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# 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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## 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

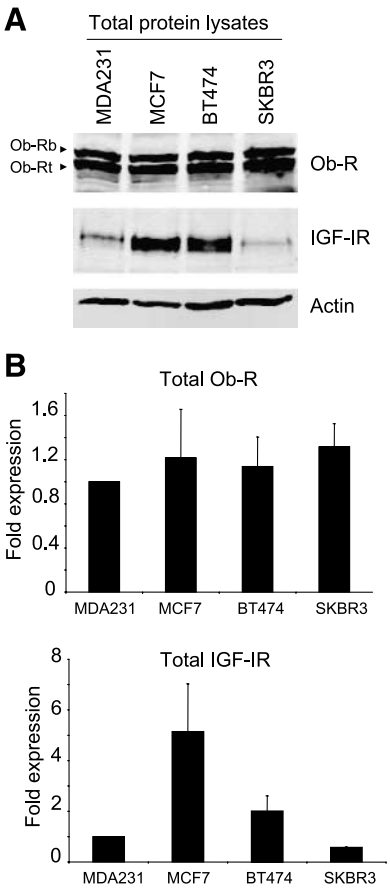


**FIGURE 1.** Expression of IGF-IR and Ob-R in breast cancer lines. The breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were lysed for total protein. **A.** Total protein lysates were immunoblotted for Ob-R using the H-300 polyclonal antibody, which recognizes both the long Ob-Rb isoform 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.

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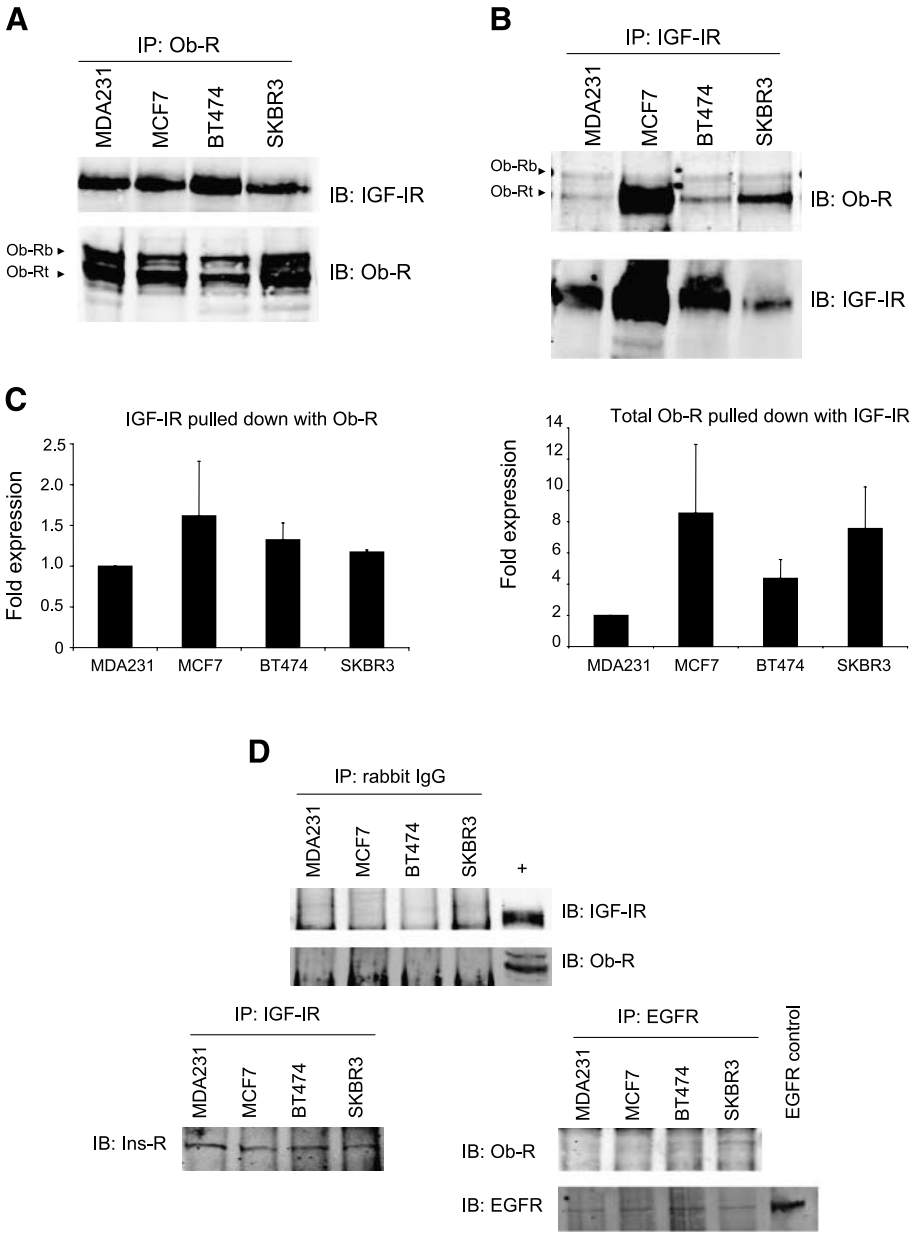


**FIGURE 2.** Interaction between IGF-IR and Ob-R in breast cancer. The breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were lysed for total protein. Ob-R (**A**) and IGF-IR (**B**) were immunoprecipitated (*IP*; 1 μg of antibody) from total protein extracts (200 μg) and immunoblotted (*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.

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## 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

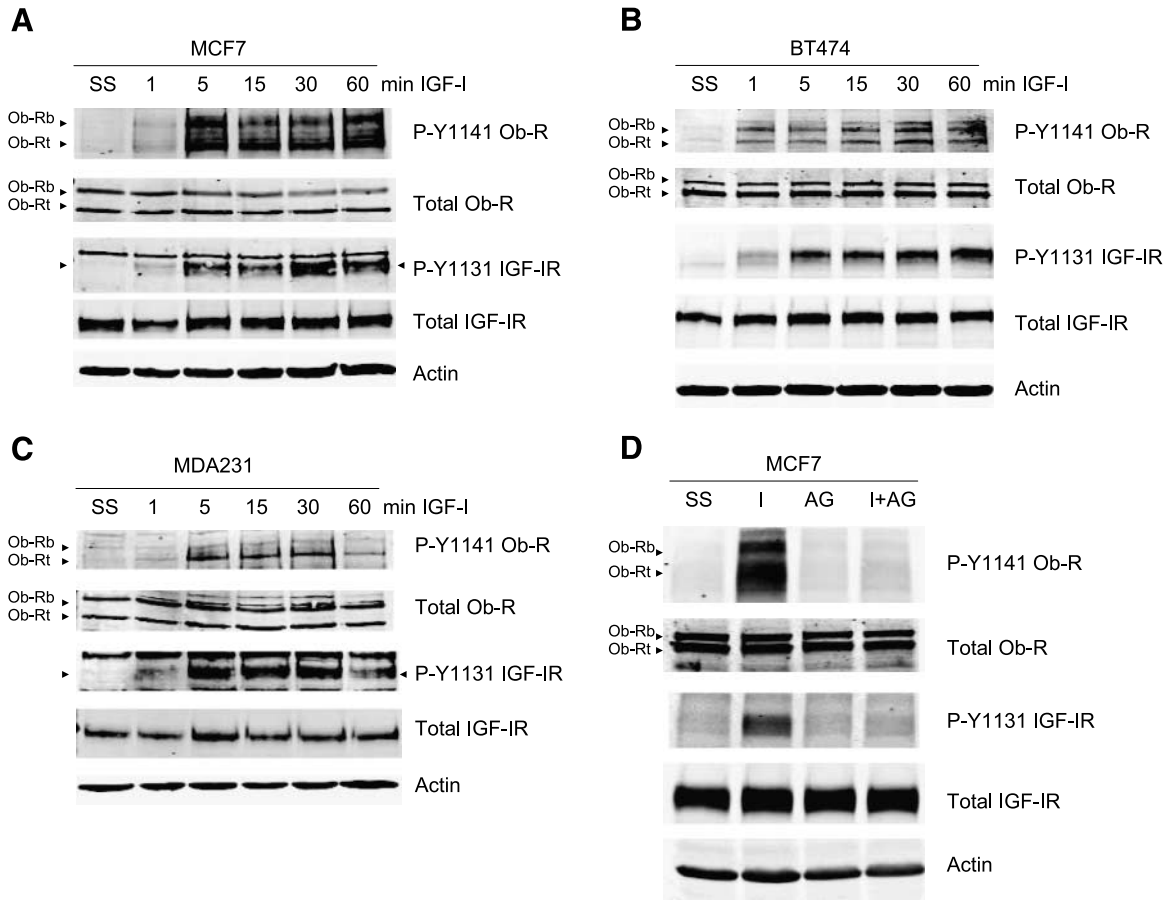


**FIGURE 3.** Evidence of cross-talk from IGF-IR to Ob-R. IGF-I induces phosphorylation of Ob-R, which is blocked by IGF-IR kinase inhibition. MCF7 (**A**), BT474 (**B**), and MDA231 (**C**) cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min. Cells 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.

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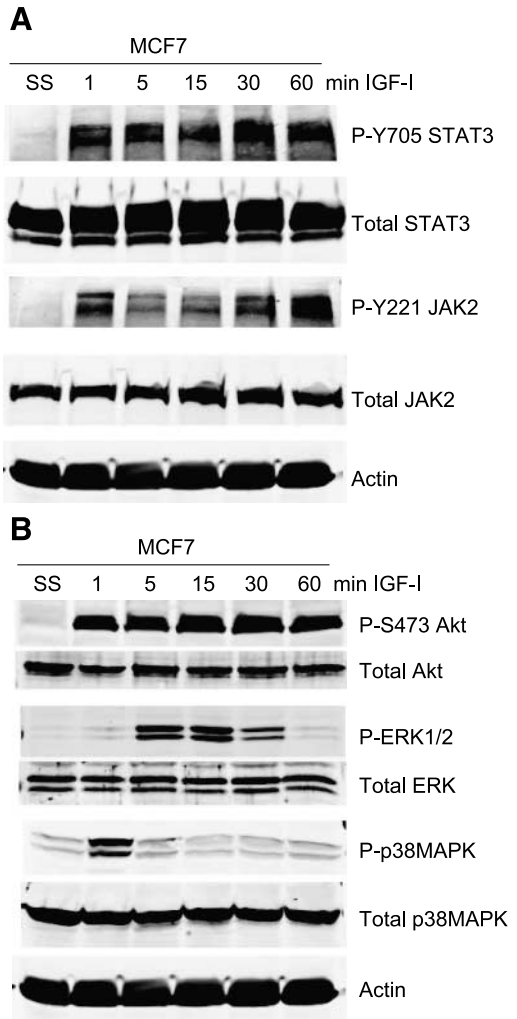


**FIGURE 4.** IGF-I activates downstream signaling. MCF7 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min. Total protein extracts (50 μg) were immunoblotted for the downstream leptin signaling molecules p-STAT3 ($Tyr^{705}$), total STAT3, p-JAK2 ($Tyr^{221}$), 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

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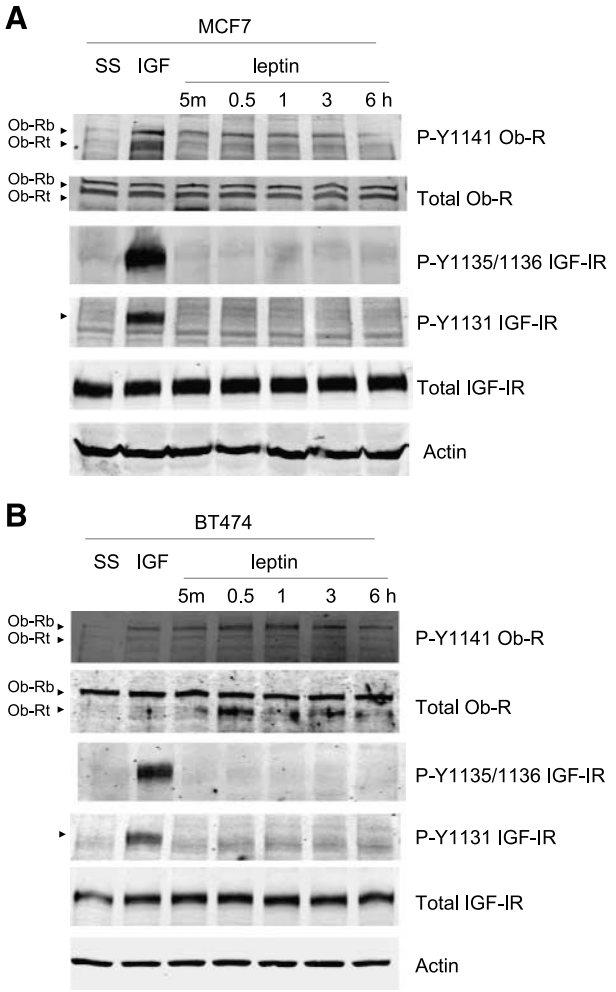


**FIGURE 5.** Evidence of unidirectional cross-talk. Leptin does not induce phosphorylation of IGF-IR. MCF7 (**A**) and (**B**) BT474 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 5 min or leptin (1,000 ng/mL) for 5 min or 0.5, 1, 3, or 6 h. Cells were lysed for protein, 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

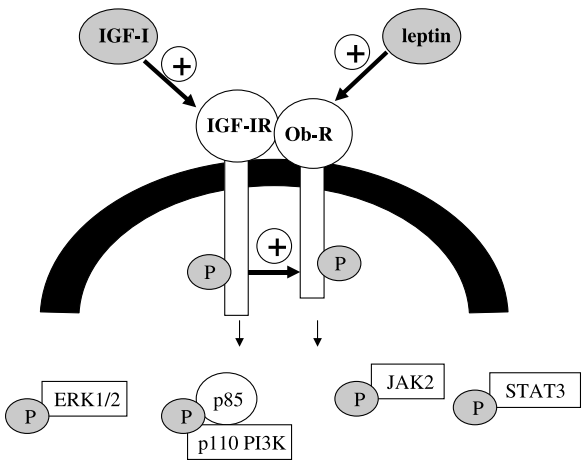


**FIGURE 6.** A novel unidirectional cross-talk from IGF-IR to Ob-R in breast cancer. Our results indicate that the IGF-I and leptin receptors interact in human breast cancer cells. Furthermore, cross-talk occurs from IGF-IR to Ob-R, such that IGF-I stimulation induces phosphorylation and activation 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.

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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-p$Thr^{180}$/$Tyr^{182}$ 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.

## References

1. Reinier KS, Vacek PM, Geller BM. Risk factors for breast carcinoma *in situ* versus invasive breast cancer in a prospective study of pre- and post-menopausal women. Breast Cancer Res Treat 2007;103:343–48.

2. Chlebowski RT, Aiello E, McTiernan A. Weight loss in breast cancer patient management. J Clin Oncol 2002;20:1128–43.

3. Calle EE, Thun MJ. Obesity and cancer. Oncogene 2004;23:6365–78.

4. Garofalo C, Surmacz E. Leptin and cancer. J Cell Physiol 2006;207:12–22.

5. Garofalo C, Koda M, Cascio S, et al. Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: possible role of obesity-related stimuli. Clin Cancer Res 2006;12:1447–53.

6. Saxena NK, Sharma D, Ding X, et al. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. Cancer Res 2007;67:2497–507.

7. Wauters M, Considine RV, Van Gaal LF. Human leptin: from an adipocyte hormone to an endocrine mediator. Eur J Endocrinol 2000;143:293–311.

8. Bahrenberg G, Behrmann I, Barthel A, et al. Identification of the critical sequence elements in the cytoplasmic domain of leptin receptor isoforms required for Janus kinase/signal transducer and activator of transcription activation by receptor heterodimers. Mol Endocrinol 2002;16:859–72.

9. Bjorbaek C, Uotani S, da Silva B, Flier JS. Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem 1997;272:32686–95.

10. Yin N, Wang D, Zhang H, et al. Molecular mechanisms involved in the growth stimulation of breast cancer cells by leptin. Cancer Res 2004;64:5870–5.

11. Garofalo C, Sisci D, Surmacz E. Leptin interferes with the effects of the antiestrogen ICI 182,780 in MCF-7 breast cancer cells. Clin Cancer Res 2004;10:6466–75.

12. Ishikawa M, Kitayama J, Nagawa H. Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. Clin Cancer Res 2004;10:4325–31.

13. Cleary MP, Phillips FC, Getzin SC, et al. Genetically obese MMTV-TGF-α/Lep(ob)Lep(ob) female mice do not develop mammary tumors. Breast Cancer Res Treat 2003;77:205–15.

14. Surmacz E. Function of the IGF-I receptor in breast cancer. J Mammary Gland Biol Neoplasia 2000;5:95–105.

15. Kahan Z, Gardi J, Nyari T, et al. Elevated levels of circulating insulin-like growth factor-I, IGF-binding globulin-3 and testosterone predict hormone-dependent breast cancer in postmenopausal women: a case-control study. Int J Oncol 2006;29:193–200.

16. Jones RA, Campbell CI, Gunther EJ, et al. Transgenic overexpression of IGF-IR disrupts mammary ductal morphogenesis and induces tumor formation. Oncogene 2007;26:1636–44.

17. Sachdev D, Yee D. Disrupting insulin-like growth factor signaling as a potential cancer therapy. Mol Cancer Ther 2007;6:1–12.

18. Knowlden JM, Hutcheson IR, Barrow D, Gee JM, Nicholson RI. Insulin-like growth factor-I receptor signaling in tamoxifen-resistant breast cancer: a supporting role to the epidermal growth factor receptor. Endocrinology 2005;146:4609–18.

19. Nahta R, Yuan LX, Zhang B, Kobayashi R, Esteva FJ. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. Cancer Res 2005;65:11118–28.

20. Novosyadlyy R, Dudas J, Pannem R, Ramadori G, Scharf JG. Crosstalk between PDGF and IGF-I receptors in rat liver myofibroblasts: implication for liver fibrogenesis. Lab Invest 2006;86:710–23.

21. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. J Biol Chem 2002;277:39684–95.

22. Schaffler A, Scholmerich J, Buechler C. Mechanisms of disease: adipokines and breast cancer-endocrine and paracrine mechanisms that connect adiposity and breast cancer. Nat Clin Pract Endocrinol Metabol 2007;3:345–54.

23. Hadsell DL, Murphy KL, Bonnette SG, Reece N, Laucirica R, Rosen JM. Cooperative interaction between mutant p53 and des(1-3)IGF-I accelerates mammary tumorigenesis. Oncogene 2000;19:889–98.

24. Bates P, Fisher R, Ward A, Richardson L, Hill DJ, Graham CF. Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II). Br J Cancer 1995;72:1189–93.

25. Wu Y, Cui K, Miyoshi K, et al. Reduced circulating insulin-like growth factor I levels delay the onset of chemically and genetically induced mammary tumors. Cancer Res 2003;63:4384–8.

26. Cao Q, Mak KM, Lieber CS. Leptin represses matrix metalloproteinase-1 gene expression in LX2 human hepatic stellate cells. J Hepatol 2007;46:124–33.

27. Li S, Ferber A, Miura M, Baserga R. Mitogenicity and transforming activity of the insulin-like growth factor-I receptor with mutations in the tyrosine kinase domain. J Biol Chem 1994;269:32558–64.

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