**Leptin Mediates Tumor–Stromal Interactions That Promote the Invasive Growth of Breast Cancer Cells**

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**Abstract**

Obesity confers risks to cancer development and progression but the mechanisms underlying these risks remain unclear. In this study, we identify a role for the obesity cytokine leptin, which has been implicated previously in breast cancer development, as a determinant for the tumor-promoting activity of cancer-associated fibroblasts (CAF) in both wild-type (WT) and K303R mutant estrogen receptor-α (ERα)–expressing breast cancer cells. Human CAFs stimulated a greater increase in the proliferation and migration of breast cancer cells expressing the K303R-ERα hyperactive receptor than WT-ERα–expressing cells. A concomitant increase was seen in leptin receptor isoform expression and activation of the leptin signaling pathway in cells expressing K303R-ERα compared with WT-ERα, correlating with leptin effects on cell growth, motility, and invasiveness in mutant cells. Epidermal growth factor and other factors secreted by K303R-ERα cells stimulated CAF proliferation, migration, and subsequent leptin secretion. Moreover, K303R-ERα expression generated a leptin hypersensitive phenotype in vivo. Together, our results reveal a bidirectional cross-talk between breast cancer cells and “educated” CAFs that drives tumor progression via leptin signaling. In elucidating a mechanism that connects obesity and cancer, these findings reinforce the concept that blocking cancer–stromal cell communication may represent an effective strategy for targeted therapy of breast cancer. *Cancer Res; 72(6); 1416–27. ©2012 AACR.*

**Introduction**

For the past 3 decades, cancer research focused predominantly on the characteristics of breast cancer cells. Recently, clinical and experimental studies revealed that both tumor initiation and progression are related to the complex interactions that transpire within the tumor microenvironment. The stromal compartment is composed of mesenchymal cells (fibroblasts, adipocytes, blood cells) and extracellular matrix (ECM; lamin, fibronectin, collagen, proteoglycans, and so on), and signals from these cells come as soluble secreted factors, ECM components, or direct cell–cell contacts. Growth factors, cytokines, adipokines, proteases, and vascular-stimulating factors are involved in stroma-mediated procarcinogenic activities (1–4). The chemokines CXCL12, CXCL14, and CCL7 stimulated tumor cell proliferation and invasion in vitro and in vivo and increased tumor angiogenesis and macrophage presence at tumor sites (5–7). The interleukins (IL)-1 and -8 induced cancer progression by enhancing metastasis and cachexia (8, 9). As important adipocyte-derived endocrine and paracrine mediator, the adipokine leptin has been correlated with breast cancer occurrence. Indeed, leptin synthesis and plasma levels increase with obesity, a pandemic condition that influences both risk and prognosis of breast cancers (10).

The processes of heterotypic signaling involve a constant bidirectional cross-talk between stromal cells and malignant cells. Stromal cells influence tumor invasiveness and malignancy, whereas at the onset and during breast cancer progression, the microenvironment is reorganized by cancer cells (11). Tumors recruit stromal fibroblasts in a process referred to as the desmoplasmic reaction, and these carcinoma-associated fibroblasts (CAF) are reprogrammed to produce growth factors, cytokines, and ECM-remodeling proteins, that acting in autocrine and paracrine fashion support tumor proliferation and invasion into surrounding tissues (4). Moreover, a variety of these factors may activate estrogen receptor-α (ERα; ref. 12). Estrogens and its receptor play a crucial role in regulating breast cancer growth and differentiation. Variant forms of ERα due to alternative splicing or gene mutation have been reported, but their clinical significance is still unresolved (13, 14). A naturally occurring mutation at nucleotide 908, introducing a lysine to arginine transition at residue 303 within the hinge domain of the receptor (K303R-ERα), was identified in one third of premalignant breast hyperplasias and one half of...
invasive breast tumors. This mutation correlated with poor outcomes, older age, larger tumor size, and lymph node–positive disease (15, 16). Other studies did not detect the mutation in invasive cancers (17–20), but our studies suggest that the detection method used might be insensitive. However, K303R expression was found at low frequency in invasive breast tumors by Conway and colleagues (21). K303R mutation allows ERα to be more highly phosphorylated by different kinases, and it alters the dynamic recruitment of coactivators and corepressors (22–24). Mutant overexpression in MCF-7 breast cancer cells increased sensitivity to subphysiologic levels of estrogen and decreased tamoxifen responsiveness when elevated growth factor signaling was present (15, 25). K303R-ERα mutation also conferred resistance to the aromatase inhibitor anastrozole (23, 26), suggesting a pivotal role for this mutation in more aggressive breast cancers.

The aim of this study was to elucidate the mechanisms underlying tumor–stroma interaction in ERα-positive breast cancer cells. First, we investigated how tumoral microenvironment pressure, exerted by CAFs, impacts breast cancer cell proliferation, migration, and invasiveness in relation to the expression of wild-type (WT) or the K303R-ERα. We then defined the effect that a single factor leptin has on stroma-mediated breast cancer progression. Finally, we examined the bidirectional interactions between CAFs and breast cancer cells, leading to increased malignancy.

Materials and Methods

Reagents and antibodies

The following reagents and antibodies were used: leptin, 17β-estradiol, and epidermal growth factor (EGF) from Sigma; IC1182780 from Tocris Bioscience; AG1490, AG1478, PD98059, and LY294002 from Calbiochem; ERα, ERβ, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ObRb, ObRs, Ob, Akt, and pAktSer437 antibodies from Santa Cruz Biotechnology; and phosphate dehydrogenase (GAPDH), ObRb, ObRs, Ob, Akt, and pAktSer437 antibodies from Santa Cruz Biotechnology; and mitogen-activated protein kinase (MAPK), Janus-activated kinase (JAK)2, STAT3, pMAPKThr202/Tyr204, pJAK2Tyr1007/1008, pSTAT3Ser118, and pERαSer167 from Cell Signaling Technology.

Plasmids

Generation of yellow fluorescent protein (YFP)-tagged expression constructs, YFP-WT and YFP-K303R-ERα, was done as described in the work of Cui and colleagues (22). XETL plasmid, containing an estrogen-responsive element, was provided by Dr. Picard, University of Geneva, Geneva, Switzerland.

Cell culture

MCF-7 and SKBR3 cells were acquired in 2010 from American Type Culture Collection where they were authenticated, stored according to supplier’s instructions, and used within 4 months after frozen aliquots resuscitations. YFP-WT and YFP-K303R-ERα stably expressing MCF-7 cells, MCF-7 and SKBR3 pools stably transfected with YFP-WT and YFP-K303R-ERα were generated as described earlier (23, 26). Immortalized normal human foreskin fibroblasts BJ1-hTERT were provided by Dr Lisanti, Jefferson University, Philadelphia, PA. Every 4 months, cells were authenticated by single tandem repeat analysis at our Sequencing Core; morphology, doubling times, estrogen sensitivity, and mycoplasma negativity were tested (MycAlert, Lonza).

CAF isolation

Human breast cancer specimens were collected in 2011 from primary tumors of patients who signed informed consent. Following tumor excision, small pieces were digested (500 IU collagenase in Hank’s balanced salt solution; Sigma; 37°C for 2 hours). After differential centrifugation (90 × g for 2 minutes), the supernatant containing CAFs was centrifuged (500 × g for 8 minutes), resuspended, and cultured in RPMI-1640 medium supplemented with 15% FBS and antibiotics. CAFs between 4 and 10 passages were used, tested by mycoplasma presence, and authenticated by morphology and fibroblast activation protein (FAP) expression.

Conditioned medium systems

CAFs were incubated with regular full media (48–72 hours). Conditioned media were collected, centrifuged to remove cellular debris, and used in respective experiments. Alternatively, conditioned media were collected from WT- and K303R-ERα–expressing MCF-7 cells incubated in 5% charcoal-stripped FBS (72 hours).

Expression microarray analysis

Expression profiles were determined with Affymetrix GeneChip Human Genome U133 plus 2.0 arrays. Data quality and statistical analyses were conducted as described in the work of Barone and colleagues (23). Microarray study followed MIAME (Minimum Information About a Microarray Experiment) guidelines. All data are available in our previous publication (23).

Immunoblot analysis

Protein extracts were subjected to SDS-PAGE as described (27). Immunoblots show a single representative of 3 separate experiments.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, permeabilized with PBS + 0.2% Triton X-100 followed by blocking with 5% bovine serum albumin (1 hour at room temperature), and incubated with anti-ObR antibody (4°C, overnight) and with fluorescein isothiocyanate–conjugated secondary antibody (30 minutes at room temperature). IgG primary antibody was used as negative control. 4’,6-Diamidino-2-phenylindole (DAPI; Sigma) staining was used for nuclei detection. Fluorescence was photographed with OLYMPUS BX51 microscope, 100× objective.

Reverse transcription and real-time reverse transcriptase PCR assays

The gene expression of FAP, Ob, cyclin D1, pS2, cathepsin D, and 36B4 was evaluated by reverse transcription PCR (RT-PCR) method as described in the work of Catalano and...
The gene expression of ObRl, ObRs, CXCR4, insulin receptor (IR), IL-2RB, IL-6R, EGF receptor (EGFR), insulin-like growth factor-1 receptor (IGF1R), fibroblast growth factor receptor 3 (FGFR3), ERa, EGF, IL-6, and insulin was assessed by real-time RT-PCR, using SYBR Green Universal PCR Master Mix (Bio-Rad). Each sample was normalized on 18S mRNA content. Relative gene expression levels were calculated as described (28). Primers are listed in Supplementary Table S1.

**ERa transactivation assays**

ERa transactivation assays were conducted as described in the work of Catalano and colleagues (29).

**Cell proliferation assays**

**MTT assays.** After 4 days of treatment, cell proliferation was assessed by MTT (Sigma) and expressed as fold change relative to vehicle-treated cells.

**Trypan blue cell count assays.** After 4 days of treatment, cell numbers were evaluated by trypsin suspension of samples followed by microscopic evaluation using a hemocytometer.

**Soft agar growth assays.** Anchorage-independent growth assays were conducted as described in the work of Barone and colleagues (26).

Data represent 3 independent experiments carried out in triplicate.

**Wound-healing scratch assays**

Cell monolayers were scraped and treated as indicated. Wound closure was monitored over 24 hours; cells were fixed and stained with Coomassie brilliant blue. Images represent 1 of 3 independent experiments (10× magnification, phase contrast microscopy).

**Transmigration assays**

Cells treated with or without leptin were placed in the upper compartments of Boyden chamber (8-mm membranes; Corning Costar). Bottom well contained regular full media. After 24 hours, migrated cells were fixed and stained with Coomassie brilliant blue. Migration was quantified by viewing 5 separate fields per membrane at 20× magnification and expressed as the mean number of migrated cells. Data represent 3 independent experiments, assayed in triplicate.

**Invasion assays**

Matrigel-based invasion assay was conducted in invasion chambers (8-mm membranes) coated with Matrigel (BD Biosciences; 0.4 μg/mL). Cells treated with or without leptin were seeded into top Transwell chambers, whereas regular full medium was used as chemoattractant in lower chambers. After 24 hours, invaded cells were evaluated as described for transmigration assays. Data represent 3 independent experiments, assayed in triplicate.

**Tumor xenografts**

In vivo studies were conducted as described in the work of Mauro and colleagues (30).

**Leptin measurement by radioimmunoassay**

Leptin was measured by a competitive in-house immunoassay (Chematil) following manufacturer’s protocol. Results are presented as nanograms per cell.

**Leptin-immunodepleted conditioned media**

Protein G-agarose beads were incubated with anti-leptin or IgG antibodies. Antibody–beads complexes were incubated with CAF conditioned media and centrifuged. Leptin immunodepletion was verified by radioimmunoassay (RIA).

**Statistical analysis**

Data were analyzed for statistical significance with 2-tailed Student t test using GraphPad Prism 4. SDs are shown. Survival curves were computed by Kaplan–Meier method and compared using 2-sided log-rank tests.

**Results**

**Tumor–stroma interactions stimulate cell proliferation and motility**

Epithelial–stromal interactions support tumor cell proliferation and invasion. Thus, we first investigated the role of tumoral microenvironment in influencing breast cancer phenotype in relation to the expression of WT- or K303R-ERa mutant receptor. We used ERa-positive MCF-7 cells stably transfected with YFP-WT or YFP-K303R-ERa expression vectors as experimental models for breast cancer. We chose this approach because wild-type receptor was present along with K303R-ERa in invasive breast tumors (16). Stable clones were screened for ERa expression using immunoblot analysis (Fig. 1A). Two clones stably expressing YFP-WT (WT1-2) or YFP-K303R-ERa (K303R1-2) are shown along with wild-type or mutant receptor stable pools (WT P and K303R P). As stromal cells, we used CAFs isolated from biopsies of primary breast tumors. CAFs possessed the basic fibroblast characteristics with long and spindle-shaped morphology and highly expressed the FAP (Fig. 1B). To create in vitro conditions that can mimic the complex in vivo microenvironment, we used coculture experiments. Breast cancer cells were incubated with regular full media, CAF-derived conditioned media, or normal fibroblasts conditioned media, and growth was evaluated by soft agar assays (Fig. 1C). As previously shown (23, 26), control basal growth of mutant-expressing cells was elevated compared with wild-type–expressing cells. CAF-derived conditioned media significantly increased colony numbers in both WT- and K303R-ERa–expressing cells; however, CAF-derived conditioned media enhanced K303R-expressing cell growth at a higher extent than wild-type–expressing cells. We then examined the ability of CAF-derived conditioned media to promote WT- and K303R-expressing cell movement in wound-healing scratch assays (Fig. 1C). The mutant cells moved the farthest in either direction to close the gap compared with wild-type–expressing cells. CAF-derived conditioned media promoted net movement of wild-type–expressing cells compared with full media, but K303R-expressing cells exposed to CAF-derived conditioned media moved at higher rate to close the
Bidirectional CAF–Breast Cancer Cell Interaction

Figure 1. CAF-induced breast cancer cell growth and motility. A, immunoblotting for ERα expression in YFP-WT and YFP-K303R-ERα stably expressing MCF-7 cells and WT P and K303R P stable pools. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control. B, CAF morphology in monolayer growth using phase contrast microscopy. RT-PCR for FAP and 36B4 (internal standard). NC, negative control. C, soft agar and scratch assays in cells treated with regular full media (FM), CAF-derived conditioned media (CAF-CM), or normal fibroblasts-derived conditioned media (NF-CM). D, soft agar and scratch assays in cells treated with FM, CAF-CM with or without AG490 (AG, 10 μmol/L) or ICI182780 (ICI, 1 μmol/L). *, P < 0.05; **, P < 0.005.

Given the gene expression profile identified in the microarray study, we defined the impact that a single factor leptin expression induced several genes potentially involved into tumor–stroma interactions; however, the leptin receptor (ObR) gene was the most highly induced (2.4-fold; Table 1). We also observed increased expression of different leptin signaling downstream effectors such as JAK2, the transcription factors fos, and STAT, as well as the suppressor of cytokine signaling 3 (Supplementary Table S3). To validate the microarray study, YFP-WT and YFP-K303R-ERα–expressing cells were evaluated for a panel of genes using real-time PCR (Fig. 2A). K303R-associated induction could be confirmed for all of them, and again, the gene encoding the long and short leptin receptor isoforms (ObRl/ObRs) was the most highly upregulated in mutant-expressing cells. However, we did not detect any differences in IGF1R mRNA expression levels between the 2 cells, although microarray analysis showed a significant decrease of IGF1R. ERα RNA levels were similar between WT- and K303R-ERα–expressing cells.

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K303R-ERα–overexpressing cells exhibit increased leptin signaling activation

Given the gene expression profile identified in the microarray study, we defined the impact that a single factor leptin
may have on K303R-ERα breast cancer cell progression. First, time course–response studies were conducted to analyze phosphorylation of leptin downstream effectors using immunoblot analysis (Fig. 2D). WT-expressing cells exhibited low basal levels of phosphorylated JAK2, STAT3, Akt, and MAPK that were increased in a time-dependent manner after leptin treatment. In contrast, K303R-expressing cells showed elevated constitutive phosphorylation of these signaling molecules in control vehicle conditions, that was slightly increased after leptin treatment. Thus, the mutant ERα expression was associated with increased leptin signaling activation.

Leptin directly activates ERα in the absence of its own ligand in MCF-7 breast cancer cells (29). As a consequence of the enhanced leptin signaling, we found increased ERα transcriptional activity and upregulated mRNA levels of the classical ERα target genes cyclin D1, pS2, and cathepsin D in both control and leptin-treated conditions in K303R-ERα-expressing cells. In addition, the mutant exhibited elevated pS118 and pS167 YFP-K303R-ERα levels (Supplementary Fig. S1).

### K303R-ERα mutation and leptin hypersensitivity

We next used these stably transfected clones as model systems to study leptin sensitivity, in relation to mutant receptor expression. First, we evaluated leptin effects on growth using anchorage-dependent growth assays (Supplementary Fig. S2). As expected, in both WT- and K303R-expressing cells, treatment with leptin (100 ng/mL) increased cell proliferation. However, low leptin treatment (10 ng/mL) significantly enhanced cell viability only in K303R-expressing clones. We also evaluated leptin-mediated proliferative effects in anchorage-independent growth assays (Fig. 3A). Leptin treatments at 100 and 1,000 ng/mL concentrations enhanced colony numbers in all 4 clones tested, although to a higher extent in mutant-expressing cells. Again, leptin at 10 ng/mL increased anchorage-independent growth only in K303R cells. The increase in colony numbers induced by leptin was reversed by the JAK2/STAT3 inhibitor AG490 (Fig. 3B). We also used the antiestrogen ICI182780 and found that this treatment suppressed anchorage-independent growth of both cell lines, indicating that ER expression remains important in growth regulation of these cells (Fig. 3B).

We next evaluated the ability of increasing doses of leptin to influence cell migration in wound-healing scratch assays (Fig. 3C). Again, the mutant cells moved the farthest in either direction to close the gap compared with WT-expressing cells. Leptin treatments at 100 and 1,000 ng/mL promoted cell motility in both WT- and K303R-expressing cells, although to a higher extent in mutant cells. Interestingly, leptin at 10 ng/mL stimulated migration only in K303R-expressing cells. Then, the capacity of cells to migrate across uncoated membrane in

### Table 1. Gene expression profile of the different receptors of CAF-secreted factors among WT- and K303R-ERα-expressing MCF-7 breast cancer cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Parametric P</th>
<th>Fold change in K303R clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin receptor</td>
<td>ObR/LepR</td>
<td>&lt;1e-07</td>
<td>2.4</td>
</tr>
<tr>
<td>Interleukin 28 receptor α</td>
<td>IL28RA</td>
<td>&lt;1e-07</td>
<td>1.9</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>CXCR4</td>
<td>&lt;1e-07</td>
<td>1.9</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>IR</td>
<td>1.7e-06</td>
<td>1.68</td>
</tr>
<tr>
<td>Interleukin 17 receptor C</td>
<td>IL17RC</td>
<td>7e-07</td>
<td>1.6</td>
</tr>
<tr>
<td>Insulin-like growth factor 2 receptor</td>
<td>IGF2R</td>
<td>1e-07</td>
<td>1.57</td>
</tr>
<tr>
<td>Interleukin 15 receptor α</td>
<td>IL15RA</td>
<td>3e-07</td>
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</tr>
<tr>
<td>Macrophage stimulating receptor 1</td>
<td>MSR1</td>
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<td>1.39</td>
</tr>
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<td>IL1R1</td>
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<td>1.38</td>
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<tr>
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<td>1.3</td>
</tr>
<tr>
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<td>Interleukin 10 receptor β</td>
<td>IL10RB</td>
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<td>Interleukin 6 receptor</td>
<td>IL6R</td>
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<td>Fibroblast growth factor receptor 3</td>
<td>FGFR3</td>
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</table>

NOTE: Representative probe sets from pathway analysis showing gene expression changes in the receptor family of CAF-secreted factors along with the P value and the fold change of K303R-expressing cells compared with wild-type cells studied by microarray analysis. In cases in which the same genes were deemed significant across multiple probe sets, only one is shown.
transmigration assays or to invade an artificial basement membrane Matrigel in invasion assays was tested in the presence of leptin (Fig. 3D). Although wild-type cells exhibited little motility and no invasive behavior in vitro, our data clearly showed that mutant receptor expression increased both motility and invasion of cells. High doses of leptin increased the number of migrated and invaded cells in both clones and again low doses of leptin stimulated motility and invasion only of cells expressing the K303R receptor. As expected, treatment with 100 ng/mL, 5 and 10 minutes), GAPDH, loading control. Numbers represent the average fold change between phospho-, total, and GAPDH levels. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

from cells with K303R-ERα expression doubled in size within 6 weeks of treatment, whereas none of xenografts from WT-ERα–expressing cells doubled in size during this experiment (Fig. 3F). Thus, expression of the mutant generated a leptin hypersensitive phenotype in vitro and in vivo.

Leptin is responsible for CAF-induced cell growth and motility

We next assessed the role of leptin in the context of heterotypic signaling working in tumor–stroma interactions. First, RIA measurement in CAF-derived conditioned media showed that leptin secretion varied from 10 ± 4.5 ng per 200,000 cells. RT-PCR evidenced Ob mRNA expression in CAFs; CAFs also expressed ObR long isoform, but they did not express ObR short isoform, α-ERα, or ERβ (Supplementary Fig. S3). Leptin was then immunodepleted from CAF-derived conditioned media by leptin-depleted conditioned media +
LepAb) significantly decreased growth- and migration-promoting activities of CAF-derived conditioned media, particularly in K303R-expressing cells (Fig. 4A). Conditioned media treated with a nonspecific mouse IgG had no effects, suggesting that the neutralizing effects of leptin antibodies were specific. Our results identify leptin as a main molecular player that mediates CAF effects on tumor cell growth and migration.

Leptin activates via JAK2 the MAPK and phosphoinositide 3-kinase (PI3K)/Akt pathways (31). Thus, we investigated the specific signaling involved in the CAF–breast cancer cells interaction and found that the PEAK/Akt inhibitor LY294002 was more effective in inhibiting CAF-induced proliferation and migration than the MEK1 inhibitor PD98059 (Fig. 4B).

Tumor-stroma interactions in SKBR3 breast cancer cells

To extend the results obtained, we generated pools of YFP-WT and YFP-K303R-ERα stable transfectants in ERα-negative SKBR3 breast cancer cells (Supplementary Fig. S4). As previously shown for MCF-7 cells, we found a significant increase in both long and short leptin receptor isoforms mRNA in mutant-expressing cells. Again, treatment with leptin at 100 and 1,000 ng/mL significantly increased colony numbers of wild-type clones and to a higher extent of K303R-expressing cells; however, 10 ng/mL of leptin enhanced migration only in K303R-expressing SKBR3 pools. Leptin-immunodepleted conditioned media strongly reduced conditioned media proliferative and migratory promoting activities on K303R cells, confirming that leptin hypersensitive phenotype...
was associated with K303R-ERα expression in different cellular backgrounds.

Effects of breast cancer cell–secreted factors on CAF phenotype

CAFs and tumor cells cross-talk via different soluble factors, where the effects on both subpopulations determine the final outcome of the tumorigenic process. Thus, as a final step of this study, we defined the effects of conditioned media from WT- and K303R-ERα–expressing breast cancer cells on CAF phenotype. Treatment with K303R-derived conditioned media elicited a dramatic alteration in the shape of CAFs in vitro, accompanied by an increased FAP mRNA expression (Fig. 5A). K303R-derived conditioned media also stimulated CAF viability and motility compared with WT-derived conditioned media effects (Fig. 5B), suggesting that how soluble K303R-ERα cell–secreted factors may generate a more activated CAF phenotype. Because leptin synthesis is influenced by different humoral factors (32–34), we evaluated the effects of breast cancer–derived conditioned media in modulating leptin secretion from CAFs. Incubation of CAFs with K303R-derived conditioned media increased leptin mRNA expression and leptin release compared with WT-derived conditioned media, whereas no differences were detected in leptin levels among WT- and K303R-derived conditioned media (Fig. 5C). Finally, to investigate the paracrine factor by which breast cancer cells may affect CAF phenotype, we used microarray analyses to measure the expression of different genes known to be associated with CAFs and/or leptin secretion (11, 32–35). Our results showed that the genes encoding for EGF (2.8-fold), IL-6 (1.2-fold), and insulin (1.2-fold) were induced in mutant cells, and real-time PCR confirmed that the EGF gene was most highly upregulated (Fig. 5D). Thus, we evaluated the role of EGF. First, addition of EGF in WT-derived conditioned media mimicked the induction of K303R-derived conditioned media on CAF motility, and the EGFR signaling inhibitor (AG1478) reduced K303R-derived conditioned media effects (Fig. 5E). Second, treatment with AG1478 reversed K303R-derived conditioned media stimulated FAP mRNA expression (Fig. 5F). Third, Ob mRNA expression and leptin secretion from CAFs cocultured with K303R-derived conditioned media were significantly decreased in the presence of AG1478 (Fig. 5F).

Our data show that K303R-ERα-expressing breast cancer cells through their soluble secreted factors may take advantage of the plastic nature of reactive surrounding cell populations, as CAFs, to generate a tumor-enhancing microenvironment.

Discussion

ERα expression has important implications for breast cancer biology and therapy. Fuqua and colleagues identified a lysine to arginine transition at residue 303 of ERα (K303R-ERα) in 30% of breast hyperplasias and in 50% of invasive breast cancers (15, 16), although using another detection method, the mutation was identified in only 6% of tumors (21); thus the frequency is still unresolved. This mutation was associated with older age, larger tumor size, lymph node positivity, and shorter time to recurrence—all features related to a more aggressive breast cancer phenotype. Because of the recently recognized importance of tumor–stroma cross-talk in promoting breast cancer progression and metastasis, it is imperative to elucidate the molecular events occurring between cancer cells and adjacent stroma at the site of primary tumors to provide new treatment options for breast cancer.

Here, we elucidated the complex interactions between peri-tumoral tissue, locally derived factors, and neoplastic cells in dependency of ERα status, with a special focus on leptin effects.
in influencing the behavior of breast cancer cells bearing the naturally occurring K303R-ERα mutation. We proposed a model in which leptin, secreted from CAFs, binds to its receptor, activates K303R-ERα, and promotes proliferation, migration, and invasiveness of K303R-ERα-expressing breast cancer cells. In turn, K303R cells release factors as EGF that "educate" CAFs to enhance secretion of leptin, which, acting back on malignant cells, may establish a positive feedback loop between cancer and stromal cells to further support breast tumor progression (Fig. 6).

**CAFs promote breast cancer cell malignancy through leptin signaling**

The phenotype of malignant cells appears regulated not only by cell autonomous signals but also is dependent on heterotypic signals coming from surrounding stromal cells, able to create a specific local microenvironment to tightly control breast cancer proliferation and differentiation (36–38).

We defined the molecular interactions between stromal fibroblasts isolated from biopsies of primary breast tumors (CAFs), WT-, and K303R-ERα-expressing MCF-7 breast cancer cells. The initial conditioned media experiments showed that the entire complement of secretory proteins released by CAFs have more profound effects on K303R-ERα-expressing cell proliferation and migration than on WT-ERα cells. We evidenced an important role for JAK2/STAT3 and ERα signaling pathways in conditioned media-mediated effects. Our microarray study pointed to the regulation of several important transcriptional programs of growth factors and cytokines receptors that, acting as mediators of stromal–epithelial interactions, are potentially involved in carcinoma progression. Among them, the gene encoding for leptin receptor was the most highly induced in K303R-expressing breast cancer cells.

Leptin is primarily synthesized from adipocytes but is also produced by other cells, including fibroblasts (39–41). We showed, for the first time, Ob mRNA expression and leptin secretion in CAFs. CAFs expressed ObR long isoforms, implying that an autocrine feedback loop may exist. Leptin immunodepletion from CAF-derived conditioned media substantially reduced the growth- and migration-promoting activities of CAFs. As one of the leptin downstream effectors (31), we found that the PI3K/Akt inhibitor LY294002 was effective in inhibiting CAF-induced effects.

Because fibroblasts are the principal cellular component of the stroma, our results suggest that in the breast microenvironment CAFs through leptin signaling may become the main actor in influencing tumor cell behavior, especially in K303R-ERα-expressing breast cancer cells.

**Cross-talk between leptin and K303R-ERα signaling pathways in breast cancer**

Leptin, a pleiotropic molecule that regulates food intake, hematopoiesis, inflammation, cell differentiation, and proliferation, is also required for mammary gland development and tumorigenesis. Indeed, leptin and its receptor isoforms (ObRs) have been detected in mammary epithelium and breast cancer.
cell lines and are overexpressed in cancer tissue compared with healthy epithelium, with a positive correlation between ObR and ERα expression (42, 43). Real-time PCR, immunoblotting, and immunofluorescent experiments revealed an increase in mRNA and protein expressions of ObR long and short isoforms in K303R-ERα-expressing cells. We also showed that the mutant expression was associated with enhanced leptin signaling activation and increased sensitivity to leptin stimulation on growth, motility, and invasiveness. Moreover, a significant increase in the growth of leptin-treated mutant tumors was observed in vivo.

Leptin is a potent modulator of the estrogen signaling pathway (29, 44). On the contrary, estradiol modulates ObR expression in rat brain, through a putative estrogen-responsive element in its promoter (45, 46), and others showed that estradiol induces leptin and ObR expression in MCF-7 breast cancer cells (43). Thus, leptin and estrogen might cooperate in sustaining estrogen-dependent breast carcinoma growth. We showed an increased S167 and S118 phosphorylation of the K303R receptor, an enhanced K303R-ERα transactivation, and a more pronounced upregulation of classical estrogen-regulated genes in K303R-expressing cells. Indeed, the pure antiestrogenICI182760 drastically suppressed leptin-stimulated anchorage-independent growth and motility of mutant cells.

These results suggest that the mutation may potentiate the role of ERα as an effector of leptin intracellular signal transduction, which may enhance cell proliferation, migration, and invasiveness, contributing to the more aggressive phenotype of K303R-associated breast cancers.

**K303R-ERα cell–derived factors contribute to CAF tumor-promoting activities**

In the same way as tumor microenvironment plays active roles in shaping the fate of a tumor, cancer cells actively recruit fibroblasts into the tumor mass, in particular, the subpopulation named CAFs. This cell type is defined on the basis of the morphologic characteristics or expression of markers as the FAP (1–4).

Studies addressing these issues are heterogeneous in terms of cell systems used, tumor cell types, and fibroblast sources. Experimental systems have used different tumor-derived conditioned media to stimulate CAFs, and others have cocultured tumor cells with normal fibroblasts or mesenchymal stem cells and measured chemokines levels in the resulting conditioned media. For instance, fibroblasts growth with tumor cells resulted in increased production of chemokines whose source is in CAFs themselves. Chemokines produced under these "mixed" conditions promoted tumor promalignancy activities (6, 9, 47). We showed increased leptin mRNA expression and secretion by CAFs in response to soluble K303R-ERα cell–secreted factors compared with WT-derived conditioned media, suggesting that K303R cells have the ability to instruct their surrounding fibroblasts to augment leptin production, thereby enhancing tumor growth. This further indicates that interactions between the 2 subpopulations are actually bidirectional.

These interactions become more productive when tumor cells have a higher aggressiveness phenotype (47–49). CAFs exposed to K303R cell–derived conditioned media acquired a more activated phenotypic characteristic, as revealed by an altered morphology, an increased FAP mRNA expression, and enhanced proliferative and migratory capabilities. We identified the epidermal growth factor, known to affect CAFs phenotype and leptin secretion (11, 32–35), as the factor responsible of the paracrine activation of the surrounding stroma.

Thus, a positive feedback loop is established which leads to the development and growth of the tumor.

**Conclusions**

Our study highlights the functional importance of tumor–host cross-talk in impacting malignant cell behavior and implies several clinical implications. First, because K303R
mutation was identified in breast premalignant hyperplasia, it is tempting to speculate that this specific mutation hyper-
sensitive to leptin signaling may promote or accelerate the
development of cancers from premalignant breast lesions,
 further increasing risk in obese women. Second, understanding
the key genes involved differently in relation to ERα status in
stroma–stroma interactions may help to identify novel biomar-
kers for breast cancer. Finally, our findings support the de-
svelopment of new therapeutics targeting stroma signaling com-
ponents (e.g., leptin) to be implemented in the adjuvant
therapy for improving clinical care and reducing mortality
from breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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