Evidences that Leptin Up-regulates E-Cadherin Expression in Breast Cancer: Effects on Tumor Growth and Progression

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Abstract

Leptin, a cytokine mainly produced by adipocytes, seems to play a crucial role in mammary carcinogenesis. In the present study, we explored the mechanism of leptin-mediated promotion of breast tumor growth using xenograft MCF-7 in 45-day-old nude mice, and an in vitro model represented by MCF-7 three-dimensional cultures. Xenograft tumors, obtained only in animals with estradiol (E2) pellet implants, doubled control value after 13 weeks of leptin exposure. In three-dimensional cultures, leptin and/or E2 enhanced cell-cell adhesion. This increased aggregation seems to be dependent on E-cadherin because it was completely abrogated in the presence of function-blocking E-cadherin antibody or EGTA, a calcium-chelating agent. In three-dimensional cultures, leptin and/or E2 treatment significantly increased cell growth, which was abrogated when E-cadherin function was blocked. These findings well correlated with an increase of mRNA and protein content of E-cadherin in three-dimensional cultures and in xenografts. In MCF-7 cells both hormones were able to activate E-cadherin promoter. Mutagenesis studies, electrophoretic mobility shift assay, and chromatin immunoprecipitation assays revealed that cyclic AMP–response element binding protein and Sp1 motifs, present on E-cadherin promoter, were important for the up-regulatory effects induced by both hormones on E-cadherin expression in breast cancer MCF-7 cells. In conclusion, the present study shows how leptin is able to promote tumor cell proliferation and homotypic tumor cell adhesion via an increase of E-cadherin expression. This combined effect may give reasonable emphasis to the important role of this cytokine in stimulating primary breast tumor cell growth and progression, particularly in obese women.

Introduction

Leptin is an adipocyte-derived hormone (1) that, in addition to the control weight homeostasis by regulating food intake and energy expenditure (2, 3), is implicated in the modulation of many other processes such as reproduction, lactation, hematopoiesis, immune responses, cell differentiation, and proliferation (4, 5). The activities of leptin are mediated through the transmembrane leptin receptor (ObR; refs. 6, 7) by activation of the Janus-activated kinase/signal transducers and activators of transcription (STAT) and mitogen-activated protein kinase (MAPK) pathways (8, 9).

Epidemiologic studies show a positive association between obesity and an increased risk of developing different cancers (10, 11). Several lines of evidence suggest that leptin and ObR are involved in the development of normal mammary gland and in mammary carcinogenesis (12–14). It has been recently reported that in primary breast tumors, leptin was detected in 86.4% of cases examined, and its expression was highly correlated with ObR (15). This indicates that leptin can influence breast cancer cells not only by endocrine and/or paracrine actions but also through autocrine pathways.

In epithelium and epithelium-derived tumors, cell-cell adhesion and tumor mass mostly depend on E-cadherin, a 120-kDa transmembrane molecule (16, 17). As it might be expected, E-cadherin seems to have a major influence on primary cancer development and evolution. Alteration in the function of E-cadherin and the cadherin-catenin complex has been implicated in cancer progression (18), invasion (19–21), and metastasis (22, 23).

In this study, we explored a new aspect of the involvement of leptin in initial steps of mammary tumorigenesis. Specifically, we asked whether leptin can affect primary tumor mass either in vivo in MCF-7 cell tumor xenograft or in vitro in MCF-7 three-dimensional cultures. Our results showed that leptin is able to promote tumor cell proliferation and homotypic tumor cell adhesion via an increase of E-cadherin expression. This combined effect may give reasonable emphasis to the important role of this cytokine in stimulating local primary breast tumor cell growth and progression, particularly in obese women.

Materials and Methods

Plasmids. The plasmids containing the human E-cadherin promoter or its deletions were given by Dr. Y.S. Chang (Chang-Gung University, Republic of China; ref. 24). pHEGO plasmid containing the full length of estrogen receptor α (ERα) cDNA was provided by Dr. D. Picard (University of Geneva). pSG5 vector containing the cDNA-encoding dominant-negative STAT3, which is a variant of the transcription factor STAT3 lacking an internal domain of 50 bp located near the COOH terminus (STAT3Δ), was given by Dr. J. Turkson (University of South Florida, College of Medicine, Tampa, FL). pCMV/myc vector containing the cDNA-encoding dominant-negative extracellular signal-regulated kinase 2 K52R (ERK2Δ) was provided by Dr. M. Cobb (Southwestern Medical Center, Dallas, TX).

Site-directed mutagenesis. The E-cadherin promoter plasmid bearing the cyclic AMP–responsive element binding protein (CREB)–mutated site (CREB mut) was created by site-directed mutagenesis using Quick Change kit (Stratagene, La Jolla, CA). We used as template the human E-cadherin.
promoter, and the mutagenic primers were as follows: 5'-AGGTTGTGAT- CACCTGATACGAGGAGGTCAGGACC-3' and 5'-GCGTTGTCGCAACTTG- TTGTGATACGAGGAGGTCAGGACC-3'. The constructed reporter vector was confirmed by DNA sequencing.

**Cell lines and culture conditions.** MCF-7, HeLa, and BT-20 cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 and HeLa cells were maintained in DMEM/F-12 containing 5% calf serum and BT-20 cells were cultured in MEM supplemented with 10% fetal bovine serum, 1% Eagle's nonessential amino acids, and 1% sodium pyruvate (Sigma, Milan, Italy). Cells were cultured in phenol red-free DMEM (serum-free medium), containing 0.5% bovine serum albumin, 24 h before each experiment. All media were supplemented with 1% t-glutamine and 1% penicillin/streptomycin (Sigma).

**In vivo studies.** The experiments in vivo were done in 45-day-old female nude mice (nu/nu Swiss; Charles River, Milan, Italy). At day 0, the animals were fully anesthetized by i.m. injection of 1.0 mg/kg Zoletil (Virbac) and 0.12% Xylor (Xylazine) to allow the s.c. implantation of estradiol (E$_2$) pellets (1.7 mg per pellet, 60-day release; Innovative Research of America, Sarasota, FL) into the intrascapular region of mice. The day after, exponentially growing MCF-7 cells (5.0 × 10$^6$ per mouse) were inoculated s.c. in 0.1 mL of Matrigel (BD Biosciences, Bedford, MA). Leptin treatment was started 24 h later, when animals were injected i.p. with either solutions: recombinant human leptin (230 μg/kg) diluted in saline + 0.3% bovine serum albumin (BSA) or saline + 0.3% BSA only (control). The treatment was done for 5 days a week until the 13th week. Tumor development was followed twice a week and 5 days after, tumor volume (V) was estimated by the following formula: V = L × W$^2$/2. At the time of killing (13 weeks), tumors were dissected out from the connecting nutritive tissue, frozen in nitrogen, and stored at −80°C. All the procedures involving animals and their care have been conducted in conformity with the institutional guidelines at the Laboratory of Molecular Oncogenesis, Regina Elena Cancer Institute in Rome.

**Three-dimensional spheroid culture and cell growth.** The cells were plated in single-cell suspension in 2% agar–coated plates and untreated or treated with 1,000 ng/mL leptin and/or E2 (100 nmol/L) for 48 h and then plated on six-well plates coated with 1.5 μg/mL recombinant human E-cadherin/Fc chimera. Before the experiment, the wells were blocked with 1% BSA for 3 h at 37°C and then washed with PBS.

After washing out nonadherent cells, adherent cells were incubated 3 h in medium containing 500 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution. The reaction product was measured at 570 nm.

**Total RNA extraction and reverse transcription-PCR assay.** Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA). Reverse transcription was done using RETROscript kit (Ambion, Austin, TX). The cDNAs were amplified by PCR using the following primers: 5'-TCTAAGATGGAGGACCTATG-3' and 5'-CCGGTGATAGGACAGGAGT- TGTT-3' (cyclin D1), 5'-TGGAATCCAGGAGAATTGC-3' and 5'-TATGTTGC- CAAAGCTTCTATTCGCA-3' (E-cadherin), and 5'-CTCAATCATCCTCCCCTTTCCTC-3' and 5'-CAATTCCTTGCGGAGGG-3' (36B4). The PCR was done for 30 cycles for cyclin D1 (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) and E-cadherin (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) and 15 cycles (94°C for 1 min, 59°C for 1 min, and 72°C for 2 min) to amplify 36B4, in the presence of 1 μL of first-strand cDNA, 1 μmol/L each of the primers mentioned above, deoxynucleotide triphosphate (0.5 mmol/L), Taq DNA polymerase (2 units per tube; Promega, Madison, WI) in a final volume of 25 μL.

**Western blot analysis.** Equal amounts of total protein were resolved on an 8% to 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with the appropriated antibody. The antigen-antibody complex was detected by incubation of the membrane at room temperature with a peroxidase-coupled goat anti-mouse or anti-rabbit IgG and revealed using the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

**Figure 1.** Effect of leptin on growth of MCF-7 cell tumor xenografts. A, xenografts were established with MCF-7 cells in female mice implanted with E2 pellet. One group was treated with 230 μg/kg leptin (p, n = 5) and a second group with vehicle (○, n = 5). *, P < 0.05, treated versus control group. Representative Western blot on protein extracts from xenografts excised from control mice and mice treated with leptin showing MAPK (B) and STAT3 (C) activation. The immunoblots were stripped and reprobed with total MAPK and STAT3, which serve as the loading control. pMAPK, phosphorylated MAPK; pSTAT, phosphorylated STAT. Columns, mean of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%; bars, SE. *, P < 0.05.
Leptin-enhanced cell-cell adhesion and proliferation depend on E-cadherin function. A, E-cadherin–positive MCF-7 cells were seeded in 2% agar–coated plates and cultured as three-dimensional spheroids (a–d). To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; α-E-cad) or EGTA (4 mmol/L). Cells were untreated (a) or treated with leptin (b), E2 (c), and leptin plus E2 (d) for 48 h and then photographed under phase-contrast microscopy. Bar, 50 μm. B, six-well plates were coated with E-cadherin/Fc recombinant protein, and binding of cells were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Columns, mean of five wells; bars, SE. C, proliferation of MCF-7 cells treated with leptin and/or E2 for 48 h in the absence or presence of E-cadherin antibody (1:100 dilution; α-E-cad) or EGTA (4 mmol/L). Columns, average of three experiments; bars, SE. Representative results. *, P < 0.05.

**Transfection assay.** MCF-7 cells were transfected using the FuGENE 6 reagent (Promega) with the mixture containing 0.5 μg of human E-cadherin promoter constructs. HeLa cells were transfected with E-cadherin promoter (0.5 μg per well) in the presence or absence of HEGO (0.2 μg per well) or cotransfected with STAT3 or ERK2 dominant negative (0.5 μg per well). Twenty-four hours after transfection, the cells were treated with 1,000 ng/mL leptin and/or 100 nmol/L E2 for 48 h. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK Renilla luciferase plasmid (5 ng per well) was used. Firefly and Renilla luciferase activities were measured by Dual Luciferase kit. The firefly luciferase data for each sample were normalized based on the transfection efficiency measured by Renilla luciferase activity.

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared from MCF-7 as previously described (25). The probe was generated by annealing single-stranded oligonucleotides, labeled with [γ-32P]ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors are as follows: CRE, 5′-TGAGGTCAGGAGTTCCAGACC-3′; Sp1, 5′-ATCACG-GGTACGGGGGGGGGTGTCCCGGGG-3′. In vitro translated and labeled CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system (Promega). The protein-binding reactions were carried out in 20 μL of buffer [20 mmol/L HEPES (pH 8), 1 mmol/L EDTA, 50 mmol/L KCl, 10 mmol/L DTT, 10% glycerol, 1 mg/mL BSA, 50 μg/mL poly(dI-dC) with 50,000 cpm] of labeled probe, 20 μg of MCF-7 nuclear protein or an appropriate amount of CREB protein or Sp1 human recombinant protein (Promega), and 5 μg of poly(dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. The specificity of the binding was tested by adding to the mixture reaction–specific antibodies (anti-CREB and anti-Sp1). Mithramycin A (100 μmol/L; ICN Biomedicals, Inc., Costa Mesa, CA) was incubated with the labeled probe for 30 min at 4°C before the addition of nuclear extracts. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× Tris borate-EDTA for 3 h at 150 V.

**Chromatin immunoprecipitation assay.** We followed chromatin immunoprecipitation (ChIP) methodology described by Morelli et al. (26). MCF-7 cells were untreated or treated with 1,000 ng/mL leptin and/or 100 nmol/L E2 for 1 h. The cells were then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleaved with sonicated salmon DNA/protein A agarose (Upstate Biotechnology, Inc., Lake Placid, NY) and immunoprecipitated with anti-CRE or anti-Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Pellets were washed as reported (26), eluted from MCF-7 as previously described (25). The probe was generated by annealing single-stranded oligonucleotides, labeled with [γ-32P]ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors are as follows: CRE, 5′-TGAGGTCAGGAGTTCCAGACC-3′; Sp1, 5′-ATCACG-GGTACGGGGGGGGGTGTCCCGGGG-3′. In vitro translated and labeled CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system (Promega). The protein-binding reactions were carried out in 20 μL of buffer [20 mmol/L HEPES (pH 8), 1 mmol/L EDTA, 50 mmol/L KCl, 10 mmol/L DTT, 10% glycerol, 1 mg/mL BSA, 50 μg/mL poly(dI-dC) with 50,000 cpm] of labeled probe, 20 μg of MCF-7 nuclear protein or an appropriate amount of CREB protein or Sp1 human recombinant protein (Promega), and 5 μg of poly(dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. The specificity of the binding was tested by adding to the mixture reaction–specific antibodies (anti-CREB and anti-Sp1). Mithramycin A (100 μmol/L; ICN Biomedicals, Inc., Costa Mesa, CA) was incubated with the labeled probe for 30 min at 4°C before the addition of nuclear extracts. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× Tris borate-EDTA for 3 h at 150 V.

**Table 1.** Effect of leptin on cell aggregation in MCF-7 breast cancer cells

<table>
<thead>
<tr>
<th>Spheroids</th>
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<tr>
<td>25 ≤ 50 μm</td>
<td>50 ≤ 100 μm</td>
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<tr>
<td>Control</td>
<td>30 ± 1.2</td>
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<tr>
<td>Leptin</td>
<td>6 ± 0.8</td>
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<tr>
<td>E2</td>
<td>7 ± 0.6</td>
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<tr>
<td>Leptin + E2</td>
<td>3 ± 0.9</td>
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NOTE: MCF-7 cells were cultured as three-dimensional spheroids in serum-free medium. The extent of aggregation was scored by measuring the spheroid diameters with an ocular micrometer. The values represent a sum of spheroids in 10 optical fields under ×10 magnification. The results are mean ± SE from at least three experiments. Representative three-dimensional cultures are shown in Fig. 2A.
with elution buffer (1% SDS and 0.1 mol/L NaHCO₃), and digested with proteinase K (26). DNA was obtained by phenol/chloroform extractions and precipitated with ethanol; 5 μL of each sample were used for PCR with CREB primers (5'-TGTAATCCAACACTTCAGGAGG-3' and 5'-TTGAGAGTCTCGCTCT-3') and Sp1 primers (5'-TAGCAACTCCAGGCTAGAGG-3' and 5'-AECTGACTTTCCGCAAGCTACA-3'). The PCR conditions were 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min for 30 cycles.

Statistical analysis. Data were analyzed by ANOVA using the STATPAC computer program. Statistical comparisons for in vivo studies were made by Wilcoxon-Mann-Whitney test.

Figure 3. Leptin up-regulates E-cadherin expression in MCF-7 spheroids and xenografts. Reverse transcription-PCR of E-cadherin mRNA was done in MCF-7 three-dimensional cultures stimulated for 48 h with 1,000 ng/mL leptin and/or 100 nmol/L E₂ (A) as well as in xenografts (B). 36B4 mRNA levels were determined as a control. C, RNA sample without the addition of reverse transcriptase (negative control). Protein extracts obtained from MCF-7 spheroids (A) and xenografts (B) were immunoblotted with a specific antibody against human E-cadherin. Representative results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Columns, mean of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%; bars, SE. MCF-7 cells were transiently transfected with a luciferase reporter plasmid containing the human E-cadherin promoter full-length p-1008/+49 (C) or mutated in the CREB site (CREB mut; D). Schematic representation of human E-cadherin promoter constructs. The +1 position represents the transcriptional initiation site. The cells were left untreated (control) or treated in the presence of 1,000 ng/mL leptin and/or 100 nmol/L E₂. Columns, mean of three separate experiments; bars, SE. In each experiment, the activities of the transfected plasmid were assayed in triplicate transfections. *, P < 0.05; **, P < 0.01 compared with control.
Results

Effects of leptin on breast cancer cell tumor growth. To determine in vivo the influence of leptin on breast cancer cell tumor growth, we used 45-day-old female nude mice bearing, into the intrascapular region, MCF-7 cell tumor xenografts with or without estrogen pellets. Tumors were obtained only in animals with estrogen pellet implants, which were in general larger in animals treated with leptin at the dose of 230 μg/kg (Fig. 1A). Particularly, 13 weeks of leptin parenteral administration increased the tumor volume to 100% the size of E2 treatment. Besides, leptin significantly enhanced phosphorylation of tumor-derived MAPK and STAT3, suggesting that concentration and dosing schedule of leptin were appropriated for in vivo stimulation (Fig. 1B and C).

Leptin enhances cell-cell adhesion and cell proliferation. We did three-dimensional MCF-7 cultures to evaluate in vitro the effects of leptin on cell aggregation. It has been reported that multicellular spheroid culture can more closely mimic some effects of leptin on cell aggregation. It has been reported that studies (27-30).

Our results evidenced that leptin and/or E2 treatment for 48 h enhances cell-cell adhesion of MCF-7 cells compared with untreated cells (Fig. 2A). The combined exposure to both hormones switches cell aggregation towards the formation of spheroids exhibiting prevalently a diameter larger than 100 μm (Table 1).

E-cadherin is a major type of adhesion molecule, which forms Ca\(^{2+}\)-dependent homophilic ligations to facilitate cell-cell contact in epithelial cells (16, 17). Thus, to study whether E-cadherin was responsible for leptin-enhanced cell-cell adhesion, we supplemented the cell culture medium with function-blocking E-cadherin antibody or EGTA, a calcium-chelating agent. As shown in Fig. 2A, in the presence of the antibody, MCF-7 cells formed small aggregates showing limited intercellular contact, whereas EGTA treatment prevented cell-cell adhesion, and cells remained rounded and singed suspended.

In addition, the role of E-cadherin was confirmed using an adhesion assay in which cells were allowed to adhere to E-cadherin Fn-coated dishes. This assay showed a greater binding of cells pretreated with leptin and/or E2 for 48 h with respect to untreated cells (Fig. 2B). The adhesion was blocked using either a function-blocking E-cadherin antibody or EGTA (data not shown). Thus, the increased aggregation observed in the presence of leptin and/or E2 was dependent on E-cadherin.

In three-dimensional cultures, we also observed a significant increase of cell growth upon leptin and/or E2 treatment. The leptin-induced cell proliferation was completely abrogated when E-cadherin function was blocked (Fig. 2C).

Furthermore, in MCF-7 spheroids and in xenografts, we observed an increase of cyclin D1, a regulator of cell cycle progression, in terms of mRNA and protein content in the presence of leptin and/or E2 (Supplementary Fig. S1).

Leptin up-regulates E-cadherin expression. To investigate if an enhanced expression of E-cadherin occurred in the above-mentioned conditions, we did reverse transcription-PCR and Western blotting analysis. Our results showed that either leptin or E2 and, in higher extent, the exposure to both hormones increased expression of E-cadherin in terms of mRNA and protein content (Fig. 3A). The latter results were also evident in MCF-7 xenografts (Fig. 3B).

To evaluate whether hormones were able to activate E-cadherin promoter, we transiently transfected MCF-7 cells with human E-cadherin promoter plasmid (p-1008/+49). A significant increase in promoter activity was observed in the transfected cells exposed to leptin and/or E2 for 48 h (Fig. 3C).

In contrast, we observed that leptin was unable to activate the constructs containing different deleted segments of human E-cadherin promoter (p-164/+49 and p-83/+49) with respect to E2. The latter results were also evident in MCF-7 cells exposed to both hormones with respect to the full length, whereas E2 induced activation in the presence of p-164/+49 construct (Supplementary Fig. S2).

Leptin enhances CREB-DNA and Sp1-DNA binding activity to E-cadherin promoter. The role of leptin and E2 on the transcriptional activity of the E-cadherin gene was explored analyzing the nucleotide sequence of the E-cadherin gene promoter. We evidenced, upstream to the initiation transcription site, one CRE (−925/−918) and two Sp1 (−144/−132 and −51/−39) as putative effectors of leptin and estrogens. For instance, in MCF-7 cells transiently transfected with E-cadherin promoter plasmid-bearing CREB-mutated site (CREB mut), we observed that the stimulatory effect of leptin was abrogated, whereas the activation of E2 still persisted, although in a lower extent with respect to the intact promoter (Fig. 3D).

To characterize the role of these motifs in modulating E-cadherin promoter activity, we did electrophoretic mobility shift assay (EMSA). Nuclear extracts from MCF-7 cells, using as probe a CRE-response element, showed two protein-DNA complexes (Fig. 4A, lane 1), which were abolished by the addition of a nonradiolabeled competitor (Fig. 4A, lane 2). Leptin treatment induced a strong increase in CREB DNA-binding activity (Fig. 4A, lane 3), which was immunodepleted in the presence of CREB antibody (Fig. 4A, lane 4). Using transcribed and translated in vitro CREB protein, we obtained two bands migrating at the same level as that of MCF-7 nuclear extracts (Fig. 4A, lane 9). In the presence of the MAPK inhibitor PD98059, the complex induced by leptin treatment was reduced (Fig. 4B, lanes 5 and 9). These findings addressed a specific involvement of leptin signaling in the up-regulation of E-cadherin expression.

Using a DNA probe containing an Sp1 site, we observed in MCF-7 nuclear extracts, a specific protein-DNA complex that was slightly enhanced by leptin, increased upon E2 exposure and furthermore by the combined treatments (Fig. 4C, lanes 1, 3, 5, and 7). In the presence of Sp1 human recombinant protein, we observed a single complex that causes the same shift with respect to the band revealed in MCF-7 nuclear extracts (Fig. 4C, lane 10). The addition...
of mithramycin A (100 μmol/L), which binds to GC boxes and prevents sequential Sp1 binding, to nuclear extracts treated with leptin and E2 blocked the formation of DNA-Sp1 complexes (Fig. 4C, lane 9). The original band DNA-protein complex was supershifted by Sp1 antibody (Fig. 4C, lanes 4, 6, and 8). In all hormonal treatments done, the pure antiestrogen ICI 182,780 reduced the Sp1-DNA binding complex (Fig. 4D, lanes 5, 7, and 9), evidencing that leptin induced an activation of ERα, as we previously showed (25).

**Effects of leptin on CREB and Sp1 recruitment to the E-cadherin promoter.** To corroborate EMSA results, we did ChIP assay. We found that the stimulation of MCF-7 cells with leptin increased the recruitment of CREB to E-cadherin gene promoter (Fig. 5A). Furthermore, we observed that leptin or E2 stimulated the recruitment of Sp1 to the E-cadherin promoter, and the combined treatment induced an additive effect (Fig. 5B). The latter event suggests that leptin and E2 may converge in activating ERα to recruit Sp1 on E-cadherin promoter.

**Involvement of ERα in the leptin-induced up-regulation of E-cadherin expression.** Stimming from the data provided by EMSA and ChIP assays, we evaluated the involvement of ERα in the enhanced E-cadherin expression induced by leptin. Our results showed that in three-dimensional cultures, in the presence of the pure antiestrogen ICI 182,780, the up-regulatory effect of leptin on E-cadherin protein expression still persisted, whereas the stimulatory effect of E2 was abrogated (Fig. 6A).

In addition, the specific role of leptin signaling in up-regulating E-cadherin expression was also confirmed by functional studies in ERα-negative HeLa cells. We evidenced that leptin was able to activate E-cadherin promoter (Fig. 6B), which was abrogated in the presence of ERK2 and STAT3 dominant negative (Fig. 6C), sustaining furthermore the involvement of leptin signaling. It is worth to note how the ectopic expression of ERα in HeLa cells was able to potentiate the effect of leptin (Fig. 6B). To test the activity of the transfected ERα, we did Western blotting analysis for phosphorylated ERα, whereas for dominant-negative ERK2 and STAT3 genes, we evaluated the expression of c-fos, as target of both pathways (31–33). Moreover, in BT-20 cells lacking of ERα, leptin-enhanced E-cadherin protein content was reduced in the presence of either ERK2 or STAT3 dominant negative. In the same cells, cotransfected with ERα and ERK2 or STAT3 dominant negative, E2 alone or in combination with leptin was unable to maintain the up-regulatory effect on E-cadherin expression (Supplementary Fig. S3).

**Discussion**

Leptin stimulates cell growth, counteracts apoptosis, and induces migration and angiogenic factors in different cellular cancer models (10). For instance, hyperleptinemia is a common feature of obese women who have a higher risk of breast cancer than women with normal weight (34), but the association between circulating leptin and breast cancer is still not clear. It has been reported that in interstitial fluid of the adipose tissue, leptin concentration is higher than the circulating levels (35). Thus, we may reasonably assume that in the presence of an abundant adipose tissue surrounding epithelial breast cancer cells, the paracrine leptin effects become crucial in affecting local and primary tumor progression.

The aim of this study was to evaluate whether leptin can influence local primary breast cancer development and progression, using an in vivo model of MCF-7 xenografts implanted in female nude mice and an in vitro system represented by MCF-7 three-dimensional cultures. Our results showed in MCF-7 xenografts that leptin treatment significantly potentiated the E2-induced increased tumor size. In the same view, in vitro studies revealed that the combined exposure to both hormones enhanced cell-cell aggregation with respect to the separate treatments.

E-cadherin is an intercellular adhesion molecule generally implicated as tumor suppressor in several types of epithelial tumors, based on findings that the expression of this homotypic adhesion molecule is frequently lost in human epithelial cancers (18, 20, 21). However, it has well been shown in ovarian epithelial tumors that E-cadherin expression is much more elevated than normal ovaries, suggesting that E-cadherin can play a role in the development of ovarian carcinomas (36). For instance, it is worth to mention that E-cadherin may serve not only as an intercellular adhesion molecule, but it may also trigger intracellular activation of proliferation and survival signals (37).
In our study, the increased cell-cell aggregation, observed in MCF-7 three-dimensional cultures upon leptin and/or E2 treatments, seems to be dependent on E-cadherin molecule that has an indispensable role in this process. Indeed, the addition of a function-blocking E-cadherin antibody or a calcium-chelating agent (EGTA) blocked cell-cell adhesion induced by both hormones. Besides, we showed by adhesion assay a greater binding of cells pretreated with leptin and/or E2 on E-cadherin/Fc protein–coated dishes.

In the same experimental conditions, an increased proliferative rate was observed upon leptin or E2 exposure, which was completely abrogated when E-cadherin function was blocked.

An important cell cycle regulator, such as cyclin D1, resulted to be up-regulated in three-dimensional cultures and in xenografts. Besides, in both models, we showed that leptin and/or E2 enhanced E-cadherin expression in terms of mRNA, protein content, and promoter activity.

The analysis of E-cadherin promoter sequence revealed the presence of CRE and Sp1 sites as potential target of leptin and E2 signals. It is well documented how leptin and E2 through nongenomic effects are able to activate the MAPK pathway that induces activation of CREB kinase, a member of the p90RSK family that corresponds to RSK2 and thereby phosphorylates CREB Ser133 (38–40). This well fits with our functional studies showing that leptin was no longer able to activate the E-cadherin gene promoter mutated in the CREB site, whereas E2 maintained an activatory effect although in a lower extent with respect to the intact promoter. The latter data suggest that the activatory effect of E2 may persist through its binding to Sp1-DNA complex.

The important role of the Sp1-responsive element in activating E-cadherin promoter was shown by EMSA and ChIP assays. Our results evidenced that E2, as extensively documented, acts in a nonclassic way through the interaction of ERα with Sp1 (41–45). It is worth to note that upon leptin exposure, we also observed an increase in Sp1-DNA binding activity, clearly reduced in the presence of the pure antiestrogen ICI 182,780, as well as an enhanced recruitment of Sp1 to E-cadherin promoter. These observations are supported by our previous findings reporting that leptin is able to transactivate, in a unliganded-dependent manner, ERα through MAPK signal (25).

A cross-talk between leptin and E2 has been well documented in neoplastic mammary tissues and breast cancer cell lines (15, 25, 46, 47). For instance, E2 up-regulates leptin expression in MCF-7 cells (15), whereas leptin is an amplifier of E2 signaling through a double mechanism: an enhanced aromatase gene expression (46) and a direct transactivation of ERα (25). Thus, we investigated whether the up-regulatory effect induced by leptin on E-cadherin expression can be modulated by ERα. We found that E-cadherin protein seems up-regulated still by leptin in the presence of the pure antiestrogen ICI 182,780. Moreover, in HeLa cells, leptin was able to activate E-cadherin promoter, which was abrogated in the presence of ERK2 or STAT3 dominant negative, suggesting that

Figure 6. Influence of ERα on leptin-induced upregulation of E-cadherin expression. A, MCF-7 spheroids were preincubated with 1 μmol/L ICI 182,780 (ICI) for 1 h and then treated with leptin (1,000 ng/mL) and/or E2 (100 nmol/L) for 48 h. Total proteins (50 μg) were immunoblotted with a specific antibody against human E-cadherin. GAPDH serves as loading control. B, ER-negative HeLa cells were transfected with a plasmid containing E-cadherin promoter or cotransfected with E-cadherin promoter and pHEGO. Transfected cells were treated with leptin (1,000 ng/mL) and/or E2 (100 nmol/L) for 48 h. Columns, means of three separate experiments; bars, SE. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. Inset, Western blot analysis for phosphorylated ERα (pERα) using anti–phosphorylated ERα (Ser118). *, P < 0.05; **, P < 0.01, compared with control. C, HeLa cells were transiently transfected with dominant-negative ERK2 or STAT3 plasmid and then treated for 48 h with leptin. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. Inset, Western blot analysis for c-fos. *, P < 0.05, compared with control.
The presence of ectopic ERα may interact with Sp1 and bind DNA in a nonclassic way. Both CREB and Sp1 transactivation factors bind on E-cadherin promoter at specific responsive sequences and induce an enhanced E-cadherin expression.

The up-regulatory effect induced by E2 on E-cadherin expression in breast cancer is shown in Fig. 7. Leptin through MAPK activation may phosphorylate CREB and induce its transactivation. For instance, CREB phosphorylated at Ser333 is often reported not only as an index of PKA but also as an effector of MAPK activation (49). Concomitantly, leptin in the presence of E2 may potentiate the transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. On the other hand, it is well known that ERα, in the presence of its natural ligand, interacts with Sp1.

Thus, we may reasonably propose that upon leptin exposure, the increased E-cadherin-mediated cellular adhesion and activation of proliferation signals may enhance the transformation of normal epithelial cells to neoplastic cells and then stimulate the growth of tumor mass. Distinct from its role as a tumor suppressor, E-cadherin may function as tumor enhancer in the development of primary breast cancer.

In conclusion, all these data address how leptin and E2 signaling may represent a target of combined pharmacologic tools to be exploited in the novel therapeutic adjuvant strategies for breast cancer treatment particularly in obese women.

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Figure 7. Hypothesized model of leptin signaling in modulating E-cadherin expression in breast cancer. Interaction of leptin (Lep) with its specific receptor (ObR) induces, through MAPK activation, phosphorylation of CREB and its transactivation. Leptin may potentiate the transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. Both CREB and Sp1 transactivation factors bind on E-cadherin promoter at specific responsive sequences and induce an enhanced E-cadherin expression.

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Consequence of the enhanced expression of leptin receptor upon E2 exposure (15), which may have an impaired signaling on E-cadherin expression. An additional explanation, which could coexist with the previous one, may be that both ERK2 and STAT3 dominant negative could interfere with ERα-Sp1 interaction at level of E-cadherin gene transcription (48).

A hypothetical model of the possible mechanism through which leptin and E2 may functionally interact in modulating E-cadherin expression in breast cancer is shown in Fig. 7. Leptin through MAPK activation may phosphorylate CREB and induce its transactivation. For instance, CREB phosphorylated at Ser333 is often reported not only as an index of PKA but also as an effector of MAPK activation (49). Concomitantly, leptin in the presence of E2 may potentiate the transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. On the other hand, it is well known that ERα, in the presence of its natural ligand, interacts with Sp1.

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