL) stimulation for 15 min. Protein lysates were immunoblotted for phosphorylated and total Src, IGF-IR, and Ob-R (**Figure 8**). Inhibition of Src kinase appeared to partially reduce IGF-I-mediated phosphorylation of the IGF-IR and Ob-R, suggesting that Src kinase activity is important to IGF-IR kinase activity and to its cross talk to Ob-R. We also pre-treated MCF7

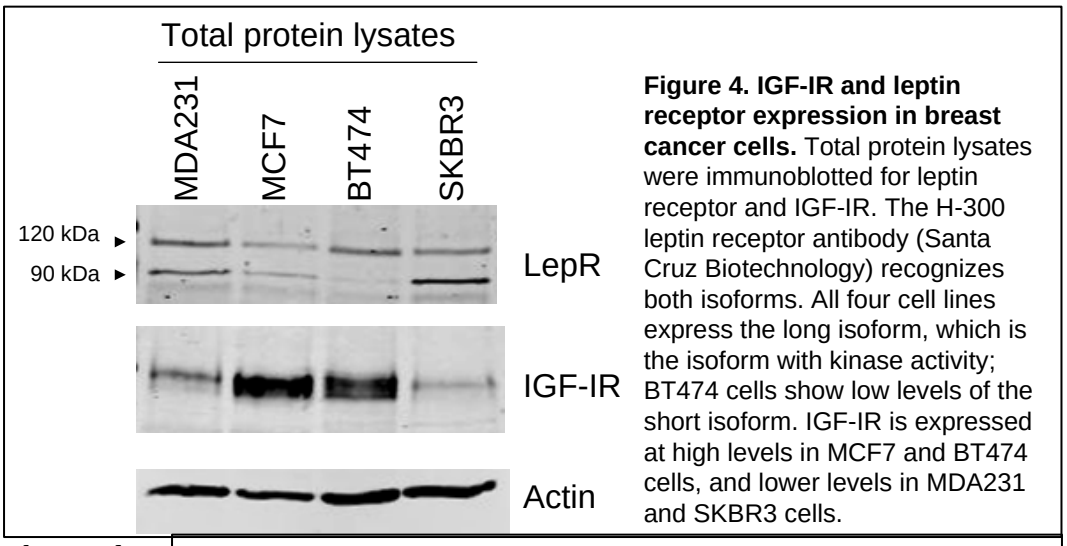

Figure 4. IGF-IR and leptin receptor expression in breast cancer cells. Total protein lysates were immunoblotted for leptin receptor and IGF-IR. The H-300 leptin receptor antibody (Santa Cruz Biotechnology) recognizes both isoforms. All four cell lines express the long isoform, which is the isoform with kinase activity; BT474 cells show low levels of the short isoform. IGF-IR is expressed at high levels in MCF7 and BT474 cells, and lower levels in MDA231 and SKBR3 cells.

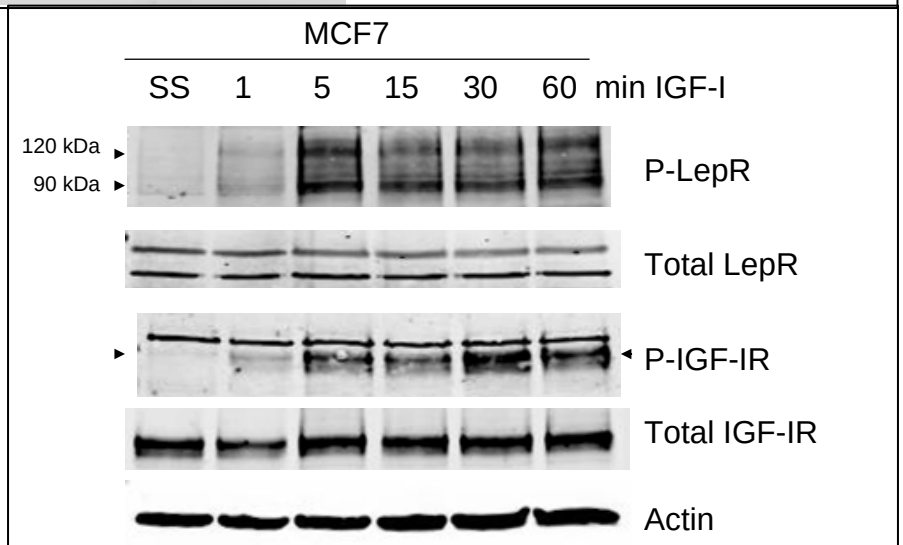

Figure 5A. IGF-I stimulates phosphorylation of leptin receptor in MCF7 breast cancer cells: evidence of receptor cross talk. MCF7 cells were serum starved overnight, and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 minutes (min). SS indicates the serum starved, non-stimulated control. Within 5 minutes of stimulation, IGF-IR was phosphorylated as expected. Total IGF-IR levels were unchanged. Interestingly, leptin receptor (LepR) was also phosphorylated within 5 minutes, indicating that activation of IGF-IR corresponds with activation of LepR, suggesting cross talk from IGF-IR to leptin receptor. Total levels of both isoforms of LepR were unchanged.

cells for 1 hour with inhibitors of Src, Jak2, PI3K, or MAPK prior to IGF-I stimulation and examined Ob-R phosphorylation (**Figure 9**). The short-term treatment with inhibitors did not inhibit IGF-IR cross talk to Ob-R, in contrast to the overnight treatment with Src inhibitor I (**Figure 8**). Since insulin receptor shares homology with IGF-IR, we inhibited insulin receptor with 10 µM HNMPA (EMD Chemicals) overnight followed by IGF- I stimulation and immunoblotted for phosphorylated and total IGF-IR and Ob-R (**Figure 10**). IGF-I mediated phosphorylation of Ob-R in the presence of HNMPA, suggesting that IGF-I stimulation of insulin receptor does not mediate the cross talk to Ob-R. However, stimulation with insulin does phosphorylate Ob-R (**Figure 11**), suggesting that insulin may be activating IGF-IR which then cross talks to Ob-R.

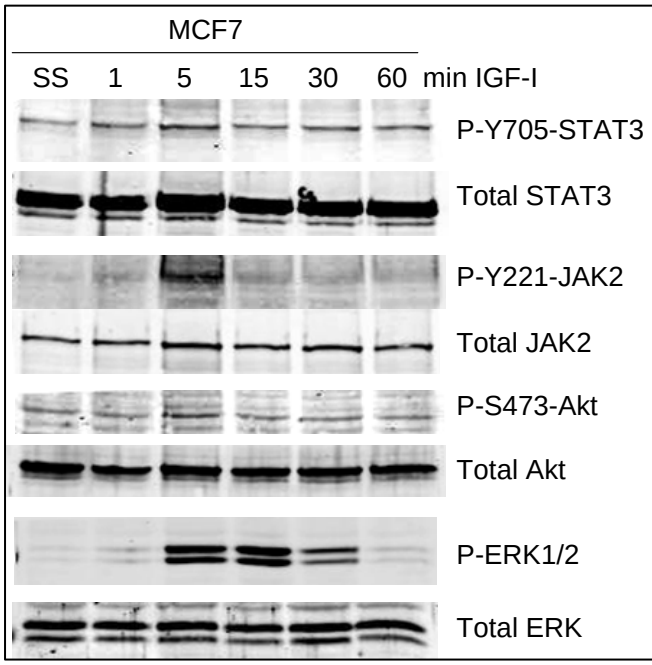

Figure 5B. IGF-I activates leptin receptor signaling in MCF7 breast cancer cells: evidence of receptor cross talk. MCF7 cells were serum starved overnight, and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 minutes (min). SS indicates the serum starved, non-stimulated control. JAK2 and, to a lesser extent, STAT3 were phosphorylated within 5 min of IGF-I stimulation, as were ERK1 and ERK2 and, to a lesser extent, Akt. Thus, the main downstream molecules activated were JAK2 and ERK1/2.

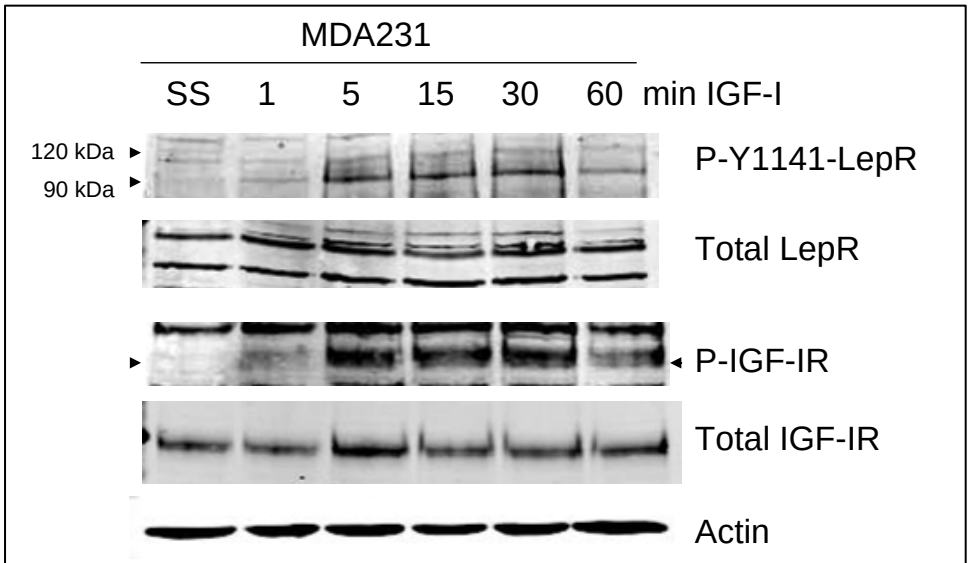

Figure 6A. IGF-I stimulates phosphorylation of leptin receptor in MDA231 breast cancer cells: evidence of receptor cross talk. MDA231 cells were serum starved overnight, and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 minutes (min). SS indicates the serum starved, non-stimulated control. Within 5 minutes of stimulation, IGF-IR was phosphorylated as expected. Total IGF-IR levels were unchanged. Interestingly, leptin receptor (LepR) was also phosphorylated within 5 minutes, indicating that activation of IGF-IR corresponds with activation of LepR, suggesting cross talk from IGF-IR to leptin receptor. Total levels of both isoforms of LepR were unchanged. These results are similar to what was observed in MCF7 cells.

<u>Task 3.</u> Demonstrate that Ob-R signaling activated by leptin or IGF-I contributes to taxane resistance.

MCF7, BT474, and SKBR3 cells were either untreated, treated with 100ng/mL leptin for 36 hours (h), 5nM docetaxel for 24 h, or treated with a combination of leptin and docetaxel, where cells were pre-treated with leptin for 12 hours and then docetaxel was added to the media for an additional 24 hours. DNA fragmentation as a measure of apoptosis was examined using the Cell Death ELISA Plus Kit (Roche Applied Science) per manufacturer instructions. With this particular experimental design, very little change in DNA fragmentation was observed in MCF7 and BT474 cells (**Figure 12**). In SKBR3 cells docetaxel alone caused a 2-fold increase in DNA fragmentation, and the added contribution of leptin was negligible. We then treated MCF7 cells with IGF-I and docetaxel, and examined cytotoxicity

using a clonogenic assay. MCF7 cells were treated with 100ng/mL IGF-I +/- 10nM docetaxel for 1 week, with media plus IGF-I or drug changed daily. Photographs of cultures were taken to assess colony growth (**Figure 13**). IGF-I partially reduced docetaxel sensitivity, as a higher colony count was observed in the IGF-I + docetaxel cultures. Dose-response profiles were established for MCF7 and MDA231 cells treated with paclitaxel (**Figure 14**). The cell lines responded similarly to paclitaxel, with both lines showing 50% inhibition of proliferation (IC50) at approximately 10-20 nM. In our initial DoD application, we hypothesized that obesity-associated hormones will reduce taxane sensitivity. Thus, we treated MCF7 cells with DMSO control or 10 nM docetaxel while being maintained in regular cell culture DMEM media containing 10% fetal calf serum, conditioned media (CM) from 3T3 mouse adipocytes, or CM from human abdominal omental adipocytes. MTS proliferation assays were performed after 6 days (**Figure 15**); all treatments were done in 6 replicates. Our results indicated that 3T3 CM reduced response to docetaxel, but that omental CM did not change response. In addition, we hypothesized in the initial application that inhibition of Ob-R and IGF-IR signaling will increase taxane sensitivity. We were unable to identify effective siRNA or shRNA against Ob-R. Thus, we used pharmacologic inhibition of downstream

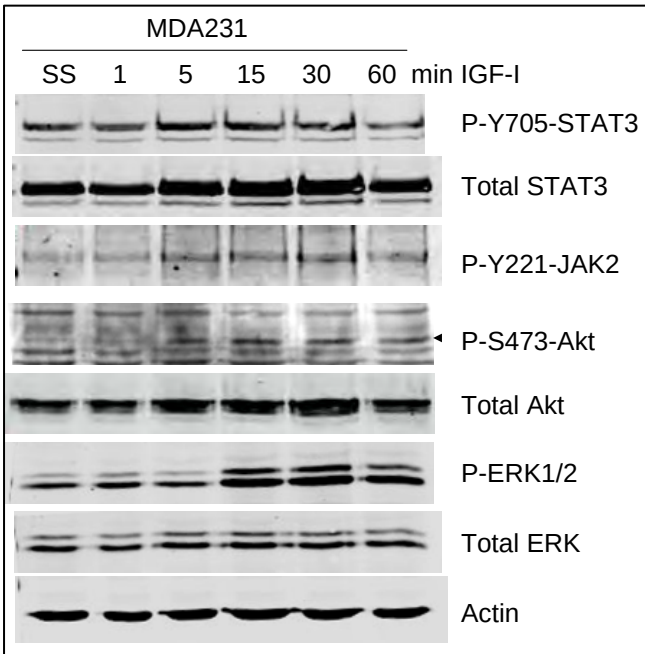

Figure 6B. IGF-I activates leptin receptor signaling in MDA231 breast cancer cells: evidence of receptor cross talk. MDA231 cells were serum starved overnight, and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 minutes (min). SS indicates the serum starved, non-stimulated control. JAK2 and, to a lesser extent, STAT3 were phosphorylated within 5 min of IGF-I stimulation, as were ERK1 and ERK2 and, to a lesser extent, Akt. Thus, the main downstream molecules activated were JAK2 and ERK1/2, similar to results seen in MCF7 cells.

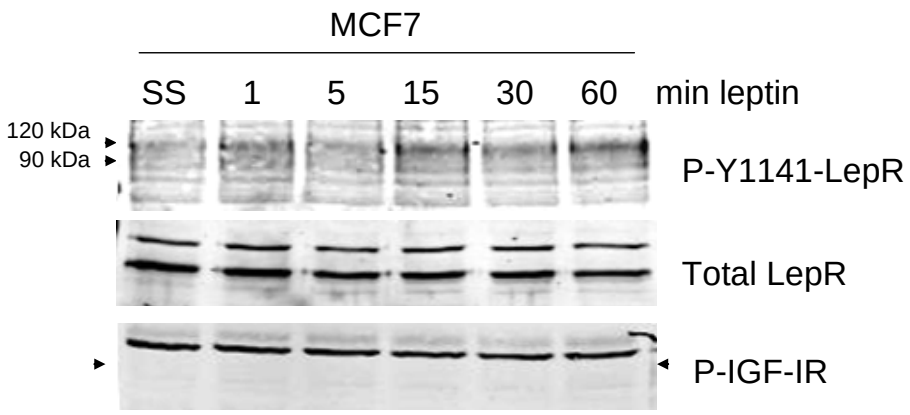

Figure 7. Leptin does not induce phosphorylation of IGF-IR: evidence of potential unidirectional receptor cross talk. MCF7 cells were serum starved overnight, and then stimulated with leptin (100 ng/mL) for 1, 5, 15, 30, or 60 minutes (min). SS indicates the serum starved, non-stimulated control. No change in IGF-IR phosphorylation status was observed. As discussed in the text, the quality of this preliminary blot is weak and must be repeated several times. In addition, a positive control is required for p-IGF-IR to ensure that leptin is actually not inducing phosphorylation of IGF-IR.

JAK/STAT signaling to determine if inhibition of Ob-R signaling affects taxane response. HCC1806, MDA231, and MDA468 breast cancer cells were treated with 2-fold serial dilutions of WP1066, a JAK2/STAT3 inhibitor, and proliferation was measured after 6 days (**Figure 16**). All cells showed similar response with IC50 approaching 8uM. We treated MCF7 with the IGF-IR inhibitor PPP plus JAK2 inhibitor (**Figure 17**), and have not observed any additive or synergistic effects. Interestingly, despite having high endogenous levels of IGF-IR, MCF7 cell proliferation is unaffected by IGF-IR inhibition alone. In contrast, cells appear to be partially dependent upon JAK2 signaling, as JAK2 inhibition produced a dose-dependent decline in cell proliferation. The IC50 of JAK2 inhibitor in MCF7 cells was consistent with the IC50s observed in response to WP1066 in HCC1806, MDA231, and MDA468. Finally, since we have not found an effective siRNA against leptin receptor, we tested a mutant recombinant human leptin peptide. Mutant leptin did not increase inhibition of MCF7 cell proliferation achieved by the IGF-IR inhibitor PPP (**Figure 18**). These results are consistent with data shown in Figure 17, indicating that PPP does not increase the effect of JAK2 inhibition on MCF7 cell proliferation. Interestingly, in Figure 18, PPP alone reduced proliferation, in contrast to Figure 17. Thus, combined IGF-IR and Ob-R/JAK2 inhibition may not be beneficial, but rather JAK2 inhibition or IGF-IR inhibition alone may inhibit proliferation of breast cancer cells. In

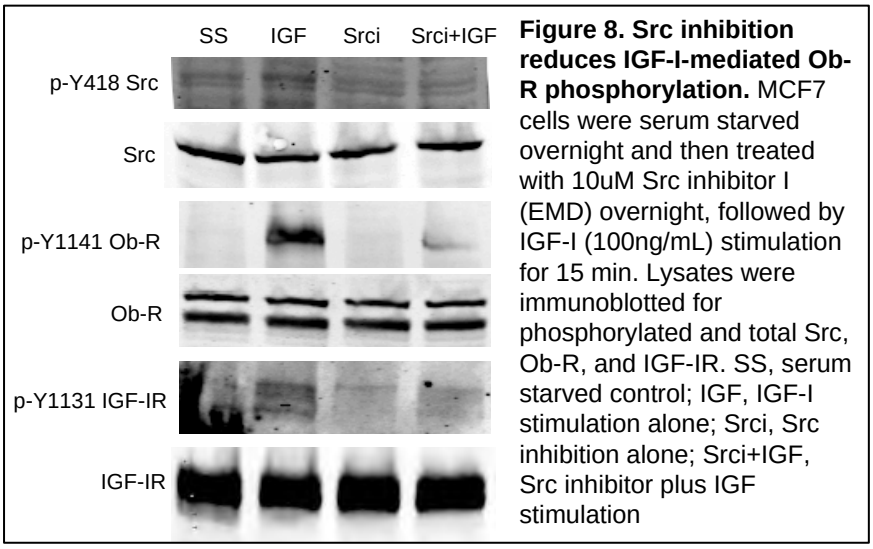

Figure 8. Src inhibition reduces IGF-I-mediated Ob-R phosphorylation. MCF7 cells were serum starved overnight and then treated with 10uM Src inhibitor I (EMD) overnight, followed by IGF-I (100ng/mL) stimulation for 15 min. Lysates were immunoblotted for phosphorylated and total Src, Ob-R, and IGF-IR. SS, serum starved control; IGF, IGF-I stimulation alone; Srci, Src inhibition alone; Srci+IGF, Src inhibitor plus IGF stimulation

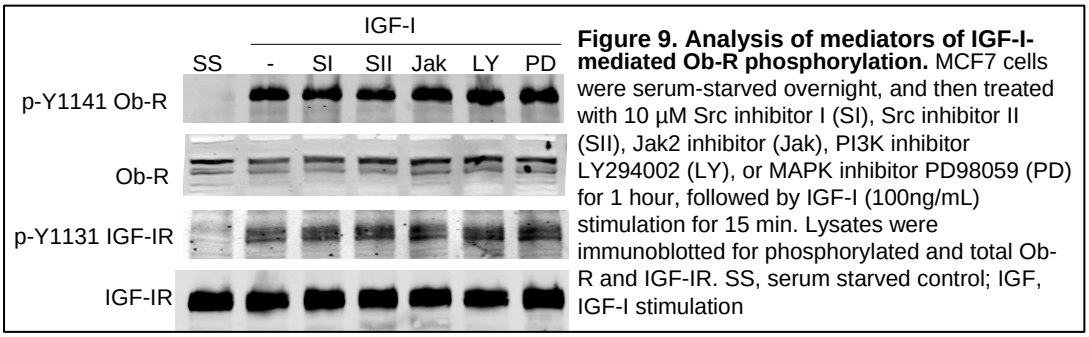

Figure 9. Analysis of mediators of IGF-I-mediated Ob-R phosphorylation. MCF7 cells were serum-starved overnight, and then treated with 10 µM Src inhibitor I (SI), Src inhibitor II (SII), Jak2 inhibitor (Jak), PI3K inhibitor LY294002 (LY), or MAPK inhibitor PD98059 (PD) for 1 hour, followed by IGF-I (100ng/mL) stimulation for 15 min. Lysates were immunoblotted for phosphorylated and total Ob-R and IGF-IR. SS, serum starved control; IGF, IGF-I stimulation

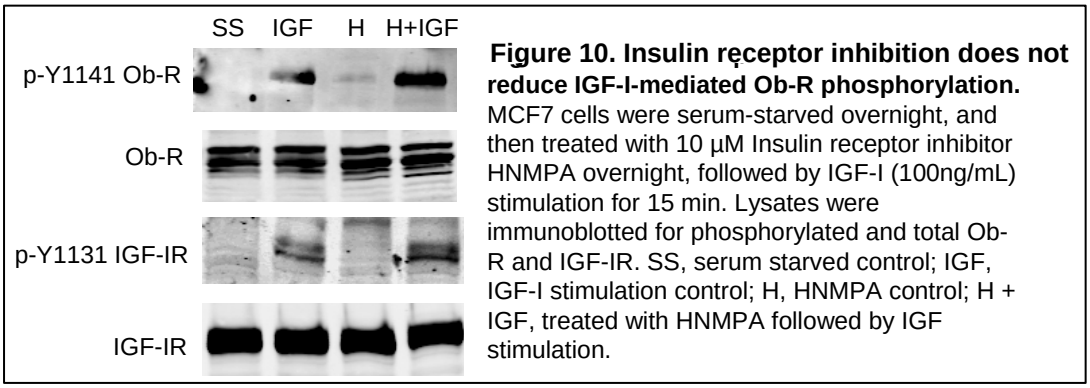

Figure 10. Insulin receptor inhibition does not reduce IGF-I-mediated Ob-R phosphorylation. MCF7 cells were serum-starved overnight, and then treated with 10 µM Insulin receptor inhibitor HNMPA overnight, followed by IGF-I (100ng/mL) stimulation for 15 min. Lysates were immunoblotted for phosphorylated and total Ob-R and IGF-IR. SS, serum starved control; IGF, IGF-I stimulation control; H, HNMPA control; H + IGF, treated with HNMPA followed by IGF stimulation.

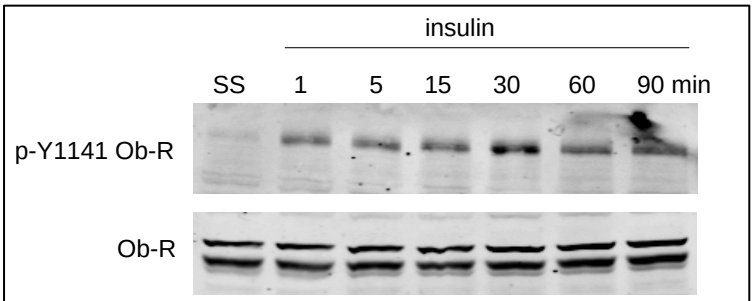

Figure 11. Insulin stimulates Ob-R phosphorylation. MCF7 cells were serum-starved overnight, and then treated with insulin (100ng/mL) over a time course for 1 min to 90 min. Lysates were immunoblotted for phosphorylated and total Ob-R. SS, serum starved control

our initial DoD application, we hypothesized that the obesity-associated hormones leptin and IGF-I will reduce taxane sensitivity. MDA231 ER-negative breast cancer cells and MCF7 ER-positive breast cancer cells were pre-treated for 24 h with 100ng/mL leptin or solvent control, and then treated with a combination of leptin 100ng/mL (or control) plus two-fold serial dilutions of docetaxel ranging from 0nM to 100nM. After 6 days, proliferation was measured using the MTS colorimetric assay (Promega). Co-treatment with leptin reduced response to docetaxel in MDA231 and MCF7 cells (**Figure 19**), with statistically significant results observed at the middle doses of docetaxel. The values shown in the graph reflect average fold proliferation relative to control cells treated with DMSO +/- leptin. Error bars represent the standard deviation between 6 replicates per group. Thus, increased exogenous exposure to leptin, which may occur during obesity, may result in reduced response to the chemotherapeutic drug docetaxel in breast cancer. In contrast, co-treatment of HER2-overexpressing breast cancer cells with leptin plus either the HER2-targeted therapy trastuzumab or lapatinib resulted in reduced response to HER2-targeted therapies, but results did not reach statistical significance. For this experiment, SKBR3 HER2-overexpressing breast cancer cells were treated with 1000 ng/mL leptin for 24 h, and then with either 10 μg/mL trastuzumab or 0.1 μM lapatinib for an additional 72 h. Cells were trypsinized, stained with trypan blue, and counted under a microscope. The percentage of viable cells is shown relative to untreated cells (**Figure 20**). Error bars represent standard deviation between duplicate cultures. These results indicate that the antagonistic effects of leptin on drug response may be specific for docetaxel, as significant effects were not observed with trastuzumab or lapatinib. As another comparison, we tested the effects of another adipocyte-secreted factor called GDF15 on response to docetaxel. HCC1806 ER-negative breast cancer cells were treated with 1nM, 10nM, or 100nM docetaxel in the absence or presence of

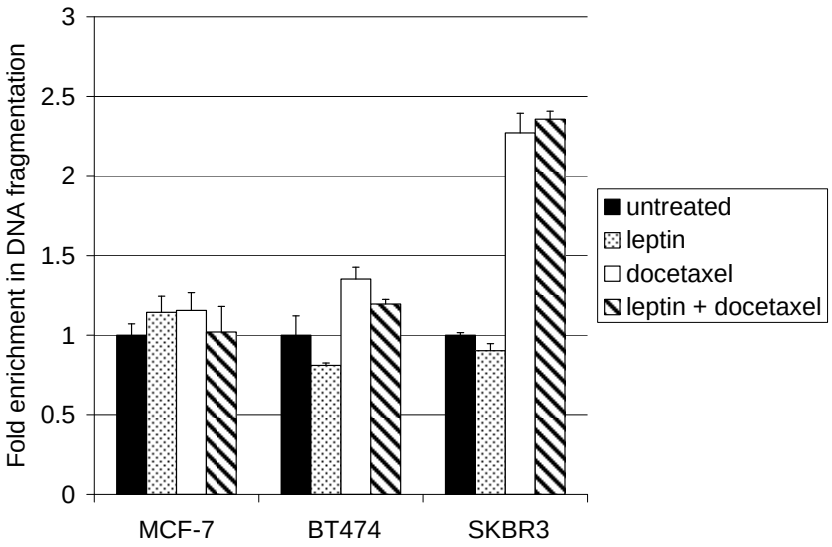

Figure 12. Effect of leptin on taxane-stimulated DNA fragmentation. MCF7, BT474, and SKBR3 cells were stimulated with leptin (100 ng/mL) for 12 h (overnight). Docetaxel (5nM) was then added for 24 h. Cell lysates were examined for DNA fragmentation as a measure of apoptosis (Cell Death ELISA Plus, Roche). Fold enrichment in DNA fragmentation using untreated cells as a control is shown for each cell line. Leptin did not inhibit apoptosis. However, docetaxel did not induce significant apoptosis except for a 2-fold increase in SKBR3 cells. Hence, the experimental design may need to be improved to accurately test our hypothesis. We will examine different doses and time points and use the other assays for measuring apoptosis described in the original proposal.

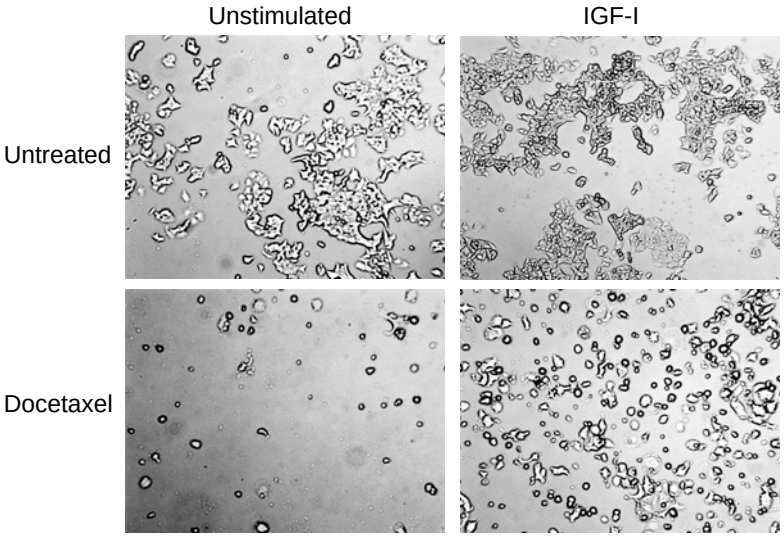

Figure 13. Effect of IGF-I on cytotoxicity of docetaxel. MCF7 cells were treated with docetaxel (10 nM) in the absence or presence of 100ng/mL IGF-I. Media was changed every 24 hours for 1 week. Representative photographs are shown.

100ng/mL recombinant human GDF15. After 6 days, proliferation was measured using the MTS colorimetric assay (Promega). A dose of 10nM docetaxel resulted in 50% inhibition of proliferation, which was not altered by co-treatment with GDF15 (**Figure 21**). Thus, whereas leptin reduced response to docetaxel, another obesity-associated cytokine did not show similar effects on docetaxel sensitivity.

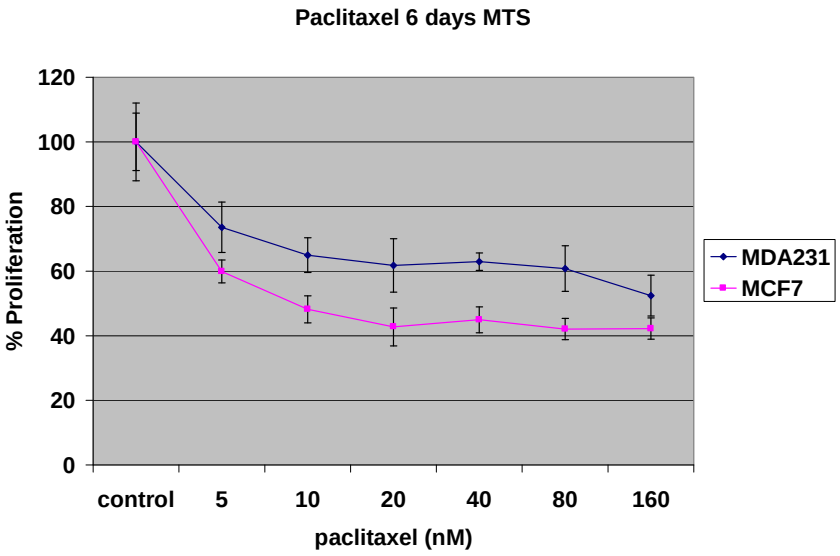

Figure 14. Dose-response to paclitaxel. MCF7 and MDA231 cells were treated with 2-fold serial dilutions of paclitaxel ranging from 5nM to 160nM for 6 days in 6 replicates per drug concentration. MTS assays were then performed and % proliferation is shown relative to control. Control reflects cells treated with the paclitaxel solvent DMSO at amount present in the highest concentration of paclitaxel (160 nM).

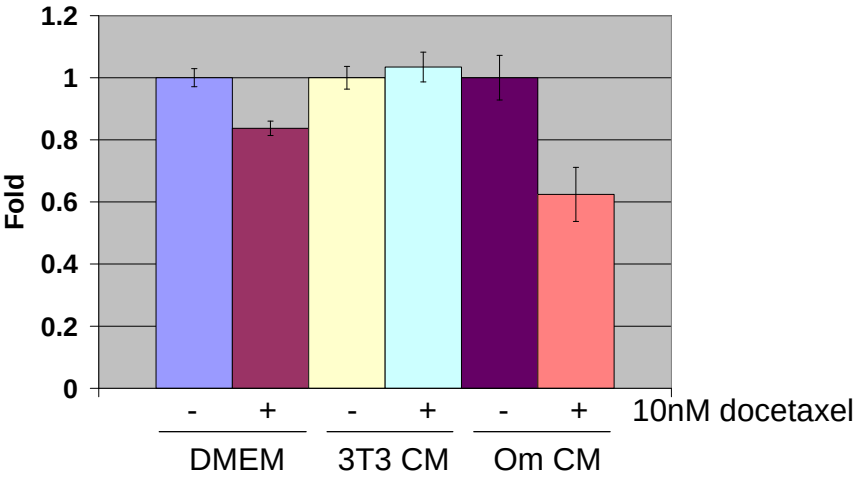

Figure 15. Response of MCF7 cells to docetaxel in adipocyte conditioned media. MCF7 cells were plated in DMEM + 10% FCS (regular complete media), conditioned media (CM) from mouse 3T3 adipocytes, or CM from human abdominal omental cells (Om). Cells were untreated or treated for 6 days in 6 replicates per group with 10 nM docetaxel. MTS assays were performed and % proliferation is shown relative to DMSO control.

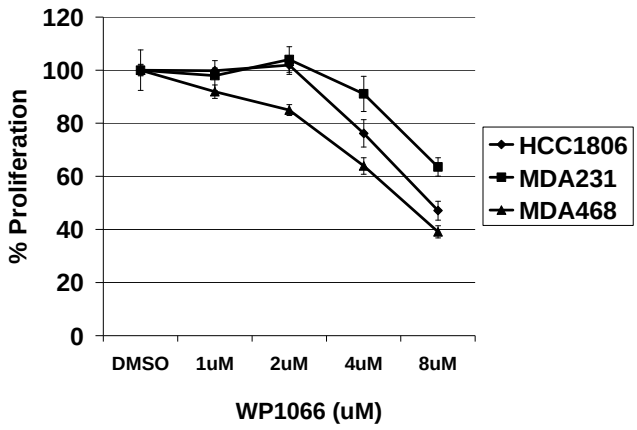

Figure 16. Dose-response to JAK2/STAT3 inhibitor WP1066. HCC1806, MDA231, and MDA468 cells were treated with 2-fold serial dilutions of WP1066 ranging from 1uM to 8uM for 6 days in 6 replicates per drug concentration. MTS assays were then performed and % proliferation is shown relative to DMSO control.

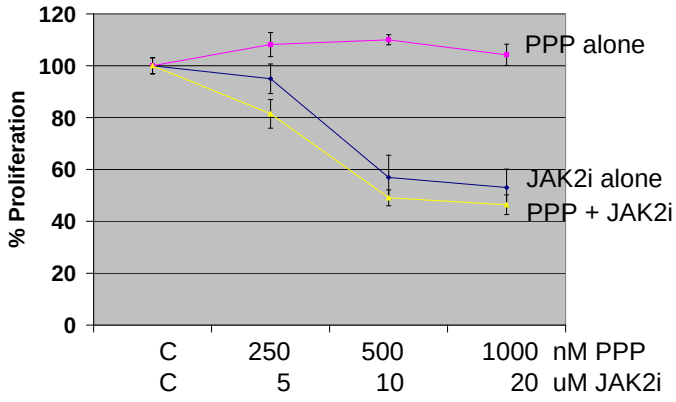

Figure 17. Effect of combined inhibition of IGF-IR and JAK2 on MCF7 cell proliferation. MCF7 cells were treated for 6 days in 6 replicates per group with DMSO (C), IGF-IR inhibitor PPP ranging from 250nM to 1000nM, JAK2 inhibitor ranging from 5uM to 20uM, or a combination of the two inhibitors. MTS assays were performed and % proliferation is shown relative to DMSO control.

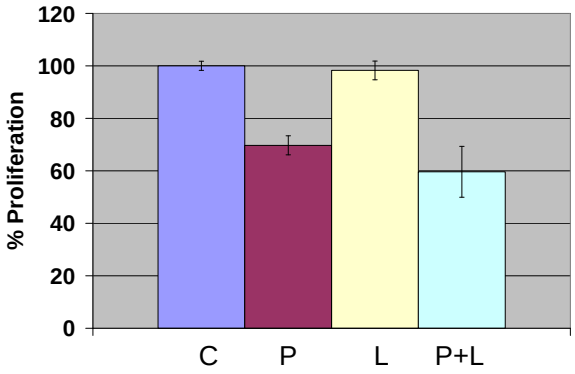

Figure 18. Leptin mutant did not inhibit MCF7 cell proliferation. MCF7 cells were treated for 6 days in 6 replicates per group with DMSO (C), 250 nM IGF-IR inhibitor PPP (P), 2.5 ug/mL recombinant human leptin mutant (L), or a combination of P+L. MTS assays were performed and % proliferation is shown relative to DMSO control.

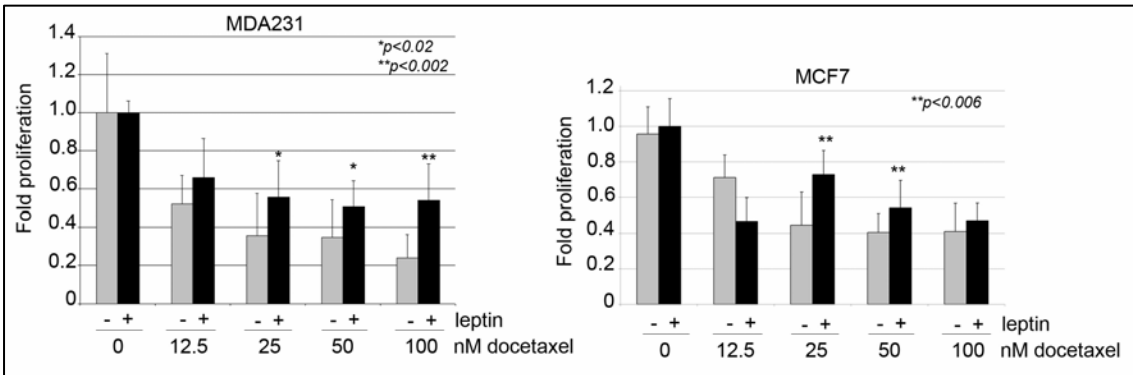

Figure 19. Leptin reduces response to docetaxel in breast cancer cells.

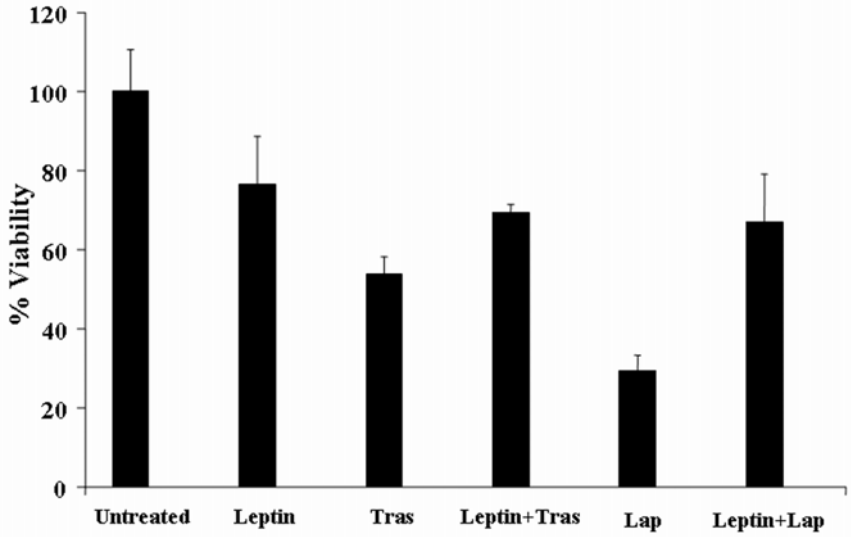

Figure 20. Leptin does not significantly alter response to HER2-targeted therapies.

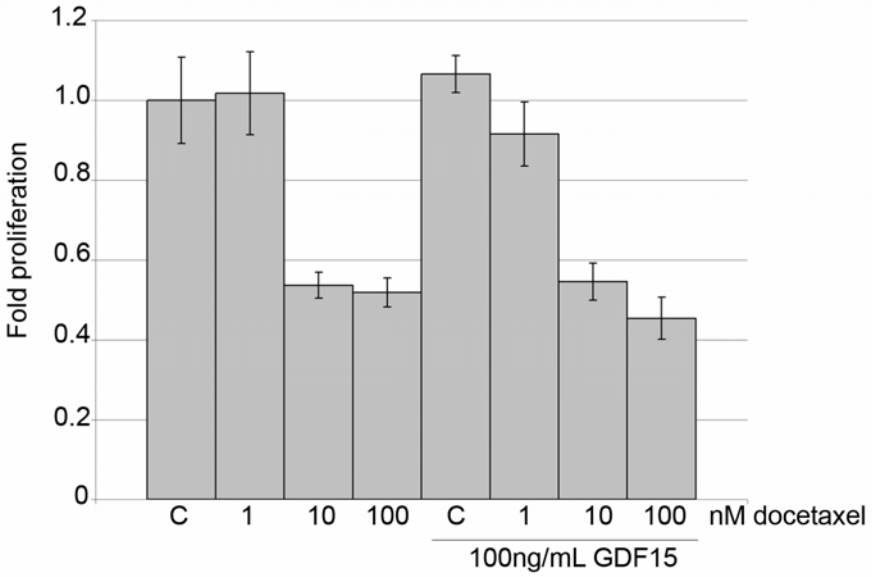

Figure 21.Adipocytokine GDF15 does not reduce response to docetaxel in HCC1806 breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

Key accomplishments during the entire funding period of this project were:

(1) Discovery that IGF-IR and leptin receptor interact [published in ref. 22]
(2) Discovery that IGF-I induces leptin receptor phosphorylation [published in ref. 22]
(3) Discovery that IGF-IR kinase inhibition blocks signaling to leptin receptor [published in ref. 22]
(4) Discovery that IGF-IR cross talk to Ob-R is unidirectional [published in ref. 22]
(5) Potential role of Src kinase as mediator of IGF-IR cross talk to Ob-R
(6) Finding that insulin stimulates phosphorylation of Ob-R
(7) Demonstration that IGF-I reduces toxicity of docetaxel in breast cancer cell cultures
(8) Paclitaxel dose-response curves were established for a panel of breast cancer cell lines.
(9) Finding that adipocyte conditioned media reduces docetaxel dose-response, demonstrating that adipocytes may reduce response to docetaxel.
(10) JAK2 inhibition produced a dose-dependent decline in MCF7 cell proliferation.
(11) Studies suggested that combined inhibition of IGF-IR and JAK2 were not beneficial, but that single agent inhibition of IGF-IR or JAK2 may be effective.
(12) Data suggested that leptin specifically reduces sensitivity to docetaxel, and not to targeted therapies trastuzumab or lapatinib. Further, an unrelated obesity-associated cytokine did not reduce docetaxel sensitivity, suggesting that chemoresistance may be specifically induced by leptin and not all adipocytokines.

REPORTABLE OUTCOMES

Receipt of this DoD Award as an indication of expertise in the area of breast cancer endocrinology and drug resistance has contributed to several opportunities for the PI.

(1) The research findings were published. The citation (listed as *in press* in first annual report, and has since been published) is as follows: Ozbay T, Nahta R. (2008) A novel unidirectional cross talk from the insulin-like growth factor-I receptor to the leptin receptor in human breast cancer cells. Mol Cancer Res 6: 1052-1058.
(2) The PI has been invited to serve on several grant review study sections: Department of Defense / CDMRP Breast Cancer Research Program (BCRP) Concept, IDEA, and Post-doc review panels, 2008-2010; 2010 New Zealand Breast Cancer CURE Research Trust grant reviewer; 2011 The Mary Kay Foundation Research Review Committee
(3) The project funded by this IDEA Award was chosen as for an oral symposium at the DoD BCRP 2008 Era of Hope meeting. The PI presented the results of this grant in the "Modifiable risk factors" session of that meeting.
(4) The PI has become an Editorial Board Member for several scientific journals (*International Scholarly Research Network (ISRN) Oncology*, *Current Pharmacogenomics and Personalized Medicine*, *Medical Hypotheses*, *Breast Cancer: Basic and Clinical Research*, and Guest Editor for *International Journal of Breast Cancer*).
(5) In addition, start-up funding has been received from Georgia Cancer Coalition Distinguished Scholars Program, and grant funding was received from The Mary Kay Foundation for a project unrelated to this IDEA Award. The honor of receiving the IDEA Award and the established funding record of the PI may have positively influenced selection of the PI for receipt of these other awards.
(6) In addition, because of her recognized expertise in the area of breast cancer drug resistance, the PI was invited to speak at the National Surgical Adjuvant Breast and Bowel Project (NSABP) Foundation Research Program Investigators Meeting on 10/09/2010 in Las Vegas, where she spoke about Mechanisms of Herceptin resistance.

CONCLUSION

As published in reference 22, we made the following discoveries. (1) The IGF-I and leptin receptors interact in human breast cancer cells. (2) Cross signaling occurs from IGF-IR to Ob-R in breast cancer. IGF-I stimulation induces phosphorylation and activation of Ob-R, while IGF-IR kinase inhibition blocks IGF-I-mediated Ob-R activation. Downstream signaling molecules JAK2, STAT3, Akt, and ERK1/2, all of which are functional in the leptin and IGF-IR pathways as well as in multiple other signaling pathways, were activated by IGF-I stimulation. (3) Cross talk is unidirectional, as leptin does not activate IGF-IR. Thus, leptin is not likely to affect IGF-IR oncogenic function in breast cancer. However, since IGF-IR cross talks to Ob-R, it is feasible that Ob-R may contribute to IGF-IR molecular or biological effects, and is worthy of further study. Thus, we have identified a novel receptor interaction and unidirectional cross talk involving the IGF-IR and leptin receptor. As discussed in a previous annual report, we have also shown the following. (4) Insulin appears to stimulate Ob-R phosphorylation as well, although inhibition of insulin receptor does not block IGF-I-mediated Ob-R phosphorylation. Insulin is known to bind and activate IGF-IR; thus, insulin may be activating Ob-R via IGF-IR. (5) Adipocyte-secreted factors found in conditioned media collected from adipocytes reduced response to taxanes. Furthermore, MCF7 cells appeared to be dependent upon JAK2 signaling, which is downstream of leptin receptor and potentially upregulated during obesity. Thus, JAK2 inhibition downstream of leptin receptor may be a potential strategy for combating obesity-associated breast cancer and possibly for improving chemosensitivity of obesity-associated breast cancers. As discussed in this report, the following additional conclusions are made. (6) Leptin and not another adipocytokine (GDF15) appeared to specifically reduce response to docetaxel, with no effects on sensitivity to HER2-targeted therapies. Thus, leptin appears to play a potential role in mediating taxane resistance in breast cancer.

All final reports must include a bibliography of all publications and meeting abstracts and a list of personnel (not salaries) receiving pay from the research effort.

Publications:

Ozbay T, Nahta R. (2008) A novel unidirectional cross talk from the insulin-like growth factor-I receptor to the leptin receptor in human breast cancer cells. Mol Cancer Res 6: 1052-1058.

Meeting Abstract:

June 2008, Invited speaker, Department of Defense Breast Cancer Research Program Era of Hope Meeting, Baltimore, MD, oral presentation: “A novel unidirectional cross talk from the insulin-like growth factor-I receptor to the leptin receptor in human breast cancer cells”

List of Personnel:

Rita Nahta, Ph.D., PI of grant
Jayashree Joshi, Post-doc

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APPENDICES

Reference [22] was a direct result of this Award and is attached.

SUPPORTING DATA

N/A

A Novel Unidirectional Cross-Talk from the Insulin-Like Growth Factor-I Receptor to Leptin Receptor in Human Breast Cancer Cells

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Departments of [1]Pharmacology and [2]Hematology/Oncology, School of Medicine, [3]Winship Cancer Institute, and [4]Molecular and Systems Pharmacology Program, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, Georgia

Abstract

Obesity is a major risk factor for the development and progression of breast cancer. Increased circulating levels of the obesity-associated hormones leptin and insulin-like growth factor-I (IGF-I) and overexpression of the leptin receptor (Ob-R) and IGF-I receptor (IGF-IR) have been detected in a majority of breast cancer cases and during obesity. Due to correlations between increased leptin, Ob-R, IGF-I, and IGF-IR in breast cancer, we hypothesized that molecular interactions may exist between these two signaling pathways. Coimmunoprecipitation and immunoblotting showed that IGF-IR and Ob-R interact in the breast cancer cell lines MDA-MB-231, MCF7, BT474, and SKBR3. Stimulation of cells with IGF-I promoted Ob-R phosphorylation, which was blocked by IGF-IR kinase inhibition. In addition, IGF-I activated downstream signaling molecules in the leptin receptor and IGF-IR pathways. In contrast to IGF-I, leptin did not induce phosphorylation of IGF-IR, indicating that receptor cross-signaling is unidirectional, occurring from IGF-IR to Ob-R. Our results show, for the first time, a novel interaction and cross-talk between the IGF-I and leptin receptors in human breast cancer cells.
(Mol Cancer Res 2008;6(6):1052–8)

Background

Obesity is an important and manageable risk factor for the development and progression of postmenopausal breast cancer (1). Increased body weight and body mass index are associated with reduced disease-free and overall survival and poorer therapeutic response rates in breast cancer patients, regardless of menopausal status or age (2). Although the exact molecular mechanisms by which obesity influences cancer biology are unknown, there is evidence suggesting that increased production and secretion of adipocyte-derived growth factors and hormones contributes to cellular transformation and tumorigenesis (3, 4). The obesity-associated hormones leptin and insulin-like growth factor-I (IGF-I) have been independently implicated in the connection between obesity and breast cancer (5).

Leptin, a product of the obese (*ob*) gene, is an adipocytokine that regulates appetite, bone formation, reproduction, cellular proliferation, and angiogenesis (6). Because of the strong association between human obesity and elevated levels of circulating leptin, this hormone has been widely studied in the fields of nutrition and weight management (7). More recently, however, leptin has emerged as a potential factor contributing to mammary tumorigenesis. *In vitro* studies showed that leptin stimulates the growth, survival, and transformation of breast cancer cells (5), primarily by activating the Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathway (8, 9) and the phosphoinositol-3-kinase/Akt and mitogen-activated protein kinase (MAPK) pathways (10). Leptin induces cell cycle progression by up-regulating cyclin D1 expression and cyclin-dependent kinase 2 activity, as well as by inactivating the retinoblastoma growth suppressing protein (11). Importantly, leptin and its receptor (Ob-R) were found to be overexpressed in a majority of breast cancer tissues, especially in high-grade tumors, but absent or expressed at very low levels in normal mammary epithelium or benign tumors (5, 12). In addition, leptin-deficient mice have a decreased incidence of spontaneous and oncogene-induced mammary tumors (13). Thus, leptin signaling seems to play an important role in breast cancer biology.

Similar to leptin, increased levels of IGF-I and its receptor are detected in sera and primary tumors of breast cancer patients (14, 15), and transgenic overexpression of IGF-I receptor (IGF-IR) has been shown to induce mammary tumor formation (16). IGF-I is an important endocrine, paracrine, and autocrine regulator of breast epithelial cell growth. Increased signaling through the IGF-IR results in increased cellular proliferation, mitogenesis, and survival and decreased apoptosis, causing resistance to numerous antineoplastic agents (14, 17). For these reasons, the IGF-IR has become an important therapeutic target for drug discovery in breast oncology (17).

Cross-talk between different growth factor receptor families is frequently observed in tumors. This mechanism allows cancer cells to enhance downstream signaling resulting in greatly increased proliferation, mitogenesis, and cell survival.

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The IGF-IR has been shown to interact and cross-talk with multiple receptors, including the epidermal growth factor receptor (EGFR; ref. 18), HER2 (19), platelet-derived growth factor receptor (20), and the estrogen receptor (14). Due to the correlations between elevated levels of leptin, IGF-I, and their associated receptors with obesity and breast cancer, we hypothesized that interactions and/or cross-talk may occur between these two signaling pathways.

Results

IGF-IR and Leptin Receptor Interact in Human Breast Cancer Cells

The human breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were examined for expression of the IGF-IR and leptin receptor (Ob-R). Immunoblotting of total protein lysates (Fig. 1A) showed that the two major isoforms of Ob-R, called Ob-Rb (longer isoform) and Ob-Rt (shorter isoform), are expressed at similar levels in all cell lines (Fig. 1B). IGF-IR is expressed at higher levels in MCF7 and BT474 cells versus SKBR3 and MDA231 cells, with highest levels observed in MCF7 cells (Fig. 1B).

Immunoprecipitation of Ob-R with subsequent immunoblotting for IGF-IR showed that Ob-Rb and Ob-Rt are both pulled down with IGF-IR in all four cell lines (Fig. 2A). Conversely, IGF-IR immunoprecipitation pulled down Ob-Rb and Ob-Rt in each cell line, with preferential interaction observed with the shorter isoform of Ob-R in MCF7, BT474, and SKBR3 cells (Fig. 2B). Quantitation showed that IGF-IR was pulled down with Ob-R to a similar extent in all four lines (Fig. 2C). Total Ob-R was pulled down with IGF-IR in all four lines; however, higher levels of Ob-R interacting with IGF-IR was observed in MCF7 cells (Fig. 2C), likely due to the higher expression level of total IGF-IR in these cells (Fig. 1B). Negative controls in which cell lysates were immunoprecipitated with rabbit IgG confirmed that IGF-IR and Ob-R were not pulled down (Fig. 2D). In addition, because IGF-IR has been shown to interact with insulin receptor (21), we blotted IGF-IR immunoprecipitates for insulin receptor as a positive control (Fig. 2D). Insulin receptor was pulled down with IGF-IR in all four lines. Finally, another tyrosine kinase receptor, EGFR, was immunoprecipitated and blotted for Ob-R in all lines (Fig. 2D). Collectively, the results of these immunoprecipitation experiments indicate that the IGF-IR and leptin receptor interact in human breast cancer cells.

IGF-IR Cross-Signals to the Leptin Receptor

To determine the effect of IGF-IR/leptin receptor interaction on receptor signaling, MCF7 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for up to 1 hour. IGF-IR phosphorylation was induced within 5 minutes (Fig. 3A), while total IGF-IR levels were unaltered. Importantly, phosphorylation of Ob-R was also induced within 5 minutes of IGF-I exposure, suggesting potential cross-signaling from IGF-IR to leptin receptor. Similarly, in BT474 cells (Fig. 3B) and MDA231 cells (Fig. 3C), IGF-I stimulation induced phosphorylation of both IGF-IR and Ob-R within 5 minutes, without affecting total levels of either receptor. To determine if IGF-I stimulates phosphorylation of the leptin receptor via the IGF-IR kinase, MCF7 cells were treated with the IGF-IR kinase inhibitor I-OMe-AG538 and stimulated with IGF-I (Fig. 3D). Immunoblotting showed that inhibition of IGF-IR kinase blocked IGF-I–stimulated phosphorylation of leptin receptor. Thus, IGF-I cross-signals to the leptin receptor via the IGF-IR kinase.

Having established that IGF-IR stimulates phosphorylation of the leptin receptor, we examined IGF-I–mediated effects on downstream receptor signaling. MCF7 cells were stimulated with IGF-I and immunoblotted for phosphorylated and total JAK2 and STAT3 (Fig. 4A) and for phosphorylated and total Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 MAPK (Fig. 4B). Significant phosphorylation of JAK2 and STAT3 was observed in response to IGF-I within 5 minutes. IGF-I also activated the phosphoinositol-3-kinase pathway, as shown by phosphorylation of Akt. Phosphorylation of ERK1/2 and p38 MAPK was rapidly activated by

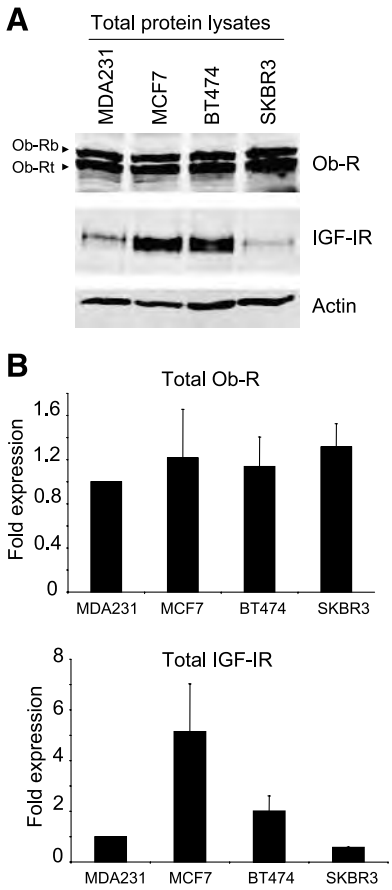

FIGURE 1. Expression of IGF-IR and Ob-R in breast cancer lines. The breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were lysed for total protein. **A.** Total protein lysates were immunoblotted for Ob-R using the H-300 polyclonal antibody, which recognizes both the long Ob-Rb isoform and the shorter Ob-Rt isoform of the leptin receptor. Immunoblotting was also done for total IGF-IR and for actin as a loading control. **B.** Bands on immunoblots were quantitated using NIH ImageJ and are expressed relative to expression levels in MDA231 cells (*lane 1*). Error bars, SD between three independent experiments. Total Ob-R levels were similar among the four lines; IGF-IR was expressed at the highest level in MCF7 cells, with BT474 cells showing moderate expression compared with the other two lines which expressed the lowest levels of IGF-IR.

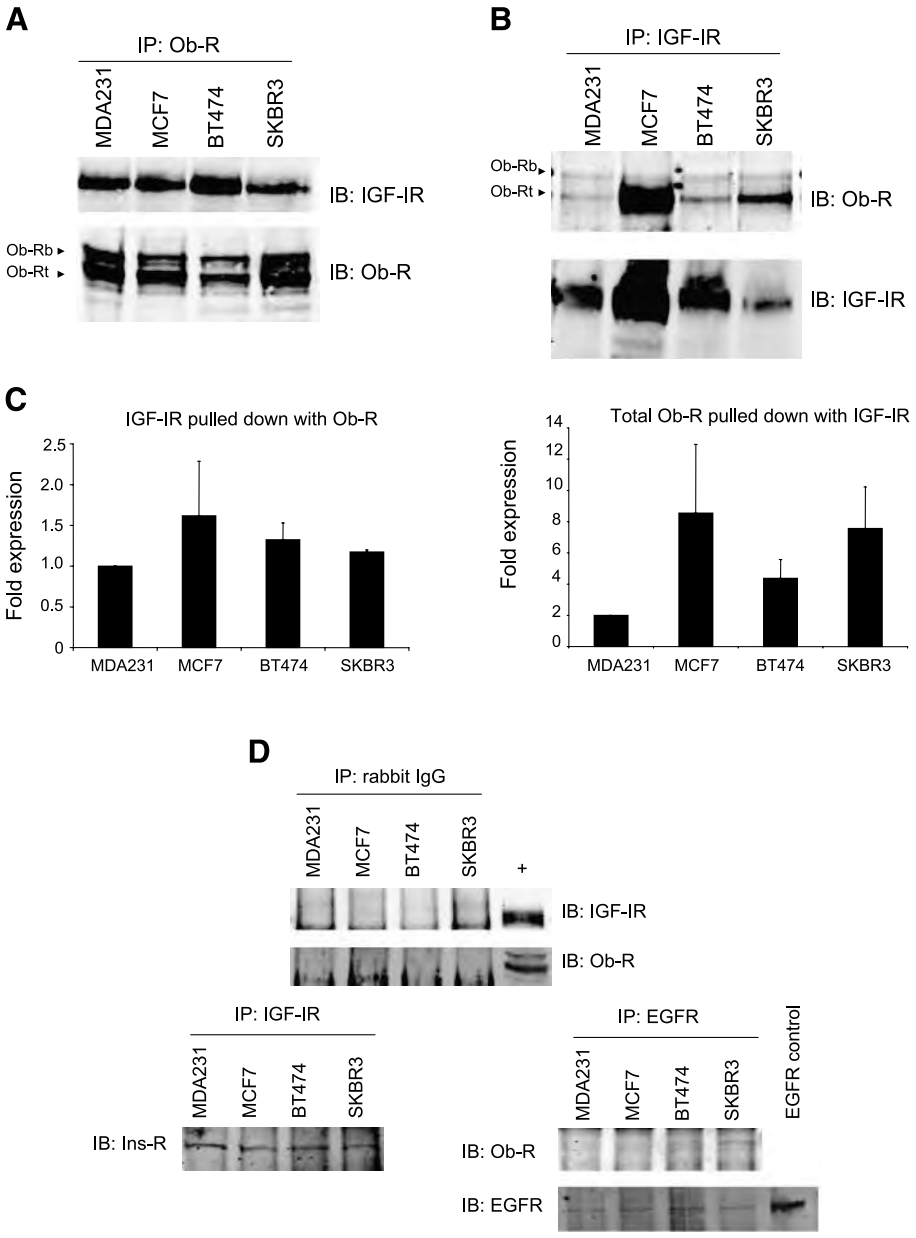

FIGURE 2. Interaction between IGF-IR and Ob-R in breast cancer. The breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were lysed for total protein. Ob-R (**A**) and IGF-IR (**B**) were immunoprecipitated (*IP*; 1 μg of antibody) from total protein extracts (200 μg) and immunoblotted (*IB*) to detect IGF-IR and Ob-R. Ob-R immunoprecipitation pulled down IGF-IR; conversely, IGF-IR immunoprecipitation pulled down Ob-R. **C.** Quantitation of immunoprecipitated experiments. Error bars, SD between three independent experiments. Values were normalized to the MDA231 cells (*lane 1*). **D.** Cell lysates were immunoprecipitated using 1 μg rabbit IgG and immunoblotted for IGF-IR and Ob-R as a negative control. On IGF-IR blot, total lysate from MCF7 cells is included as a positive control (+) for the antibody; on Ob-R blot, lysate from COLO320DM cells was purchased as a positive control (+) for the H-300 antibody from Santa Cruz. As a positive immunoprecipitated control, cell lysates were immunoprecipitated with IGF-IR antibody and blotted for insulin receptor, which is known to interact with IGF-IR. EGFR tyrosine kinase receptor was also immunoprecipitated and blotted for Ob-R, with MDA231 total cell lysate added as a positive control for EGFR. Our results show that the IGF-IR and leptin receptor form a protein complex in breast cancer cells.

transient versus other signaling pathways. Collectively, these results support the concept that IGF-I cross-activates the leptin receptor signaling pathway, although the signaling molecules examined are downstream of multiple growth factor receptors and, thus, do not strictly confirm activation of leptin receptor signaling. However, as leptin receptor phosphorylation was induced by IGF-I and blocked by IGF-IR kinase inhibitor on Tyr^{1141}, which is the phosphorylation site that binds STAT3 and activates downstream signaling, our results strongly suggest that IGF-IR induces activation of the leptin receptor.

IGF-IR/Leptin Receptor Cross-Talk Is Unidirectional

We next examined whether cross-talk occurs in the opposite direction, i.e., from the leptin receptor to IGF-IR. MCF7 cells were serum starved and stimulated with leptin (1,000 ng/mL) for up to 6 hours. Leptin induced phosphorylation of leptin receptor within 5 minutes (Fig. 5A). However, phosphorylation of IGF-IR at either Tyr^{1131} or $Tyr^{1135/1136}$ was not stimulated by leptin at these time points of up to 6 hours nor was it stimulated at shorter time point increments or longer time points of up to 24 hours or with lower doses of leptin (not shown). As a positive control, IGF-I stimulated phosphorylation of IGF-IR as expected and also induced phosphorylation of leptin receptor as previously observed (Fig. 3). Similarly, BT474 cells stimulated with leptin showed phosphorylation of leptin receptor but not of IGF-IR at either of the three sites examined (Tyr^{1131}, Tyr^{1135}, and Tyr^{1136}; Fig. 5B). Thus, our results suggest a unidirectional cross-talk from the IGF-IR to the leptin receptor in breast cancer cells.

Discussion

Epidemiologic studies estimate that obesity increases the risk of breast cancer by up to 50% (3). The molecular mechanisms guiding obesity-associated breast cancer are not well understood, but are likely to involve an increased production and secretion of obesity-associated hormones (22). IGF-I and leptin are capable of regulating mammary tissue growth at multiple levels (5). Both hormones are secreted by abdominal adipocytes, resulting in endocrine effects on various tissues, including the breast. Paracrine growth stimulatory effects occur via IGF-I and leptin released by the adipocyte component of stroma surrounding breast epithelial cells or existing breast tumor cells. In addition, an autocrine signaling component is present as breast cancer cells themselves produce and secrete IGF-I and leptin and express cell surface receptors for both ligands. Thus, IGF-I and leptin represent a molecular link between adipose tissue and mammary tissue.

The IGF-IR and Ob-R signaling pathways have each been independently implicated in the development and progression of breast cancer. High circulating levels of IGF-I have been associated with an increased risk of developing breast cancer, and patients with existing breast cancer expressed high serum levels of IGF-I (17). In addition, transgenic mouse models overexpressing IGF-I, IGF-II, or IGF-IR showed an increased incidence of mammary tumor formation (16, 17, 23, 24). Conversely, liver-specific depletion of IGF-I caused reduced circulating levels of IGF-I in mice, resulting in diminished IGF-I endocrine effects on mammary tissue and, ultimately, reduced incidence of breast tumors (25). Similar to the IGF-I signaling pathway, leptin signaling has been associated with breast cancer. Leptin and its receptor were shown by immunohistochemistry to be overexpressed in primary and metastatic breast cancers relative to noncancer tissues (5). Expression of both leptin and Ob-R was most abundant among high-grade tumors, supporting a role for this pathway in breast cancer progression. In addition, *in vivo* models showed that whereas mice that overexpress transforming growth factor-α developed mammary tumors, leptin-deficient transforming growth factor-α

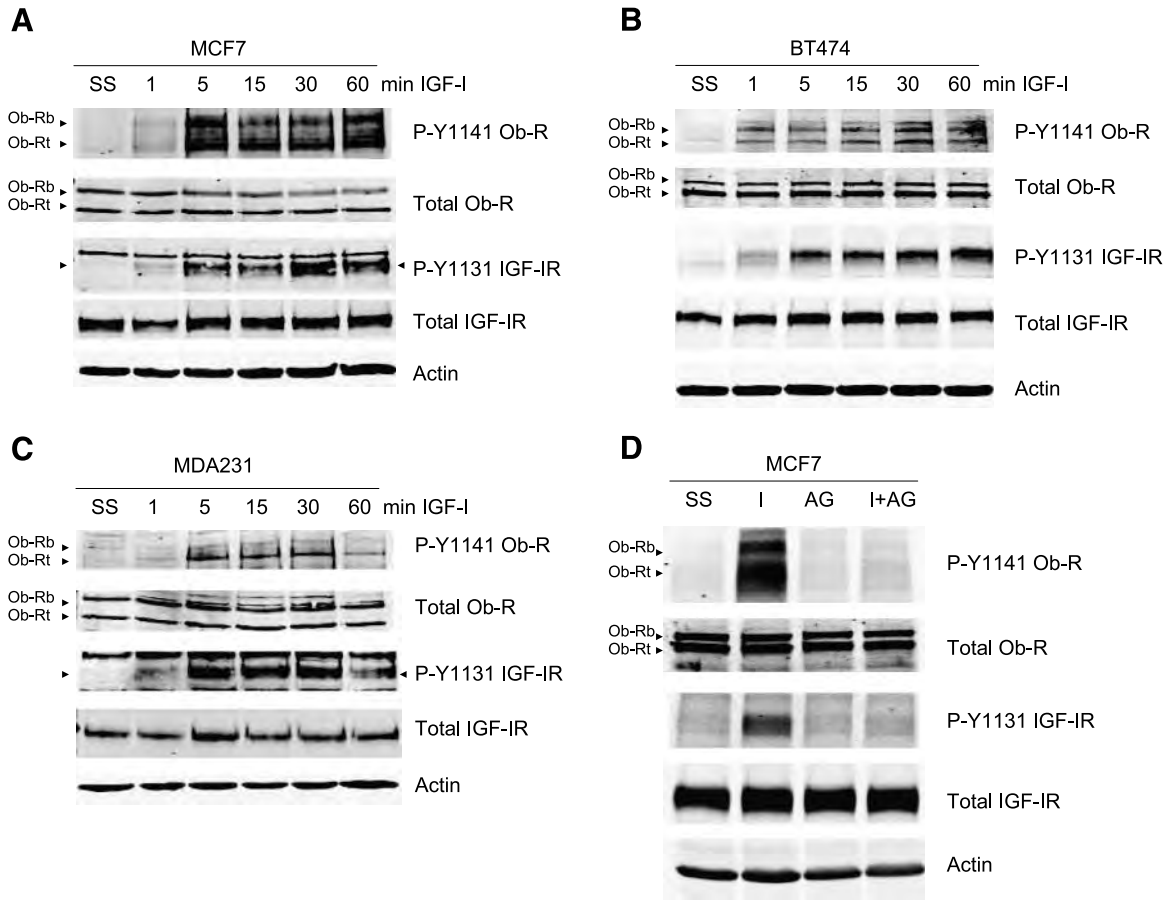

FIGURE 3. Evidence of cross-talk from IGF-IR to Ob-R. IGF-I induces phosphorylation of Ob-R, which is blocked by IGF-IR kinase inhibition. MCF7 (**A**), BT474 (**B**), and MDA231 (**C**) cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min. Cells were lysed for protein, and total protein extracts (50 μg) were immunoblotted (SS, serum-starved control) for p-Y^{1141}-Ob-R (phosphorylated Tyr^{1141} on leptin receptor), total Ob-R, p-Tyr^{1131} IGF-IR (phosphorylated Tyr^{1131} on IGF-IR), IGF-IR β, and actin as a loading control. IGF-I stimulated phosphorylation of IGF-IR within 5 min in all cell lines. Importantly, phosphorylation of the leptin receptor was also induced within 5 min of IGF-I exposure. Total receptor levels did not change. **D.** MCF7 cells were serum-starved overnight, then stimulated with IGF-I (100 ng/mL) for 5 min, and/or treated with the IGF-IR kinase inhibitor I-OMe-AG538 (10 μmol/L overnight). Total protein was immunoblotted for p-Y^{1141}-Ob-R, total Ob-R, p-Tyr^{1131} IGF-IR, and total IGF-IR. Experiments were done at least twice. Inhibition of IGF-IR kinase blocked IGF-I–mediated phosphorylation of leptin receptor, supporting cross-talk from the IGF-IR kinase to leptin receptor. SS, serum-starved control; I, IGF-I; AG, I-OMe-AG538; I + AG, IGF-I + I-OMe-AG538.

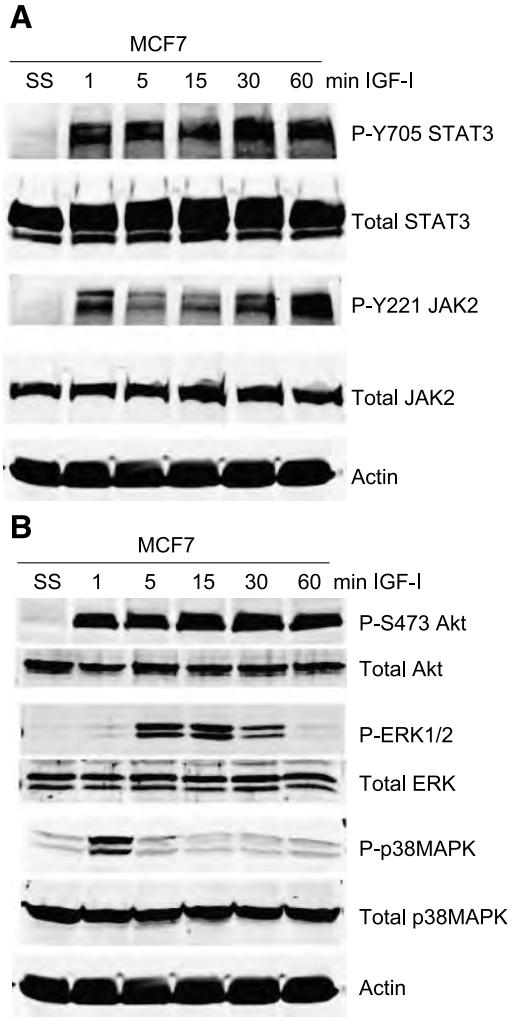

FIGURE 4. IGF-I activates downstream signaling. MCF7 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min. Total protein extracts (50 μg) were immunoblotted for the downstream leptin signaling molecules p-STAT3 (Tyr^{705}), total STAT3, p-JAK2 (Tyr^{221}), and total JAK2 (24^{B11}) (**A**) and for molecules downstream of both leptin receptor and IGF-IR, p-Akt (Ser^{473}), total Akt, p-p42/p44 MAPK (Thr^{202}/Tyr^{204}; ERK1/2), total p42/p44 MAPK (ERK1/2), p-p38 MAPK ($pThr^{180}/Tyr^{182}$), and total p38 MAPK (**B**). IGF-I induced phosphorylation of STAT3 and JAK2, consistent with IGF-I–mediated activation of leptin signaling, and also activated Akt, ERK1/2, and p38 MAPK signaling. Since the same lysates were used in **A** and **B**, the same actin blot is shown.

mice were resistant to mammary tumor development (13), illustrating the important contribution of the leptin signaling pathway to some forms of breast cancer. Hence, because IGF-I and leptin are frequently detected in the serum of breast cancer patients and both receptors are overexpressed in a majority of breast tumors, we sought to determine whether molecular interactions occur between IGF-IR and leptin receptor in breast cancer.

We showed the following novel findings (Fig. 6):

(*a*) The IGF-I and leptin receptors interact in human breast cancer cells. Of potential interest, IGF-IR may preferably associate with Ob-Rt versus Ob-Rb in MCF7, BT474, and SKBR3 cells, as more of this isoform was pulled down in the IGF-IR immunoprecipitates (Fig. 2B); total levels of both Ob-R isoforms were similar in each line (Fig. 1A).

(*b*) Cross-signaling occurs from IGF-IR to Ob-R in breast cancer. IGF-I stimulation induces phosphorylation and activation of Ob-R, whereas IGF-IR kinase inhibition blocks IGF-I–mediated Ob-R activation. Downstream signaling molecules examined included JAK2, STAT3, Akt, and ERK1/2, all of which are functional in the leptin and IGF-IR pathways, as well as in multiple other signaling pathways. Thus, the IGF-I signaling experiments do not strictly indicate that IGF-I induces activation of one particular pathway. However, our results clearly indicate that IGF-I induces phosphorylation of Ob-R on Tyr^{1141}. Phosphorylation of Tyr^{1141} is required for Ob-R to bind to the STAT3 transcription factor, which is then activated by JAK2 and translocated to the nucleus to stimulate transcription of downstream target genes (26). Thus, our results indicate that IGF-I activates Ob-R via the IGF-IR kinase.

(*c*) Cross-talk is unidirectional, as leptin does not activate IGF-IR. Whereas it is feasible that other phosphorylation sites on IGF-IR may be affected by leptin stimulation, the three sites examined here (Tyr^{1131} and $Tyr^{1135/1136}$) were not affected by leptin. These three phosphorylation sites are the critical sites known to be required for IGF-IR mitogenicity and transforming activity (27). Thus, the inability of leptin to induce phosphorylation at these sites suggests that the leptin hormone alone is not likely to affect IGF-IR oncogenic function in breast cancer. However, because IGF-IR cross-talks to Ob-R, it is feasible that Ob-R may contribute to IGF-IR molecular or biological effects and is worthy of further study.

Thus, we have identified a novel receptor interaction and unidirectional cross-talk involving the IGF-IR and leptin receptor, which has not been previously described. Interestingly, Garofalo et al. (5) showed that IGF-I can induce leptin transcript levels in MCF7 cells. Our results further support this concept of IGF-I–mediated positive regulation of the leptin pathway.

Cross-talk from IGF-IR to other signaling pathways seems to be a potentially common mechanism used by cancer cells to enhance tumor growth and supports the significance of the IGF-I system to the biology of breast cancer, as well as the relevance of IGF-IR as a therapeutic target. We previously showed that IGF-IR cross-talks to the HER2 cell surface receptor in breast cancer cells that have become resistant to the HER2-targeted agent trastuzumab (19). Others have also shown that IGF-IR is capable of cross-signaling to the EGFR (18) and to the estrogen receptor (14). Thus, understanding the mechanisms by which IGF-IR mediates activation of other growth factor signaling pathways is important to breast cancer research. We have examined the role of the Src kinase family in mediating IGF-IR cross-talk to leptin receptor and have found that Src kinase inhibition does not inhibit IGF-IR/Ob-R cross-talk (not shown). Future studies will examine the molecular mechanisms mediating this receptor cross-talk. In addition, cotargeting leptin receptor and IGF-IR as a strategy to inhibit breast cancer progression, as well as the contribution of leptin receptor to IGF-I–mediated promitogenic and antiapoptotic effects, will be examined in breast cancer cells.

In summary, our results show, for the first time, that the IGF-I and leptin receptors physically form a protein complex in

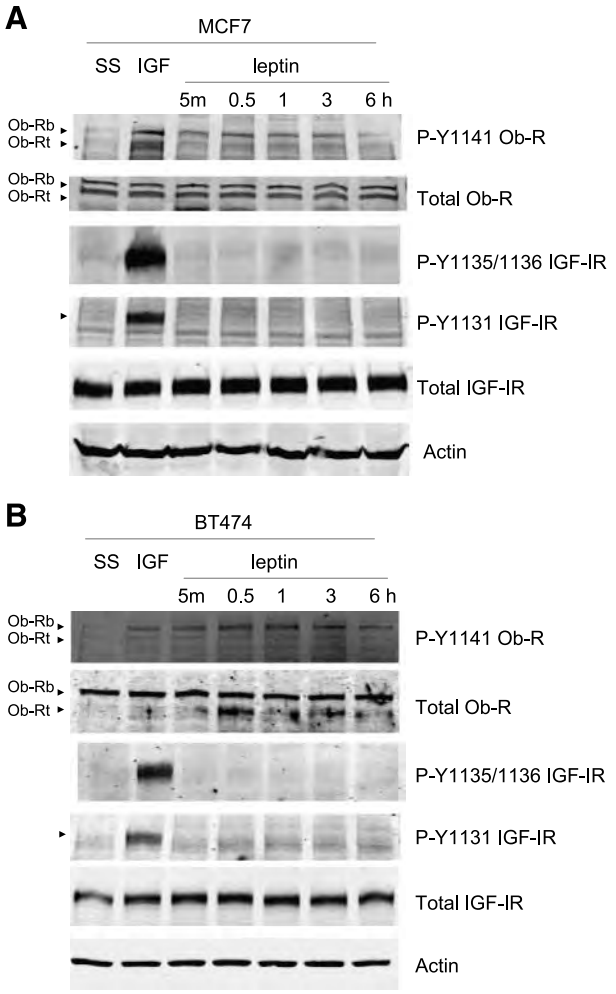

FIGURE 5. Evidence of unidirectional cross-talk. Leptin does not induce phosphorylation of IGF-IR. MCF7 (**A**) and (**B**) BT474 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 5 min or leptin (1,000 ng/mL) for 5 min or 0.5, 1, 3, or 6 h. Cells were lysed for protein, and total protein extracts (50 μg) were immunoblotted (SS, serum-starved control) for p-Y^{1141}-Ob-R, total Ob-R, p-Tyr^{1131} IGF-IR, p-$Tyr^{1135/1136}$ IGF-IR, total IGF-IR β, and actin. Experiments were done at least twice. IGF-I stimulated phosphorylation of IGF-IR and Ob-R within 5 min in both cell lines as expected and served as a positive control. Leptin stimulated phosphorylation of Ob-R in both lines but did not induce phosphorylation of IGF-IR at the phosphorylation sites examined, suggesting that receptor cross-talk is unidirectional, occurring from IGF-IR to Ob-R only.

breast cancer cell lines and, further, that there exists a one-way cross-talk whereby IGF-IR induces phosphorylation and activation of the leptin receptor in breast cancer.

Materials and Methods

Materials

Human recombinant IGF-I (Sigma) was dissolved at 100 μg/mL in PBS and used at 100 ng/mL in culture. Human recombinant leptin (EMD Biosciences) was dissolved at 1 mg/mL in PBS and used at 100 or 1,000 ng/mL. I-OMe-AG538 IGF-IR kinase inhibitor (Sigma) was dissolved at 1 mmol/L in PBS and used at 10 μmol/L in culture.

Cell Culture

MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 breast cancer cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% FCS.

Ligand Stimulation

Cells were serum starved overnight, and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min or leptin (1,000 ng/mL) for 5 min, 0.5 h, 1 h, 3 h, or 6 h. In addition, a subset of cells were serum starved, treated with the IGF-IR kinase inhibitor I-OMe-AG538 (10 μmol/L overnight), and stimulated with IGF-I (100 ng/mL).

Immunoprecipitation

Total protein lysates (200 μg) were incubated with 1 μg of Ob-R or IGF-IR antibody or 1 μg rabbit IgG, rotating for 4 h, followed by addition of protein A/G-agarose (Cell Signaling) and rotating overnight. Beads were then washed thrice in PBS containing 0.1% Tween 20 and immunoblotted to detect Ob-R (H-300, Santa Cruz), IGF-IR (polyclonal, Cell Signaling), EGFR (monoclonal 1F4, Cell Signaling), or insulin receptor β (polyclonal, Cell Signaling). Blots of immunoprecipitations were quantitated using NIH imaging software ImageJ.

Immunoblotting

Cells were lysed in buffer containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease and phosphatase inhibitor cocktails (Sigma). Total protein extracts (50 μg) were immunoblotted using the following antibodies at the indicated dilutions: IGF-IRβ (polyclonal at 1:1,000; Cell Signaling); p-Tyr^{1131}-IGF-IR/Tyr^{1146}-IR (polyclonal at 1:200; Cell Signaling); p-$Tyr^{1135/1136}$-IGF-IR/$Tyr^{1150/1151}$-IR (polyclonal at 1:200; Cell Signaling); leptin receptor (Ob-R; H-300 polyclonal at 1:200; Santa

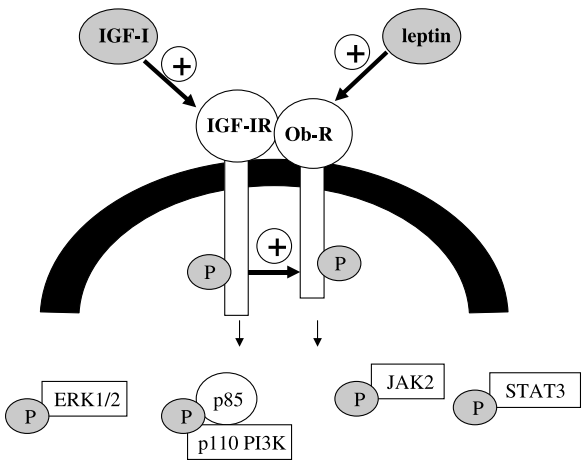

FIGURE 6. A novel unidirectional cross-talk from IGF-IR to Ob-R in breast cancer. Our results indicate that the IGF-I and leptin receptors interact in human breast cancer cells. Furthermore, cross-talk occurs from IGF-IR to Ob-R, such that IGF-I stimulation induces phosphorylation and activation of Ob-R. IGF-IR kinase inhibition blocks IGF-I–mediated Ob-R activation. Cross-talk is unidirectional, as leptin does not activate IGF-IR.

Cruz Biotechnology); p-Y^{1141}-Ob-R (polyclonal at 1:200; Santa Cruz); actin (monoclonal AC-15 at 1:5,000; Sigma Chemical); from Cell Signaling, polyclonal antibodies against p-STAT3 (Tyr^{705}), total STAT3, p-JAK2 (Tyr^{221}), total JAK2 (24B11), total Akt, p-Thr^{202}/Tyr^{204} p42/p44 MAPK (ERK1/2), total p42/p44 MAPK (ERK1/2), p-pThr^{180}/Tyr^{182} p38 MAPK, and total p38 MAPK, monoclonal 587F11 against p-Ser^{473}-Akt, each used at 1:1,000 dilution, and monoclonal 1F4 anti-EGFR used at 1:200 dilution. Secondary antibodies were chosen according to the species of origin of the primary antibody. Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences). Bands were quantitated using NIH imaging software ImageJ.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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