Leptin receptor expression and cell signaling in breast cancer

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Abstract. Obesity is considered a risk factor for many cancers, including breast cancer. Our laboratory has previously shown that leptin is mitogenic in many cancer cell lines, including breast. Information regarding the effects of high leptin levels on leptin receptor expression and signaling is lacking. The purpose of this study was to characterize leptin receptor expression in response to leptin in breast cancer cells. In addition, SOCS-3 expression (a leptin inducible inhibitor of leptin signaling), plus MAPK and PI3K signaling, were examined to determine their role in leptin-induced cell proliferation. Breast cancer cell lines, ZR75-1 and HTB-26, were treated with 0, 4, 40 or 80 ng/ml of leptin. Multiplex RT-PCR was performed to determine relative mRNA expression levels of the human short (huOB-Ra) or long (huOB-Rb) leptin receptor isoforms, or SOCS-3. MAPK and PI3K signaling was analyzed by phosphorylation of ERK and Akt, respectively, via Western blotting. Cell proliferation and inhibitor studies were analyzed by MTT assay. HTB-26 and ZR75-1 both expressed huOB-Ra, huOB-Rb and SOCS-3 mRNA; however, mRNA expression levels generally remained unchanged over time with leptin treatment. MAPK and PI3K pathways were activated in the presence of leptin over time. MAPK and PI3K inhibitors significantly blocked leptin-induced proliferation. Higher levels of circulating leptin contribute to breast cancer proliferation by activation of the MAPK and PI3K signaling pathways involved in cell growth and survival. The mitogenic effects of leptin are not a consequence of altered leptin receptor or SOCS-3 mRNA expression.

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Abbreviations: SOCS-3, suppressor of cytokine signaling; PI3K, phosphotidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JAK/STAT, Janus kinase/signal transducers and activators of transcription

Keywords: suppressor of cytokine signaling, phosphotidylinositol 3-kinase, mitogen-activated protein kinase, extracellular signal-regulated kinase, Janus kinase, signal transducers, activators of transcription

Introduction

One consequence of obesity is the increased risk of cancer and cancer progression (1). It has been well documented that being overweight increases one's risk of developing certain cancers, including colon, endometrial, breast (in post-menopausal women), esophageal and renal (2-9) The number of deaths from cancer that are attributable to being overweight range from up to 14% in men, to 20% in women (10). For breast cancer, overweight women with a body mass index (BMI) of ≥40 have a 2.12-fold higher risk of mortality from breast cancer than leaner women with BMIs of 18 (10). Furthermore, the association between higher body weight and breast cancer risk is strongly linked in postmenopausal women; however the converse is true for premenopausal women (11-14). Overall, one's BMI, distribution of body fat, and level of tumor-promoting hormones, such as estradiol and insulin, all correlate with breast cancer risk (2,15-20).

Leptin is a 16-kDa peptide hormone involved in regulating food intake, metabolism, body fat, energy expenditure and neuroendocrine function (21-24). Mature adipocytes secrete leptin, and it has been shown that serum leptin levels correlate with body weight. Obese individuals have an average of 40 ng/ml of circulating leptin as opposed to lean individuals who have an average of 4 ng/ml of circulating leptin (25,26). *In vitro* studies have shown that leptin does increase cell proliferation in certain breast, colon, prostate, endothelial, esophageal, and pancreatic cancer cell lines (4,27-33). Thus, defining the mechanism(s) involved in cancer cell proliferation in response to high leptin levels is of paramount importance.

Leptin receptor signaling pathways are important to understanding the effects of leptin on cell proliferation. Leptin binds the leptin receptor (OB-R), a type I cytokine receptor, that activates JAK/STAT (janus kinase signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) signaling pathways. Alternative mRNA splicing gives rise to different isoforms of OB-R, all of which maintain the same extracellular domain but contain variable lengths of

the intracellular signaling domain. Two main leptin receptor isoforms dominate: the short leptin receptor isoform (OB-Ra) and the long leptin receptor isoform (OB-Rb) (22,34). OB-Rb contains the full-length intracellular domain and is believed to be the main leptin signaling receptor. Ob-Ra contains a truncated intracellular domain and has been shown to participate in signaling through JAK-dependent activation of MAPK but cannot activate STAT (35-37). To date, the physiological relevance of each leptin isoform is still under investigation.

Further downstream signals activated by OB-Rb are also of interest in determining the effects of leptin on cell proliferation. SOCS-3 (a suppressor of cytokine signaling) is a known leptin inducible suppressor of cytokine signaling (38,39). In hypothalamic tissues, SOCS-3 mRNA and protein levels have been shown to increase with leptin treatment, demonstrating its function as a negative feedback loop for leptin signaling (40-44). Potential defects in SOCS-3 signaling could contribute to increased cell proliferation. Also, the altered expression of signals involved in cell growth and cell cycle progression, such as extracellular signal-regulated kinase (ERK) or phosphatidylinositol 3-kinase (PI3K), could play an important role in leptin-induced proliferation.

The purpose of this study was to determine the role of leptin in breast cancer cell proliferation. Two breast cancer cell lines, ZR75-1 and HTB-26, were assayed for the presence and expression of huOB-Ra (human OB-Ra) and huOB-Rb (human OB-Rb) mRNAs in response to leptin treatment. In addition, SOCS-3 mRNA expression, plus activation of MAPK and PI3K pathways in response to leptin treatment, were analyzed.

Materials and methods

Cell lines. Two breast epithelial cancer cell lines previously reported to respond to leptin treatment were selected for testing (4,27,29,32,45,46). ZR75-1 is an estrogen-responsive breast cancer cell line, whereas HTB-26 is an estrogen-receptor negative cell line (ATCC, Manassas, VA). ZR75-1 cells were maintained in RPMI media (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Gibco, Rockville, MD), 1% antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin; BioWhittaker), and 1% sodium pyruvate. HTB-26 cells were maintained in Leibovitz's L-15 media with 2 mM L-glutamine (ATCC) supplemented with 10% fetal bovine serum and 1% antibiotics. All cell lines were maintained at 37˚C in a 5% CO_2 atmosphere.

Leptin treatments. Cells were plated at $1x10^6$ cells per well of a 6-well plate and allowed to attach overnight. Cells were then serum-deprived for 20 h and then treated with 0, 4, 40 or 80 ng/ml of human recombinant leptin (R&D Systems, Minneapolis, MN) for 0, 0.5, 1, 2, 4, 24 or 48 h. Two independent tissue culture experiments were analyzed per cell line.

RNA isolation and RT-PCR. Total RNA was isolated from ZR75-1 and HTB-26 cells treated with leptin at each specified time point using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). RT-PCR was performed using the OneStep RT-PCR kit (Qiagen, Santa Clara, CA) to amplify the huOB-Ra, huOB-Rb or SOCS-3 mRNAs. Primers 5'-TTGTGCCAGTAATTATTT CCTCTT-3' (sense) and 5'-AGTTGGCACATTGGGTT CAT-3' (antisense) were used to amplify a 200-bp fragment specific for huOB-Ra (28). Primers 5'-CCAGAAACGTTTG AGCATCT-3' (sense) and 5'-CAAAAGCACACCACTCT CTC-3' (antisense) were used to amplify a 609-bp fragment specific for huOB-Rb (29). Primers 5'-GTCACCCACAGCA AGTTT-3' (sense) and 5'-CTGAGCGTGAAGAAGTGG-3' (antisense) were used to amplify a 245-bp fragment specific for SOCS-3 (47).

Multiplex RT-PCR was performed with an endogenous standard to determine the relative changes in mRNA expression in each sample. The 18S rRNA internal control (Ambion, Austin, TX) was amplified along with the huOB-Ra, huOB-Rb, or SOCS-3 primer sets. PCR cycle numbers were performed within the linear range of amplification. Resulting cDNAs were run on 1% agarose gels, stained with ethidium bromide, and densitometry readings were taken for all bands (EagleSight software, Stratagene, La Jolla, CA). Each leptin receptor or SOCS-3 cDNA band intensity was normalized with its corresponding 18S cDNA band (324 bp) intensity.

Western blot analysis. Cells were plated at $1x10^6$ cells per well of a 6-well plate and allowed to attach overnight. Cells were then treated with 0, 4, 40 or 80 ng/ml of human recombinant leptin (R&D Systems) for 4 h or treated with 40 ng/ml leptin for 0, 0.5, 1, 2 and 4 h. Confluent wells were washed at the appropriate times with cold 1X phosphate-buffered saline three times and then scraped and transferred to eppendorf tubes. The resulting cell suspensions were spun cold at 12,000 rpm for 10 min, supernatants were removed, and the final cell pellets were then lysed on ice for 30 min in RIPA buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Trition X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF] supplemented with 2 mM aprotinin, 2 mM leupeptin, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 2 mM pepstatin. Lysates were then spun at 14,000 rpm for 15 min and supernatants were collected and stored at -80˚C. The protein concentration of the total cellular protein extract was determined using the Bio-Rad protein assay reagent (Richmond, CA) and equal protein aliquots were loaded and separated on SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes in 20 mM Tris-HCl (pH 8.0) supplemented with 150 mM glycine plus 20% (v/v) methanol. Membranes were subsequently blocked with 5% non-fat dried milk plus 0.05% Tween-20, and antibodies against phospho-Akt (Ser473) or phospho-p44/42 MAPK (ERK). The membranes were then washed in 1X phosphate buffered saline three times and incubated with the appropriate horseradish peroxidase-linked IgG. Protein bands were visualized by chemiluminescence (NEN, Boston, MA) and analyzed using densitometry.

MTT assay. Cells were plated at $1x10^4$ cells/well in a 96-well plate and allowed to attach overnight. Cells were then serum-deprived for 20 h prior to drug treatment. Quiescent cells were then exposed to media containing 10% FBS with 20 or 40 μM LY294002 (Promega, Madison, WI), or 10 or 20 μM U0126 (Promega), in the presence or absence of human

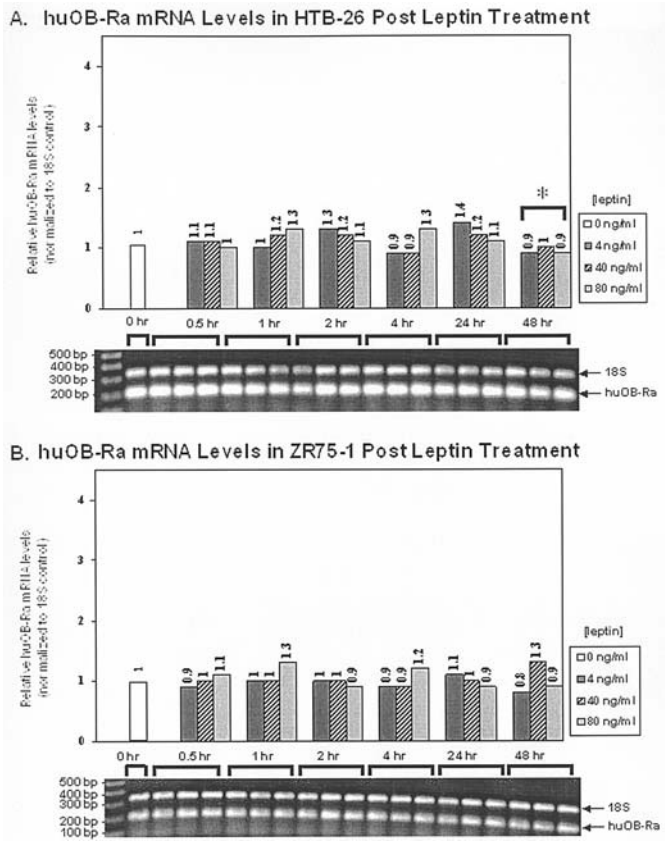

Figure 1. Expression levels of huOB-Ra mRNA in HTB-26 (A) and ZR75-1 (B) cells over time in response to treatment with human recombinant leptin. Data are representative of the average of two independent experiments. All ratios were determined by dividing the huOB-Ra cDNA band density value by its respective 18S cDNA band density value, and normalizing to the 0 h, 0 ng/ml leptin data point. *P<0.05, treated vs. control.

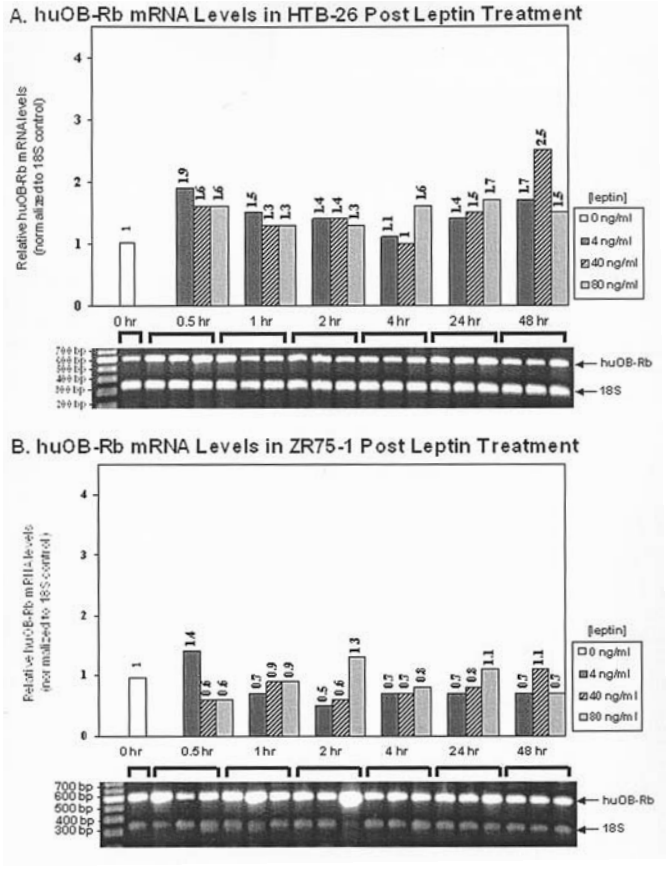

Figure 2. Expression levels of huOB-Rb mRNA in HTB-26 (A) and ZR75-1 (B) cells over time in response to treatment with human recombinant leptin. Data are representative of the average of two independent experiments. All ratios were determined by dividing the huOB-Rb cDNA band density value by its respective 18S cDNA band density value, and normalizing to the 0 h, 0 ng/ml leptin data point.

recombinant leptin (40 ng/ml) for 24, 48 or 72 h. The final total volume in each well was 200 μl. Since DMSO was a solvent for the inhibitors, a dimethyl sulfoxide (DMSO 1:500) control was included to rule out any cell death due to DMSO itself. Cell proliferation was measured by 3-(4,5-Dimethylthiazolyl)-2,5diphenyl tetrazolium bromide (MTT) colorimetric assay (13). Briefly, cells were incubated with the tetrazolium dye (20 μl/well of a 7.5 mg/ml solution; Sigma, St. Louis, MO) for 4 h at 37˚C. Mitochondrial dehydrogenase activity reduces the MTT dye to a purple formazan in living cells. The formazan crystals were solubilized with the addition of acidified isopropanol. Cell proliferation was determined by reading the absorbance per well at 570 nm on an enzyme-linked immunosorbent assay plate reader.

Statistical analysis. Determination of statistical significance was performed by analysis of variance. *Post hoc* comparisons of individual concentration means with the control were completed using the Turkey-Kramer Multiple Comparison test. Data are presented as means and standard errors.

Results

Expression of huOB-Ra mRNA in response to leptin treatment. RT-PCR of total RNA extracts from HTB-26 and ZR75-1 cell lines treated with leptin over time demonstrated that both cell lines express huOB-Ra mRNA (200 bp band; Fig. 1). The resulting huOB-Ra cDNA band densities were normalized to their respective 18S cDNA band densities, and ratios were then normalized to the 0 h, 0 ng/ml leptin control. No dose-dependent effects with respect to mRNA expression levels were seen over time in HTB-26 or ZR75-1. huOB-Ra mRNA levels in HTB-26 decreased significantly at the 48-h time point (P<0.05, treated vs control); however, no significant time effects were found in ZR75-1.

Expression of huOB-Rb mRNA in response to leptin treatment. RT-PCR of total RNA extracts from HTB-26 and ZR75-1 cell lines treated with leptin over time demonstrated that both cell lines express huOB-Rb mRNA (609-bp band; Fig. 2). The resulting huOB-Rb cDNA band densities were normalized to their respective 18S cDNA band densities, and ratios were then normalized to the 0 h, 0 ng/ml leptin control. No dose or time-dependent effects were seen with HTB-26 or ZR75-1.

Expression of SOCS-3 mRNA in response to leptin treatment. RT-PCR of total RNA extracts from HTB-26 and ZR75-1 cell lines treated with leptin over time demonstrated that both cell lines express SOCS-3 mRNA (245-bp band; Fig. 3). The resulting SOCS-3 cDNA band densities were normalized to their respective 18S cDNA band densities, and ratios were

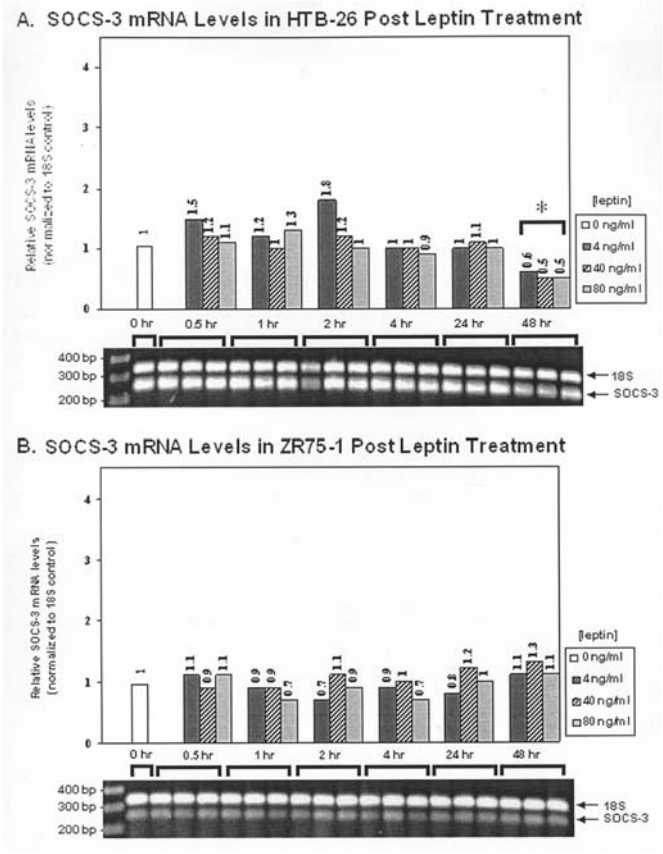

Figure 3. Expression levels of SOCS-3 mRNA in HTB-26 (A) and ZR75-1 (B) cells over time in response to treatment with human recombinant leptin. Data are representative of the average of two independent experiments. All ratios were determined by dividing the SOCS-3 cDNA band density value by its respective 18S cDNA band density value, and normalizing to the 0 h, 0 ng/ml leptin data point. *P<0.01, treated versus control.

then normalized to the 0 h, 0 ng/ml leptin control. HTB-26 and ZR75-1 cells did not exhibit any significant dose-dependent effects with leptin treatment. HTB-26 showed significant time-dependent decreases in SOCS-3 mRNA expression at 48-h time point (P<0.01 vs. 0.5- and 2-h time points).

MAPK and PI3K signaling in response to leptin treatment. HTB-26 cells activated both MAPK and PI3K pathways as assayed by the phosphorylation of ERK (p-ERK) and Akt (p-Akt), respectively (Fig. 4). All concentrations of leptin increased p-Akt (Fig. 4A) and p-ERK (Fig. 4C) levels above controls at the 4-h time point in HTB-26 cells. Time effects were observed in HTB-26 cells for both p-ERK and p-Akt (Fig. 4B and D) when treated with 40 ng/ml leptin. ZR75-1 cells demonstrated increases in p-ERK activation with all leptin treatments (Fig. 5A) and time effects were evident at 2 h post leptin (40 ng/ml) administration (Fig. 5B). Activity of p-Akt was not detected in the ZR75-1 cells (data not shown).

Inhibition of leptin-induced proliferation in HTB-26. HTB-26 cells treated with 40 ng/ml leptin exhibited a significant increase in cell proliferation at the 48-h time point (P<0.0001 vs. 10% FBS control; Fig. 6). All inhibitor treatments showed a significant decrease in cell proliferation as compared to the 10% FBS control (P<0.0001) at the 48-h time point (Fig. 6). The addition of MAPK inhibitor U0126 (20 μM) decreased the cell proliferation to 44% (absence of leptin) and 54% (presence of leptin) as compared to the 10% FBS positive control (P<0.0001; Fig. 6A) by 72 h. Likewise, the addition of PI3K inhibitor LY294002 (40 μM) decreased the cell proliferation to 65% (absence of leptin) and 72% (presence of leptin) as compared to the 10% FBS control (P<0.0001; Fig. 6A) by 72 h. The presence of DMSO, a solvent used to dissolve the inhibitors, did not significantly affect the cell proliferation.

As shown in Fig. 6B, dose-dependent effects were seen with HTB-26 cells treated only with LY294002 (20 μM vs. 40 μM; P<0.0001) or U0126 (10 μM vs. 20 μM; P<0.0001) at 48 h. No dose-dependent effects were seen at 24 h, however dose-dependent effects were seen at 72 h with U0126 treatment (10 μM vs. 20 μM; P<0.0001; data not shown). When comparing inhibitor treatments in the presence of leptin, proliferation was significantly decreased in a dose-dependent manner for HTB-26 cells treated with U0126 (10 μM vs. 20 μM) or LY294002 (20 μM vs. 40 μM) at the 48-h time point (P<0.0001; Fig. 6B). No significant dose-dependent changes were seen at 24 and 72 h (data not shown). Additionally, there were no significant changes in cell proliferation when comparing U0126 treatment vs. U0126 + leptin treatment, or LY294002 vs. LY294002 + leptin treatment at 48 (Fig. 6B), 24 or 72 h (data not shown).

Inhibition of leptin-induced proliferation in ZR75-1. ZR75-1 cells treated with 40 ng/ml leptin exhibited a significant increase in cell proliferation at the 48-h time point (P<0.0001; Fig. 7). All inhibitor treatments showed a significant decrease in cell proliferation as compared to the 10% FBS control (P<0.0001) at the 48-h time point (Fig. 7). The addition of MAPK inhibitor U0126 (20 μM) decreased the cell proliferation to 54% (absence of leptin) and 61% (presence of leptin) as compared to the 10% FBS positive control (P<0.0001; Fig. 7A) by 72 h. Likewise, the addition of PI3K inhibitor LY294002 (40 μM) decreased the cell proliferation to 49% (absence of leptin) and 50% (presence of leptin) as compared to the 10% FBS control (P<0.0001; Fig. 7A) by 72 h. Again, no significant change in ZR75-1 cell proliferation was observed in the presence of DMSO.

As shown in Fig. 7B, no dose-dependent effects were seen with ZR75-1 cells treated only with LY294002 (20 μM vs. 40 μM) or U0126 (10 μM vs. 20 μM) at 48, 24 or 72 h (data not shown). When comparing inhibitor treatments in the presence of leptin, proliferation was not significantly decreased in a dose-dependent manner for HTB-26 cells treated with U0126 (10 μM vs. 20 μM) or LY294002 (20 μM vs. 40 μM) at the 48 (Fig. 7B,) 24 or 72-h time point (data not shown). Additionally, there were no significant changes in cell proliferation when comparing U0126 treatment vs. U0126 + leptin treatment, or LY294002 vs. LY294002 + leptin treatment at 48 (Fig. 6B), 24 or 72 h (data not shown).

Discussion

Growing evidence suggests that obesity is an important risk factor for certain cancers. The role of the adipocyte hormone, leptin, in breast cancer cell proliferation is not well understood.

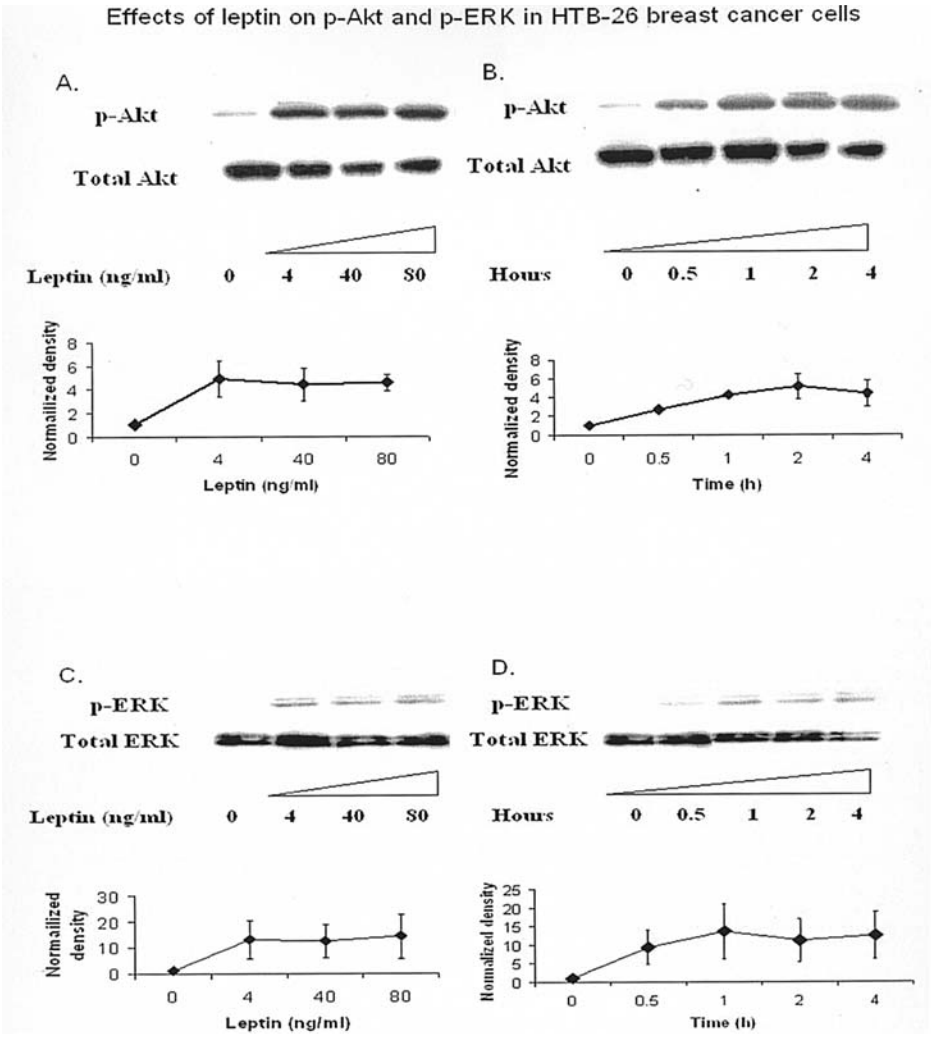

Figure 4. Leptin-activated PI3K and MAPK signal transduction pathways in HTB-26 breast cancer cells. Cells were treated with 0, 4, 40, or 80 ng/ml of leptin for 0, 0.5, 1, 2, or 4 h. Representative Western blots are shown. Activation of the PI3K pathway was verified via phosphorylation of AKT (p-Akt) with cells treated with different concentrations of leptin (A, 4-h time point shown) over different periods of time (B, 40 ng/ml treatment shown). Likewise, activation of the MAPK pathway was verified via phosphorylation of ERK (p-ERK) with cells treated with different concentrations of leptin (C, 4-h time point shown) over different periods of time (D, 40 ng/ml treatment shown). Densitometry analysis was normalized to the total AKT or total ERK in each cell lysate.

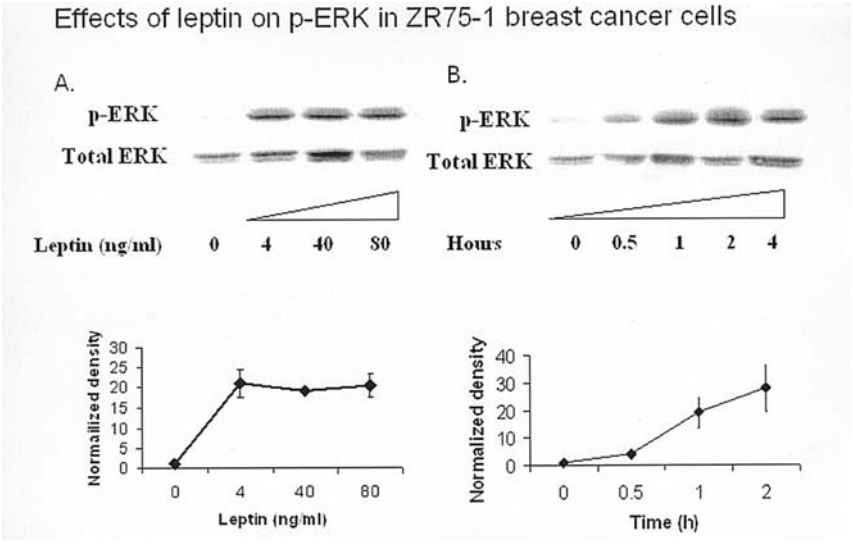

Figure 5. Leptin activates MAPK signal transduction pathways in ZR75-1 breast cancer cells. Cells were treated with 0, 4, 40, or 80 ng/ml of leptin for 0, 0.5, 1, 2, or 4 h. Representative Western blots are shown. Activation of the MAPK pathway was verified via phosphorylation of ERK (p-ERK) with cells treated with different concentrations of leptin (A, 4-h time point shown) over different periods of time (B, 40 ng/ml treatment shown). Densitometry analysis was normalized to the total ERK in each cell lysate.

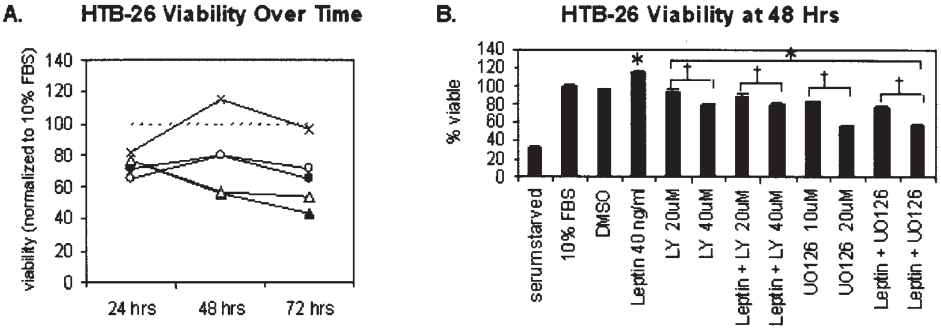

Figure 6. Effects of MAPK inhibitor U0126 and PI3K inhibitor LY294002 (LY) on leptin-induced proliferation in HTB-26 breast cancer cells. Quiescent cells were exposed to 10% FBS in the presence or absence of 40 ng/ml leptin, with or without LY294002 or U0126. Cell proliferation was determined using an MTT assay and data was normalized to the 10% FBS control. (A) Cell proliferation was evaluated at 24, 48 and 72 h post treatment: 10% FBS (---), leptin (x), 40 μM LY294002 (•), leptin + 40 μM LY294002 (◦), 20 μM U0126 (▲), leptin + 20 μM U0126 (△). (B) HTB-26 cell proliferation 48 h post leptin treatment. *P<0.0001 versus 10% FBS control. †P<0.0001 dose-dependent effects for LY294002 20 μM vs. 40 μM alone or in the presence of leptin, and U0126 10 μM vs. 20 μM alone or in the presence of leptin.

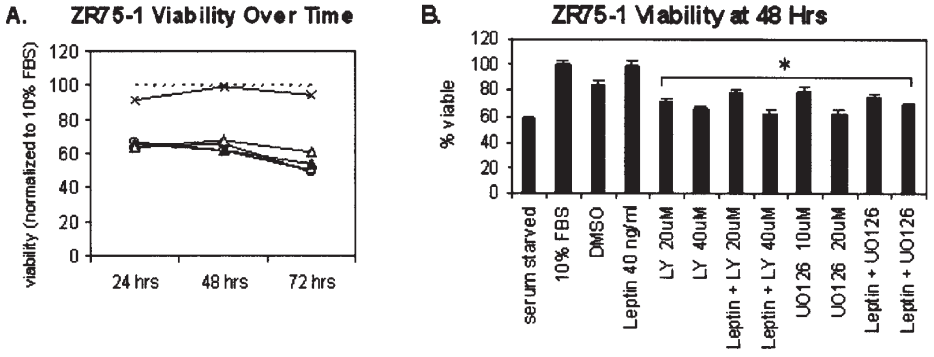

Figure 7. Effects of MAPK inhibitor U0126 and PI3K inhibitor LY294002 (LY) on leptin-induced proliferation in ZR75-1 breast cancer cells. Quiescent cells were exposed to 10% FBS in the presence or absence of 40 ng/ml leptin, with or without LY294002 or U0126. Cell proliferation was determined using an MTT assay and data was normalized to the 10% FBS control. (A) Cell proliferation was evaluated at 24, 48 and 72 h post treatment: 10% FBS (---), leptin (x), 40 μM LY294002 (•), leptin + 40 μM LY294002 (◦), 20 μM U0126 (▲), leptin + 20 μM U0126 (△). (B) ZR75-1 cell proliferation 48 h post leptin treatment. *P<0.0001 versus 10% FBS control.

In this report, we investigated the molecular basis behind leptin's mitogenic effect on breast cancer cells.

We have previously shown that leptin stimulates cell proliferation in certain breast, esophageal and prostate cancer cell lines *in vitro* (32). Additional studies have also documented the mitogenic effect of leptin in other cell lines from tissues including vascular endothelium (31,48) smooth muscle (49), and normal and neoplastic colon cells (28,50). However, *in vitro* data on leptin-induced cell proliferation in breast cancer cell lines remains controversial. Several studies have shown that MCF-7, HTB-26, and HTB131 breast cell lines fail to demonstrate any effect on cell proliferation in response to leptin treatment (32,51). Yet, other studies demonstrate that leptin has a direct effect on the growth stimulation of MCF-7 cells, as well as T47-D, HBL100 and ZR75-1 breast cancer cell lines (4,27,29,32,45,46). Discrepancies between studies could be attributed to the concentration of leptin used, measurement of cell proliferation, as well as the expression levels of leptin receptor isoforms themselves found on different cell lines. We selected the ZR75-1 cell line to study the effects of leptin because it is estrogen-positive and tamoxifen-resistent, while HTB-26 is estrogen-negative. Both responded to leptin treatment with increased cell proliferation by 48 h. A more robust response to leptin was observed with the estrogen-negative HTB-26 cell line at this time. Previous studies have shown a differential response by breast cell lines to exogenous leptin (32). These discrepancies are probably due to inherent genetic differences between breast cancer cell lines.

We hypothesized that changes in leptin concentrations would cause changes in receptor expression at the level of mRNA. HTB-26 and ZR75-1 breast cancer cells express huOB-Ra and huOB-Rb mRNA. Our data is in agreement with other findings that have also identified huOB-Ra and huOB-Rb isoforms present in a number of various breast cancer cell lines (4,27,29,51). Mice defective for the receptor, OB-Rb, do not develop oncogene-induced mammary tumors indicating that leptin and its cognate receptor may be involved in mammary tumorigenesis (52). In this study, the mRNA expression levels of huOB-Ra did not change for HTB-26 or ZR75-1 breast cancer cell lines with different concentrations of leptin over time. We did not expect huOB-Ra levels to change in response to leptin treatment since huOB-Ra is not the main signaling isoform. Although we expected the huOB-Rb mRNA levels to respond to leptin treatment, there was not a significant change for either HTB-26 or ZR75-1 breast cancer cell lines. We propose several explanations for this

observation. Firstly, the number of leptin receptor isoforms could be so low on HTB-26 and ZR75-1 cell lines that there is little detectable response to leptin treatment. Secondly, the mRNA expression levels may not change in response to leptin treatment and, instead, the stability of the mRNA is maintained or more receptor protein is produced. Antibodies specific for each isoform would need to be created to determine isoform levels. Thirdly, a more sensitive PCR assay may be needed to detect small changes in mRNA expression levels.

Since the leptin receptor levels did not change in response to leptin, we hypothesized that changes might occur at the signal transduction level. huOB-Rb is known to stimulate the JAK/STAT pathway; one consequence of this signaling cascade is the upregulation of SOCS-3 to turn off the activated leptin receptor. Therefore, we tested whether cancer cells are responsive to the mitogenic effects of leptin due to defects in leptin signaling, specifically SOCS-3 signaling. Our data indicates that, overall, no significant changes in SOCS-3 mRNA levels were observed in response to leptin treatment in HTB-26 or ZR75-1 cells. These results could be interpreted in three different ways: i) the SOCS-3 pathway is intact and functioning at a normal level and is not responsible for the mitogenic effects of leptin; ii) the huOB-Rb leptin activated pathway is not being turned off by SOCS-3, thus contributing to the proliferative effects of leptin; or iii) low huOB-Rb receptor levels on HTB-26 and ZR75-1 cells do not allow for detectable changes of SOCS-3 mRNA expression. *In vivo* studies in animals and hypothalmic tissue have reported increases in SOCS-3 expression in response to leptin (40-44). The hypothalamus contains a high percentage of huOB-Rb and therefore gives a greater detectable SOCS-3 response to leptin treatment than other cell types. Additional *in vitro* studies of cell lines transfected with the leptin receptor have also supported the *in vivo* animal studies (41). Chinese hamster ovary (CHO) cells transfected with OB-Rb exhibited increases in SOCS-3 mRNA expression in response to leptin, whereas CHO cells transfected with only OB-Ra showed no changes in SOCS-3 levels. These transfected cell lines expressed sizeable amounts of the leptin receptor. This again demonstrates that the level of receptor expression is important in detecting increases in SOCS-3 mRNA.

Interestingly, another point to consider is that breast cancer cell lines may be defective in SOCS-3 gene expression. A study by He *et al* showed that, in MCF-7 cells, the SOCS-3 gene promoter is actually silenced due to high promoter methylation (12). This was also the case with three additional breast cancer cell lines, but was not seen in normal tissue. They also found that restoring a non-methylated SOCS-3 gene promoter restored apoptosis. Our data supports this finding. We have reported previously that leptin induced proliferation works by inhibiting apoptosis in some cancer cell lines (53,54). Therefore, the absence of an increased SOCS-3 response to leptin may help to block apoptosis and increase cancer cell survival, but not directly promote cell proliferation.

Since the mRNA levels of huOB-Ra, huOB-Rb and SOCS-3 did not change in response to leptin, we hypothesized that leptin may be inducing proliferation via the PI3K and/or MAPK signaling pathways. Western analysis demonstrated that both MAPK and PI3K pathways were upregulated in response to leptin treatment over time in HTB-26 and ZR75-1 cell lines. Our data agrees with other studies that show that PI3K and MAPK pathways are activated by leptin treatment in normal cells such as epithelial colonic (28), peripheral blood mononuclear cells (55,56) and Chinese hamster ovary cells overexpressing huOB-Ra or huOB-Rb (37,41). Additionally, T47-D and MCF-7 cell lines have been shown to induce MAPK phosphorylation in response to leptin treatment (4,27,29). Leptin also activates the estrogen receptor α through MAPK pathway (57). Here we report that HTB-26 and ZR75-1 breast cancer cell lines also induce phosphorylation of MAPK, in addition to PI3K, in a time-dependent manner in response to leptin treatment. Both signaling pathways are involved in cell proliferation and cell survival, thus increased activation may contribute to the mitogenic effects of leptin.

To further prove that the mitogenic effects of leptin were working partly through the leptin-induced huOB-Rb MAPK pathway, the effects of MAPK inhibitor U0126 and PI3K inhibitor LY294002 on cell proliferation were analyzed in the presence and absence of leptin. We observed a significant decrease in cell proliferation with each inhibitor showing that the MAPK and PI3K pathways are important to leptin-stimulated growth. Our data is in agreement with other studies which show that leptin-induced proliferation and migration are inhibited by PI3K and MAPK inhibitors in colonic epithelial, kidney epithelial and gastric cells (58,59). In addition, PI3K, MAPK and JAK2/STAT3 inhibitors have been shown to block leptin-stimulated proliferation of T-47D and HBL100 breast cancer cells (4,29). Together with our data, this confirms that huOb-Rb stimulates both the JAK/STAT and MAPK (ERK) pathways, and inhibition of these pathways leads to decreased breast cancer cell growth. We have shown that MAPK and PI3K inhibitors block leptin-induced proliferation *in vitro* in HTB-26 and ZR75-1 breast cancer cells. However, inhibition was not dependent upon whether the breast cancer cells were estrogen receptor positive or negative.

Activation of PI3K has been shown to be required for numerous cell functions including cell growth and proliferation, migration, adhesion and cell survival (60). More importantly, PI3K is believed to be required for mitogen-induced inhibition of apoptosis (14). Our previous studies show that leptin-induced proliferation in cancer cells is largely due to a decrease in apoptosis (53,54). Phosphorylation of PI3K phosphorylates Akt which in turn induces the activity of apoptotic suppressors. *In vitro* and *in vivo* studies have shown that the therapeutic use of PI3K inhibitors decreases cell proliferation, increases apoptosis and decreases breast cancer cell proliferation (60-63). *In vivo* studies in mice have also shown this to be true for malignant gliomas, lung cancer and colon cancer (64-67). Drugs targeting the anti-apoptotic characteristic of PI3K may provide a successful direction in cancer therapy treatment.

In conclusion, our data demonstrate that the mitogenic effects of leptin do not involve alterations in the mRNA expression levels of huOB-Ra or huOB-Rb in breast cancer cells *in vitro*. The proliferative effects of leptin are unlikely to be due to defects in SOCS-3 signaling but are, instead, closely linked to the mitogenic MAPK and PI3K pathways. As both of these signaling pathways are involved in cell proliferation and cell survival, their activation accounts for the mitogenic effects of leptin in breast cancer cells. Future studies are ongoing to determine additional signal transduction

pathways and growth factors upregulated by leptin that contribute to the mitogenic effects of leptin in cancer cells.

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References

1. Bray GA: The underlying basis for obesity: relationship to cancer. J Nutr 132: S3451-S3455, 2002.
2. Abu-Abid S, Szold A and Klausner J: Obesity and cancer. J Med 33: 73-86, 2002.
3. Carroll KK: Obesity as a risk factor for certain types of cancer. Lipids 33: 1055-1059, 1998.
4. Hu X, Juneja SC, Maihle NJ and Cleary MP: Leptin - a growth factor in normal and malignant breast cells and for normal mammary gland development. J Natl Cancer Inst 94: 1704-1711, 2002.
5. Romero Y, Cameron AJ, Locke GR III, Schaid DJ, Slezak JM, Branch CD and Melton LJ III: Familial aggregation of gastro-esophageal reflux in patients with Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology 113: 1449-1456, 1997.
6. Rose DP, Gilhooly EM and Nixon DW: Adverse effects of obesity on breast cancer prognosis, and the biological actions of leptin (Review). Int J Oncol 21: 1285-1292, 2002.
7. Schapira DV, Kumar NB, Lyman GH and Cox CE: Obesity and body fat distribution and breast cancer prognosis. Cancer 67: 523-528, 1991.
8. Van den Brandt PA, Spiegelman D, Yaun SS, Adami HO, Beeson L, Folsom AR, Fraser G, Goldbohm RA, Graham S, Kushi L, Marshall JR, Miller AB, Rohan T, Smith-Warner SA, Speizer FE, Willett WC, Wolk A and Hunter DJ: Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. Am J Epidemiol 152: 514-527, 2000.
9. Wolf G, Chen S, Han DC and Ziyadeh FN: Leptin and renal disease. Am J Kidney Dis 39: 1-11, 2002.
10. Calle EE, Rodriguez C, Walker-Thurmond K and Thun MJ: Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med 348: 1625-1638, 2003.
11. Cleary MP and Maihle NJ: The role of body mass index in the relative risk of developing premenopausal versus post-menopausal breast cancer. Proc Soc Exp Biol Med 216: 28-43, 1997.
12. He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F and Jablons DM: SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci USA 100: 14133-14138, 2003.
13. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63, 1983.
14. Perugini RA, McDade TP, Vittimberga FJ Jr and Callery MP: Pancreatic cancer cell proliferation is phosphatidylinositol 3-kinase dependent. J Surg Res 90: 39-44, 2000.
15. Bruning PF, Bonfrer JM, van Noord PA, Hart AA, Jong-Bakker M and Nooijen WJ: Insulin resistance and breast-cancer risk. Int J Cancer 52: 511-516, 1992.
16. Deslypere JP: Obesity and cancer. Metabolism 44: 24-27, 1995.
17. Hershcopf RJ and Bradlow HL: Obesity, diet, endogenous estrogens, and the risk of hormone-sensitive cancer. Am J Clin Nutr 45: 283-289, 1987.
18. McTiernan A, Rajan KB, Tworoger SS, Irwin M, Bernstein L, Baumgartner R, Gilliland F, Stanczyk FZ, Yasui Y and Ballard-Barbash R: Adiposity and sex hormones in post-menopausal breast cancer survivors. J Clin Oncol 21: 1961-1966, 2003.
19. Tretli S and Robsahm TE: Height, weight and cancer of the oesophagus and stomach: a follow-up study in Norway. Eur J Cancer Prev 8: 115-122, 1999.
20. Ziegler RG, Hoover RN, Nomura AM, West DW, Wu AH, Pike MC, Lake AJ, Horn-Ross PL, Kolonel LN, Siiteri PK and Fraumeni JF Jr: Relative weight, weight change, height, and breast cancer risk in Asian-American women. J Natl Cancer Inst 88: 650-660, 1996.
21. Ahima RS and Flier JS: Leptin. Annu Rev Physiol 62: 413-437, 2000.
22. Friedman JM: Leptin, leptin receptors, and the control of body weight. Nutr Rev 56: S38-S46, 1998.
23. Friedman JM and Halaas JL: Leptin and the regulation of body weight in mammals. Nature 395: 763-770, 1998.
24. Mantzoros CS: The role of leptin in human obesity and disease: A review of current evidence. Ann Intern Med 130: 671-680, 1999.
25. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ and Bauer TL: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334: 292-295, 1996.
26. Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL and Murphy LJ: Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. Eur J Endocrinol 149: 331-335, 2003.
27. Dieudonne MN, Machinal-Quelin F, Serazin-Leroy V, Leneveu MC, Pecquery R and Giudicelli Y: Leptin mediates a proliferative response in human MCF7 breast cancer cells. Biochem Biophys Res Commun 293: 622-628, 2002.
28. Hardwick JC, van Den Brink GR, Offerhaus GJ, van Deventer SJ and Peppelenbosch MP: Leptin is a growth factor for colonic epithelial cells. Gastroenterology 121: 79-90, 2001.
29. Laud K, Gourdou I, Pessemesse L, Peyrat JP and Djiane J: Identification of leptin receptors in human breast cancer: functional activity in the T47-D breast cancer cell line. Mol Cell Endocrinol 188: 219-226, 2002.
30. Onuma M, Bub JD, Rummel TL and Iwamoto Y: Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase. J Biol Chem 278: 42660-42667, 2003.
31. Park HY, Kwon HM, Lim HJ, Hong BK, Lee JY, Park BE, Jang Y, Cho SY and Kim HS: Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases *in vivo* and *in vitro*. Exp Mol Med 33: 95-102, 2001.
32. Somasundar P, Yu A, Vona-Davis L and McFadden DW: Differential responses of leptin on cancer *in vitro*. J Surg Res 113: 50-55, 2002.
33. Somasundar P, McFadden DW, Hileman SM and Vona-Davis LC: Leptin is a growth factor in cancer. J Surg Res 116: 337-349, 2004.
34. Fei H, Okano HJ, Li C, Lee GH, Zhao C, Darnell R and Friedman JM: Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proc Natl Acad Sci USA 94: 7001-7005, 1997.
35. Bjorbaek C, Uotani S, Da Silva B and Flier JS: Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem 272: 32686-32695, 1997.
36. Murakami T, Yamashita T, Iida M, Kuwajima M and Shima K: A short form of leptin receptor performs signal transduction. Biochem Biophys Res Commun 231: 26-29, 1997.
37. Yamashita T, Murakami T, Otani S, Kuwajima M and Shima K: Leptin receptor signal transduction: OBRa and OBRb of fa type. Biochem Biophys Res Commun 246: 752-759, 1998.
38. Banks AS, Davis SM, Bates SH and Myers MG Jr: Activation of downstream signals by the long form of the leptin receptor. J Biol Chem 275: 14563-14572, 2000.
39. Bjorbak C, Lavery HJ, Bates SH, Olson RK, Davis SM, Flier JS and Myers MG Jr: SOCS3 mediates feedback inhibition of the leptin receptor via Tyr985. J Biol Chem 275: 40649-40657, 2000.
40. Bjorbaek C, Elmquist JK, Frantz JD, Shoelson SE and Flier JS: Identification of SOCS-3 as a potential mediator of central leptin resistance. Mol Cell 1: 619-625, 1998.
41. Bjorbaek C, El Haschimi K, Frantz JD and Flier JS: The role of SOCS-3 in leptin signaling and leptin resistance. J Biol Chem 274: 30059-30065, 1999.
42. Emilsson V, Arch JR, De Groot RP, Lister CA and Cawthorne MA: Leptin treatment increases suppressors of cytokine signaling in central and peripheral tissues. FEBS Lett 455: 170-174, 1999.
43. Tsumanuma I, Jin L, Zhang S, Bayliss JM, Scheithauer BW and Lloyd RV: Leptin signal transduction in the HP75 human pituitary cell line. Pituitary 3: 211-220, 2000.

44. Ziotopoulou M, Erani DM, Hileman SM, Bjorbaek C and Mantzoros CS: Unlike leptin, ciliary neurotrophic factor does not reverse the starvation-induced changes of serum corticosterone and hypothalamic neuropeptide levels but induces expression of hypothalamic inhibitors of leptin signaling. Diabetes 49: 1890-1896, 2000.
45. Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML and Ando S: Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. J Biol Chem 278: 28668-28676, 2003.
46. Okumura M, Yamamoto M, Sakuma H, Kojima T, Maruyama T, Jamali M, Cooper DR and Yasuda K: Leptin and high glucose stimulate cell proliferation in MCF-7 human breast cancer cells: reciprocal involvement of PKC-alpha and PPAR expression. Biochim Biophys Acta 1592: 107-116, 2002.
47. Blumenstein M, Bowen-Shauver JM, Keelan JA and Mitchell MD: Identification of suppressors of cytokine signaling (SOCS) proteins in human gestational tissues: differential regulation is associated with the onset of labor. J Clin Endocrinol Metab 87: 1094-1097, 2002.
48. Bouloumie A, Drexler HC, Lafontan M and Busse R: Leptin, the product of Ob gene, promotes angiogenesis. Circ Res 83: 1059-1066, 1998.
49. Oda A, Taniguchi T and Yokoyama M: Leptin stimulates rat aortic smooth muscle cell proliferation and migration. Kobe J Med Sci 47: 141-150, 2001.
50. Liu Z, Uesaka T, Watanabe H and Kato N: High fat diet enhances colonic cell proliferation and carcinogenesis in rats by elevating serum leptin. Int J Oncol 19: 1009-1014, 2001.
51. Oelmann E, Haghgu S, Kulimova E, Serve H, Schmitmann C and Berdel WE: Re: Leptin - a growth factor in normal and malignant breast cells and for normal mammary gland development. J Natl Cancer Inst 95: 1171-1173, 2003.
52. Cleary MP, Juneja SC, Phillips FC, Hu X, Grande JP and Maihle NJ: Leptin receptor-deficient MMTV-TGF-alpha/Lepr(db)Lepr(db) female mice do not develop oncogene-induced mammary tumors. Exp Biol Med (Maywood) 229: 182-193, 2004.
53. Somasundar P, Riggs D, Jackson B, Vona-Davis L and McFadden DW: Leptin stimulates esophageal adenocarcinoma growth by non-apoptotic mechanisms. Am J Surg 186: 575-578, 2003.
54. Somasundar P, Frankenberry K, Skinner H, Vedula G, McFadden DW, Riggs D, Jackson B, van Gilder R, Hileman SM and Vona-Davis LC: Prostate cancer cell proliferation is influenced by leptin. J Surg Res 118: 71-82, 2004.
55. Martin-Romero C and Sanchez-Margalet V: Human leptin activates PI3K and MAPK pathways in human peripheral blood mononuclear cells: possible role of Sam68. Cell Immunol 212: 83-91, 2001.
56. Sanchez-Margalet V, Martin-Romero C, Gonzalez-Yanes C, Goberna R, Rodriguez-Bano J and Muniain MA: Leptin receptor (Ob-R) expression is induced in peripheral blood mononuclear cells by *in vitro* activation and *in vivo* in HIV-infected patients. Clin Exp Immunol 129: 119-124, 2002.
57. Catalano S, Mauro L, Marsico S, Giordano C, Rizza P, Rago V, Montanaro D, Maggiolini M, Panno ML and Ando S: Leptin induces, via ERK1/ERK2 signal, functional activation of estrogen receptor alpha in MCF-7 cells. J Biol Chem 279: 19908-19915, 2004.
58. Attoub S, Noe V, Pirola L, Bruyneel E, Chastre E, Mareel M, Wymann MP and Gespach C: Leptin promotes invasiveness of kidney and colonic epithelial cells via phosphoinositide 3-kinase-, rho-, and rac-dependent signaling pathways. FASEB J 14: 2329-2338, 2000.
59. Schneider R, Bornstein SR, Chrousos GP, Boxberger S, Ehninger G and Breidert M: Leptin mediates a proliferative response in human gastric mucosa cells with functional receptor. Horm Metab Res 33: 1-6, 2001.
60. Fry MJ: Phosphoinositide 3-kinase signalling in breast cancer: how big a role might it play? Breast Cancer Res 3: 304-312, 2001.
61. Berrie CP: Phosphoinositide 3-kinase inhibition in cancer treatment. Expert Opin Investig Drugs 10: 1085-1098, 2001.
62. Lemke LE, Paine-Murrieta GD, Taylor CW and Powis G: Wortmannin inhibits the growth of mammary tumors despite the existence of a novel wortmannin-insensitive phosphatidylinositol-3-kinase. Cancer Chemother Pharmacol 44: 491-497, 1999.
63. Mills GB, Kohn E, Lu Y, Eder A, Fang X, Wang H, Bast RC, Gray J, Jaffe R and Hortobagyi G: Linking molecular diagnostics to molecular therapeutics: targeting the PI3K pathway in breast cancer. Semin Oncol 30: 93-104, 2003.
64. Boehle AS, Kurdow R, Boenicke L, Schniewind B, Faendrich F, Dohrmann P and Kalthoff H: Wortmannin inhibits growth of human non-small-cell lung cancer *in vitro* and *in vivo*. Langenbecks Arch Surg 387: 234-239, 2002.
65. Schultz RM, Merriman RL, Andis SL, Bonjouklian R, Grindey GB, Rutherford PG, Gallegos A, Massey K and Powis G: *In vitro* and *in vivo* antitumor activity of the phosphatidylinositol-3-kinase inhibitor, wortmannin. Anticancer Res 15: 1135-1139, 1995.
66. Semba S, Itoh N, Ito M, Harada M and Yamakawa M: The *in vitro* and *in vivo* effects of 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a specific inhibitor of phosphatidylinositol 3'-kinase, in human colon cancer cells. Clin Cancer Res 8: 1957-1963, 2002.
67. Su JD, Mayo LD, Donner DB and Durden DL: PTEN and phosphatidylinositol 3'-kinase inhibitors up-regulate p53 and block tumor-induced angiogenesis: evidence for an effect on the tumor and endothelial compartment. Cancer Res 63: 3585-3592, 2003.