Leptin—A Growth Factor in Normal and Malignant Breast Cells and for Normal Mammary Gland Development

Xin Hu, Subhash C. Juneja, Nita J. Maihle, Margot P. Cleary

Background: Obesity is a risk factor for breast cancer in postmenopausal women. As body weight and fat mass increase, circulating leptin increases. Leptin is an adipocyte-derived cytokine that acts through the long form of its receptor, termed OB-Rb. To investigate whether leptin is associated with breast cancer, we determined the expression of OB-Rb in human breast epithelial HBL100 cells and human breast carcinoma-derived T-47D cells, determined whether leptin influenced the proliferation of these cells, and evaluated the structure of mammary tissue in genetically obese leptin-deficient LepobLepob and leptin receptor-deficient Lepr dbLepr db mice. Methods: Cell numbers and cell colony formation by HBL100 and T-47D cells were determined by anchorage-dependent and anchorage-independent growth assays. OB-Rb expression was examined by reverse transcription–polymerase chain reaction and immunoblot analyses. Expression of leptin signaling pathway components was evaluated with immunoblot and electrophoretic mobility shift assays. Mammary gland development in lean and obese mice was investigated in whole-mount studies. All statistical tests were two-sided. Results: Leptin enhanced anchorage-dependent proliferation by 138% (95% confidence interval [CI] = 108% to 169%) in T-47D cells and 50% (95% CI = 38% to 60%) in HBL100 cells. In both cell lines, OB-Rb was expressed, and leptin increased the expression of phosphorylated signal transducers and activators of transcription 3 (STAT3), phosphorylated extracellular signal-regulated kinase (ERK), and transcription factor, termed OB-Rb. To investigate whether leptin is involved in the regulation of normal mammary gland development, we determined the expression of OB-Rb in human breast epithelial HBL100 cells and human breast tumor cell lines and breast tumors. Whether leptin is involved in the regulation of normal mammary gland development has not been examined extensively. Specifically, neither OB-R mRNA nor OB-R protein has been identified in either normal or malignant mammary tissues or in breast cancer-derived cell lines. The expression of leptin has been described, however, in normal human mammary tissues (41,42) and in human breast tumor cell lines and breast tumors (42). Leptin-deficient, genetically obese Lepob dbLepob mice (14) exhibit a decreased incidence of spontaneous mammary tumors (8). In addition, we have found that the incidence of oncogene-induced mammary tumor development in leptin-deficient obese Lepob dbLepob mice that overexpress human transforming growth factor-α (TGF-α) is statistically significantly decreased (0 of 59 mice) in contrast to that in their lean littermates (45 of 80 mice) (43). Similarly, genetically obese leptin receptor-deficient Lepr dbLepr db mice (23) that overexpress TGF-α also do not develop mammary tumors (0 of 42 mice), whereas their lean

Higher body weight appears to play a role in the development of breast/mammary tumors. Obesity has been identified as a risk factor for breast cancer in postmenopausal women (1–3), and higher body weight is associated with increased incidence of both spontaneous and chemically induced mammary tumors in rodents (4–9). The pathogenesis of obesity and its relationship to breast cancer, however, have not yet been delineated. Higher levels of circulating estrogen that have been attributed to elevated aromatase activity in expanded adipose tissue depots have been cited as a potential contributing factor (10). Higher insulin levels also are associated with obesity and may enhance cellular proliferation leading to the development of breast cancer (11). Another serum growth factor positively associated with body weight and/or body fat is leptin, i.e., circulating leptin increases as body weight and fat mass increase (12,13). Leptin is a cytokine-like protein secreted from adipose tissues (14). Initial investigations identified leptin receptors, termed OB-R, in the brain (15), which led to the conclusion that leptin is involved in a feedback system related to body weight regulation. Further studies, however, have shown that leptin receptors are expressed in many other tissues, including placenta, pancreas, stomach, adrenal gland, hematopoietic cells, liver, lung, and heart (16–20). Alternative splicing of the OB-R transcript results in six identified isoforms (21,22). It is generally accepted that the long isoform commonly identified as OB-Rb is responsible for signal transduction (23–26). In several different tissues, leptin signaling has been shown to be mediated through the activation of signal transducers and activators of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), and transcription activator protein 1 (AP-1) pathways (26–28).

Leptin may regulate cell proliferation in diverse normal and malignant tissues. The leptin receptor OB-R has been identified in malignant cells of diverse origins, including lung and gastric carcinomas and leukemic cells (29–33). Leptin stimulates the proliferation of certain normal hematopoietic and epithelial cells (34–38), the squamous lung cancer cell line SQ-5 (30), and the human colon cancer cell line HT29 (39). Leptin has also been shown to promote the invasiveness of premalignant colon and kidney epithelial cells in vitro (40).

Whether leptin is involved in the regulation of normal mammary gland development has not been examined extensively. Specifically, neither OB-R mRNA nor OB-R protein has been identified in either normal or malignant mammary tissues or in breast cancer-derived cell lines. The expression of leptin has been described, however, in normal human mammary tissues (41,42) and in human breast tumor cell lines and breast tumors (42). Leptin-deficient, genetically obese Lepob dbLepob mice (14) exhibit a decreased incidence of spontaneous mammary tumors (8). In addition, we have found that the incidence of oncogene-induced mammary tumor development in leptin-deficient obese Lepob dbLepob mice that overexpress human transforming growth factor-α (TGF-α) is statistically significantly decreased (0 of 59 mice) in contrast to that in their lean littermates (45 of 80 mice) (43). Similarly, genetically obese leptin receptor-deficient Lepr dbLepr db mice (23) that overexpress TGF-α also do not develop mammary tumors (0 of 42 mice), whereas their lean}

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counterparts develop mammary tumors with an incidence rate of 70% (56 of 79 mice) (Cleary MP: unpublished results).

These observations and epidemiologic studies implicating obesity as a risk factor in human breast tumorigenesis suggest that leptin may contribute to the malignant transformation of breast epithelial cells. The purpose of this study was to obtain experimental evidence to determine whether leptin has a direct role in proliferation of both normal and malignant breast epithelial cell lines and to determine the expression of the leptin receptor in these cell lines. Furthermore, we determined whether the presence of the leptin receptor was associated with leptin-induced activation of the downstream signaling proteins ERK, STAT3, and AP-1. In addition, we evaluated the epithelial component of mammary glands from both leptin-deficient Lep<sup>ob</sup>Lep<sup>ob</sup> and leptin-receptor-deficient Lepr<sup>ob</sup>Lepr<sup>ob</sup> mice.

**METHODS**

**Cell Culture**

The breast epithelial cell line used for this study was HBL100, which was initially derived from the milk of a nursing mother 3 days after delivery (44). The breast cancer cell line chosen was the estrogen receptor-positive T-47D cell line, which was isolated from a pleural effusion obtained from a 54-year-old female with an infiltrating ductal carcinoma of the breast (45). HBL100 cells were cultured in McCoy’s 5A medium (BioWhittaker, Inc., Walkersville, MD) with 10% fetal bovine serum. Cells were maintained at 37 °C in an incubator in a humidified atmosphere of 5% CO2. Cells were subcultured after incubation at 37 °C for 2 minutes with 0.05% trypsin and 0.02% EDTA. We evaluated 10 lots of fetal bovine serum (Multispecies Leptin RIA Kits, product XL-85K; Linco Research, Inc., St. Charles, MO) and detected no leptin. The lowest level of detection of this assay procedure is 0.2 ng/mL.

**Cell Growth Assays**

Cells were incubated overnight in 25-cm<sup>2</sup> flasks (4 × 10<sup>5</sup> cells per cm<sup>2</sup>). Cells were then incubated with leptin (100 ng/mL; ProproTech Inc., Rocky Hill, NJ) or without leptin for up to 10 days at 37 °C. Medium and leptin were renewed every 2 days, or cells were harvested and counted (Coulter Counter model ZM; Coulter Electronics Ltd., Luton, U.K.). Triplicate observations were made at 0, 2, 4, 6, 8, and 10 days after cells were originally cultured. Trypan blue exclusion indicated 99%–100% viable cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed in 96-well plates (46). Cells were incubated overnight and then treated with leptin (0, 25, 50, or 100 ng/mL) for 48 hours, and 50 µL of MTT (2 mg/mL in RPMI-1640 medium) was added. After 4 hours, 150 µL of dimethyl sulfoxide was added, and optical density was determined with an enzyme-linked immunosorbant assay (ELISA) multwell reader (Labsystems, Inc., St. Paul, MN) at 540 nm. For each leptin concentration, 24 replicate wells were used.

**mRNA and Protein Expression of OB-Rb, the Long Form of the Leptin Receptor**

Total RNA from the two cell lines was extracted (TRIzol reagent kit; Life Technologies, Rockville, MD). The primer sequences for OB-Rb and hypoxanthine phosphoribosyltransferase (HPRT) (positive control) were as described (47). Reverse transcription–polymerase chain reaction (RT–PCR) was performed (One-Step RT–PCR kit; Life Technologies), and products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Expected sizes for RT–PCR products were 388 base pairs (bp) for OB-R mRNA and 290 bp for human HPRT. For immunoprecipitation followed by western blot analysis, 500 µg of protein from cell lysates was immunoprecipitated with 10 µg of anti-leptin receptor antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C on an orbital shaker. Immunocomplexes were captured by adding 100 µL of protein–A/G PLUS–agarose beads (Santa Cruz Biotechnology, Inc.) and gently rocking on an orbital shaker for 4 hours at 4 °C. The pellet was collected by centrifugation at 1000g for 5 minutes at 4 °C. Immunoprecipitated proteins were then fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 6% gels and immunoblotted with the OB-R antibody. Controls used included the omission of cell lysates or the anti-OB-R antibody during immunoprecipitation or the use of normal rabbit serum (Santa Cruz Biotechnology, Inc.) as the primary antibody instead of the anti-OB-R antibody in western blot determinations.

**Western Blots of STAT3 and ERK**

Cells were cultured in six-well plates (25 000 cells per well) in serum-free medium for 18 hours. For STAT3 determination, cells were treated with leptin (0, 25, 50, or 100 ng/mL) at 37 °C for 5 minutes. For ERK determination, cells were incubated with leptin (100 ng/mL) for 1, 3, 5, or 10 minutes. At the end of the incubation, the cells were placed on ice, washed once with cold phosphate-buffered saline (PBS), and lysed in lysis buffer (62.5 mM Tris–HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 1% bromphenol blue). Cell lysates were heated at 95 °C for 5 minutes and fractionated by SDS–PAGE on 8% gels. Proteins were transferred to Immobilon membranes (Millipore, Bedford, MA) and probed with antibodies against phosphorylated STAT3 (Santa Cruz Biotechnology, Inc.) and phosphorylated ERK (New England Biolabs, Inc., Beverly, MA). Antibody bounds were detected by enhanced chemifluorescence (ECF substrate; Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed with the Storm 840 PhosphorImager® (Molecular Dynamics-Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The membranes were stripped in buffer (7 M guanidine hydrochloride, 50 mM glycine [pH 10.8], 0.05 mM EDTA, 0.1 M KCl, and 20 mM 2-mercaptoethanol) and probed with antibodies against phosphorylated STAT3 (Santa Cruz Biotechnology, Inc.) and ERK (New England Biolabs, Inc.) to provide a positive control, and normal rabbit and mouse serum were used instead of the primary antibody as the negative control.

**AP-1 DNA Binding**

AP-1 DNA binding was determined by electrophoretic mobility shift assay (48). Cells were cultured in 10-cm dishes, starved in serum-free medium for 18 hours, treated with leptin (0 or 25 ng/mL) at 37 °C for 12 hours, harvested, and disrupted by lysis in 500 µL of buffer A (25 mM HEPES [pH 7.8], 50 mM KCl, 0.5% Nonidet P-40, 100 µM dithiothreitol, leupetin at 10 µg/mL, aprotinin at 25 µg/mL, and 1 mM phenylmethylsulfonyl
Mice and Whole Mammary Tissue Mounts

Anchorage-Independent Cell Growth Assays

Cells (8000 per well) were plated in 0.33% agar over an underlayer of 0.5% agar. Agar contained basal medium Eagle (BME) medium containing 10% heat-inactivated fetal bovine serum, and both agar layers contained leptin at 0 or 100 ng/mL. Cells were incubated for 3 weeks, and the number of colonies was scored as described by Colburn et al. (49). Cells were incubated for 3 weeks, and the number of colonies was scored as described by Colburn et al. (49).

Mice and Whole Mammary Tissue Mounts

Homozygous lean (Lep<sup>+</sup>Lep<sup>+</sup> and Lepr<sup>+</sup>Lepr<sup>+</sup>) and obese (Lep<sup>ob</sup>Lep<sup>ob</sup> and Lepr<sup>ob</sup>Lepr<sup>ob</sup>) female mice were obtained from colonies maintained at the Hormel Institute. The Hormel Institute is accredited by the American Association for Accreditation of Laboratory Animal Care. This study was approved by the University of Minnesota Animal Care and Use Committee. Mice were anesthetized and mammary fat pads were excised, fixed in acetone, stained with hematoxylin, destained, and placed in Permount (Sigma Chemical Co., St. Louis, MO) on a glass slide with a glass coverslip.

Statistical Analysis

All determinations were repeated three times, and similar results were obtained. Representative results are presented as means with 95% confidence intervals. Statistical evaluations were made by analysis of variance or by Student’s t test (data meet the requirements for using Student’s t test). Analysis of variance was followed by Dunnett’s multiple comparison test to compare results with control values without leptin (see Fig. 1, C and D) and with the control leptin-stimulated value (see Table 1). All statistical tests were two-sided.

RESULTS

To examine whether leptin stimulates the proliferation of normal breast epithelial cells (HBL100) or their transformed counterparts (T-47D), we treated HBL100 (Fig. 1, A) and T-47D (Fig. 1, B) cells with leptin (100 ng/mL) and monitored cell proliferation over time. With both lines, we consistently observed greater cell proliferation in leptin-stimulated cultures than in leptin-unstimulated cultures (i.e., at 2, 4, 6, 8, and 10 days). In general, the response was more pronounced in the T-47D cells, as demonstrated by an increase in cell number of 138% (95% CI = 108% to 169%) for cells cultured with leptin compared with cells cultured without leptin after 10 days; a similar comparison using HBL100 cells resulted in an increase of only 50% (95% CI = 38% to 60%) with leptin versus without leptin. Similar results for cell proliferation in the presence of...
different concentrations of leptin were obtained with an MTT assay in HBL100 (Fig. 1, C) and T-47D (Fig. 1, D) cells.

To determine whether treatment with leptin activated the Janus kinase (JAK)/STAT kinase cascade and the ERK/AP-1 pathway through the long form of the leptin receptor OB-Rb, as described for other cell types, we examined both HBL100 and T-47D cell lines for the expression of OB-Rb by RT–PCR and by immunoprecipitation followed by western blot analysis. In both lines, we detected a single 338-bp band encoding OB-Rb after RT–PCR (Fig. 2, A) and two immunoreactive protein bands of 205 kd and 230 kd on western blots (Fig. 2, B). This expression pattern for OB-Rb is in agreement with previous reports (50,51) describing single leptin receptor transcript encoding 205-kd and 230-kd proteins.

It has been reported that the addition of leptin to mouse adipose tissue activated STAT3, ERK, and AP-1 pathways. Having determined that OB-Rb is expressed in HBL100 and T-47D cells, we hypothesized that these pathways also would be activated by leptin in these normal and malignant breast epithelial cell lines. First, we determined whether STAT3 phosphorylation would increase when these cells were stimulated with leptin. We examined the tyrosine phosphorylation of STAT3 by western blot analysis. After a 5-minute incubation with leptin (25, 50, or 100 ng/mL), the level of phosphorylated STAT3 was greater in leptin-stimulated HBL100 and T-47D cells than in untreated control HBL100 and T-47D cells (Fig. 3, A). Addition of AG490, a JAK2/STAT3 inhibitor, at 50 μM (data not shown) or 100 μM (Table 1) prevented leptin-stimulated proliferation of HBL100 and T-47D cells, as assessed by MTT assay.

Leptin was added to HBL100 and T-47D cell cultures, and levels of phosphorylated ERK1 and/or phosphorylated ERK2 were monitored. Levels of phosphorylated ERK1 and ERK2 began to increase 3 minutes after the addition of leptin, reached peak levels after 5 minutes, and decreased to near baseline levels at 10 minutes (Fig. 3, B). We suspect that this reflects classic desensitization kinetics that has been reported for a variety of other agonists of ERK activation. Addition of U0126, an ERK inhibitor, at 5 μM (data not shown) or 10 μM (Table 1) prevented leptin-stimulated proliferation of both cell lines. These results using specific inhibitors of JAK2/STAT3 and ERK phosphorylation, and thus their activation, indicate that leptin’s effect on the proliferation of normal and malignant breast epithelial cells is mediated through the JAK2/STAT3 and ERK pathways.

We next examined leptin-stimulated AP-1 DNA-binding activity in these two cell lines. The specificity of DNA-binding activity for AP-1 was confirmed by the observation that a 10-fold excess of unlabeled AP-1 probe inhibited the binding of the labeled probe to the nuclear proteins tested (Fig. 3, C, lane 1). AP-1 DNA-binding activity was twofold higher in leptin-treated cells than in untreated control cells (HBL100 cells: Fig. 3, C, compare lane 3 with lane 2, respectively; T-47D cells: Fig. 3, C, compare lane 5 with lane 4, respectively).

These results indicate that leptin stimulates the activation of signaling pathways previously shown to mediate leptin-dependent cell proliferation. We hypothesized, therefore, that leptin also might stimulate these cells to grow in an anchorage-independent manner. We analyzed the ability of leptin to stimulate colony formation by both cell lines in soft agar. In the absence of leptin, many more colonies (sevenfold increase) were formed by transformed T-47D cells than by HBL100 cells.

### Table 1. Chemical inhibition effects on leptin-stimulated cell proliferation as monitored with an MTT assay*

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Optical density value (HBL100)</th>
<th>Optical density value (T-47D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>0.237 (0.221 to 0.253)</td>
<td>0.224 (0.208 to 0.240)</td>
</tr>
<tr>
<td>Leptin + AG490</td>
<td>0.182 (0.174 to 0.190)</td>
<td>0.177 (0.163 to 0.183)</td>
</tr>
<tr>
<td>Leptin + U0126</td>
<td>0.173 (0.163 to 0.183)</td>
<td>0.175 (0.159 to 0.191)</td>
</tr>
<tr>
<td>No stimulation</td>
<td>0.175 (0.159 to 0.191)</td>
<td>0.186 (0.170 to 0.202)</td>
</tr>
</tbody>
</table>

*Cells were incubated overnight in 96-well plates and then treated with leptin (100 ng/mL), leptin (100 ng/mL) + G490 (100 μM), or leptin (100 ng/mL) + U0126 (10 μM) for 48 hours. Optical density values are means (95% CIs) of 24 replicate wells. MTT = 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide.

†Indicates P value compared with the cells treated with leptin alone by analysis of variance followed by Dunnett’s multiple comparison test.
Treatment with leptin at 100 ng/mL statistically significantly increased (P = .001) colony formation of T-47D cells by 81% (95% CI = 62% to 101%) but did not enhance colony formation by the normal HBL100 cells (Fig. 4).

Micrographs of whole-mount preparations of mammary tissues for leptin-deficient and leptin receptor-deficient mice are shown in Fig. 5. Ductal branching is prominent in the whole-mount preparations from the lean Lepr+Lepr+ (Fig. 5, A) and LeprLepr+ (Fig. 5, B) mice, but there is a notable lack of mammary duct formation in both strains of genetically obese mice, Lepr+Lepr+ (Fig. 5, B) and LeprLeprdb (Fig. 5, D), compared with their respective lean counterparts.

**DISCUSSION**

We present evidence that leptin can stimulate the proliferation of both normal and malignant breast epithelial cells by activating OB-Rb, the long form of the leptin receptor. Specifically, physiologic concentrations of leptin (i.e., 25–100 ng/mL) activated both JAK/STAT and ERK/AP-1 pathways in these cells. These pathways previously have been shown to mediate leptin-dependent signal transduction in other cell types (26–28).

Interestingly, leptin stimulation enhanced anchorage-independent growth of the breast cancer cell line T-47D but not the normal cell line HBL100. In mice with an absolute leptin deficiency (i.e., LepobLepob mice) or in those with a defect in leptin receptor-dependent signaling (i.e., Lepr dbLeprdb mice), all aspects of epithelial development and mammary gland morphogenesis were impaired.

The adipocyte-derived hormone leptin is an essential mediator of energy homeostasis in mice and humans. Although the initial studies implicating leptin in human obesity were controversial, recent genetic studies clearly demonstrate that even a modest decrease in functional leptin levels is compensated for by an increased fat mass in humans (52). Adipocytes, however, are not the only tissue that expresses leptin. Leptin transcripts have been identified in both placenta (53) and muscle (54), and leptin can be expressed in both normal and malignant breast tissues (41,42).

The first suggestion that leptin might play a pivotal role in mammary tumor development was based on the observation that, when transgenic leptin-deficient genetically obese Lepr+Lepr+ mice were produced that overexpress TGF-α, no mammary tumors developed (0 of 59 mice) (43). In contrast, the original transgenic strain of TGF-α mice exhibited a mammary tumor incidence rate of 30% by 15 months of age (55), and
with a large network of ductal branching. Long and short isoforms of the leptin receptor in various regions of the brain (1–3). Obesity is considered a risk factor for human breast cancer, and obese women have more aggressive disease at diagnosis with a poorer prognosis (3,56). Thus, increased serum concentrations of leptin may enhance the proliferation of leptin-responsive breast tumor cells. In this regard, we recently found that transgenic TGF-α homozygous lean Lep\textsuperscript{+Lep\textsuperscript{+}} female mice, who gain weight and become obese by consuming a high-fat diet (i.e., obesity-prone mice), developed mammary tumors at a younger age than did transgenic TGF-α homozygous lean Lep\textsuperscript{+Lep\textsuperscript{+}} mice who were fed the same high-fat diet but did not become obese (i.e., obesity-resistant mice) (Cleary MP, Jacobson TL, Grande JP, Maihle NJ: unpublished observation). Furthermore, a higher incidence of high-grade adenocarcinomas was observed in obesity-prone mice than in obesity-resistant mice. We have recently determined, in a similar protocol, that TGF-α obesity-prone mice had serum leptin levels threefold higher than obesity-resistant mice and fivefold higher than mice fed a low-fat diet in association with a shortened mammary tumor latency (Cleary MP, Grande JP, Maihle NJ: unpublished observation). These results suggest that, in genetically similar animals, the dietary effect on serum leptin and body fat levels can modulate aspects of mammary tumor development.

Our findings are consistent with the hypothesis that the elevated serum leptin levels associated with obesity may promote breast cancer development. Moreover, such a hypothesis might explain why weight gain, which is reportedly accompanied by higher than expected serum leptin concentrations (relative to the body weight attained), has been associated with increased breast cancer risk. Although estrogen receptor-positive breast tumors are usually more responsive to therapy than estrogen receptor-negative tumors, there is a report that estrogen receptor-positive breast tumor status in obese women is actually associated with a poorer prognosis than is estrogen receptor-negative status (57). In this study, we found that the T-47D cell line, an estrogen-sensitive cell line, showed a dramatic increase in anchorage-independent growth after treatment with leptin. Evidence is emerging of potential interrelationships between leptin and estrogen. For example, in 8-week-old female rats, both estrogen treatment and ovariectomy altered the relative expression of the long and short isoforms of the leptin receptor in various regions of the brain (58). In another study also using young adult female rats (59), ovariectomy initially resulted in lower serum leptin levels than those detected in mice that were ovariectomized and then treated with estradiol; however, 13 weeks after surgery, serum leptin levels in these two groups were similar. Several human studies have evaluated leptin status in women receiving hormone replacement therapy, but the results are inconclusive as to how this treatment affects serum leptin levels (60–63). Estrogen also appears to modulate leptin gene expression in adipose tissue (64,65). It is interesting to note that two recent studies

**Fig. 5.** Whole-mount preparations demonstrating ductal branching of mammary tissue from lean and genetically obese mice. Mammary ducts = black arrows, stromal tissue = black triangles, and nerve fibers = white arrows. The large oval structure seen in all tissue samples is a lymph node. A) Homozygous lean Lep\textsuperscript{+Lep\textsuperscript{+}} at 101 weeks of age with a large network of ductal branching. B) Homozygous obese Lep\textsuperscript{obLep\textsuperscript{ob}} at 44 weeks of age with very little ductal branching. C) Homozygous lean Lepr\textsuperscript{+Lepr\textsuperscript{+}} at 85 weeks of age with a large network of ductal branching. D) Homozygous obese Lepr\textsuperscript{obLepr\textsuperscript{ob}} at 86 weeks of age with very little ductal branching. Scale bar = 4 mm. transgenic TGF-α mice that were either homozygous Lep\textsuperscript{+Lep\textsuperscript{+}} or heterozygous Lep\textsuperscript{+Lep\textsuperscript{ob}} lean for the Lep gene exhibited mammary tumor incidence rates of 50% and 67%, respectively, by 24 months of age (43). Both the transgenic TGF-α homozygous Lep\textsuperscript{+Lep\textsuperscript{+}} and TGF-α heterozygous Lep\textsuperscript{+Lep\textsuperscript{ob}} mice have detectable circulating leptin levels, although as expected from their genotypes, the concentration in the TGF-α homozygous Lep\textsuperscript{+Lep\textsuperscript{+}} mice was approximately twice that in the transgenic TGF-α heterozygous Lep\textsuperscript{+Lep\textsuperscript{ob}} lean mice. We also have found that genetically obese leptin receptor-deficient Lep\textsuperscript{obLepr\textsuperscript{ob}} mice do not form mammary tumors in response to the overexpression of TGF-α (Cleary MP: unpublished observation). As shown in Fig. 5, in whole-mount preparations of mammary tissue, normal mammary gland morphogenesis is impaired in both nontransgenic genetically obese leptin-deficient and genetically obese leptin receptor-deficient mice. Similar results recently have been reported (43) for the transgenic TGF-α/Lep\textsuperscript{obLep\textsuperscript{ob}} mice. These findings suggest that both leptin and an intact leptin-signaling pathway are necessary for normal mammary gland development and, perhaps, for mammary gland tumorigenesis in vivo.

The effects of leptin on both mammary gland development and tumorigenesis are important in light of the epidemiologic studies suggesting a relationship between obesity and breast carcinoma (1–3). Obesity is considered a risk factor for human breast cancer, and obese women have more aggressive disease at diagnosis with a poorer prognosis (3,56). Thus, increased serum concentrations of leptin may enhance the proliferation of leptin-responsive breast tumor cells. In this regard, we recently found that transgenic TGF-α homozygous lean Lep\textsuperscript{+Lep\textsuperscript{+}} female mice, who gain weight and become obese by consuming a high-fat diet (i.e., obesity-prone mice), developed mammary tumors at a younger age than did transgenic TGF-α homozygous lean Lep\textsuperscript{+Lep\textsuperscript{+}} mice who were fed the same high-fat diet but did not become obese (i.e., obesity-resistant mice) (Cleary MP, Jacobson TL, Grande JP, Maihle NJ: unpublished observation). Furthermore, a higher incidence of high-grade adenocarcinomas was observed in obesity-prone mice than in obesity-resistant mice. We have recently determined, in a similar protocol, that TGF-α obesity-prone mice had serum leptin levels threefold higher than obesity-resistant mice and fivefold higher than mice fed a low-fat diet in association with a shortened mammary tumor latency (Cleary MP, Grande JP, Maihle NJ: unpublished observation). These results suggest that, in genetically similar animals, the dietary effect on serum leptin and body fat levels can modulate aspects of mammary tumor development.

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report increased serum leptin levels in women receiving tamoxifen as a treatment for breast cancer (66,67). Thus, there are many aspects of potential relationships between leptin and estrogen that can be addressed. We suggest that other breast cancer cell lines be evaluated for their response to leptin treatment, their leptin receptor status, leptin-stimulated anchorage-independent growth, and possibly their epidermal growth factor/ErbB receptor status to determine whether there is an association between estrogen receptor status and leptin sensitivity.

In conclusion, our study reveals that normal and transformed breast epithelial cells express the leptin receptor. In these cells, leptin can activate pathways involving STAT3, ERK, and AP-1, resulting in increased cell proliferation. However, anchorage-independent cell growth is enhanced only in the leptin-treated transformed breast cancer cell line T-47D. Mice deficient for leptin or for leptin receptor exhibit impaired mammary gland morphogenesis. Thus, these results suggest a novel association between leptin and estrogen product of extraglandular aromatization of plasma androstenedione. Thus, there are many aspects of potential relationships between leptin and estrogen that can be addressed. We suggest that other breast cancer cell lines be evaluated for their response to leptin treatment, their leptin receptor status, leptin-stimulated anchorage-independent growth, and possibly their epidermal growth factor/ErbB receptor status to determine whether there is an association between estrogen receptor status and leptin sensitivity.

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**NOTES**

Note added in proof: While this manuscript was under review, an article by Laud et al. (68) was published and it presented results also indicating that leptin enhances cell proliferation of T-47D cell line and confirming the presence of leptin receptors in this cell line. Leptin receptors in human breast tumor samples also were reported.

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